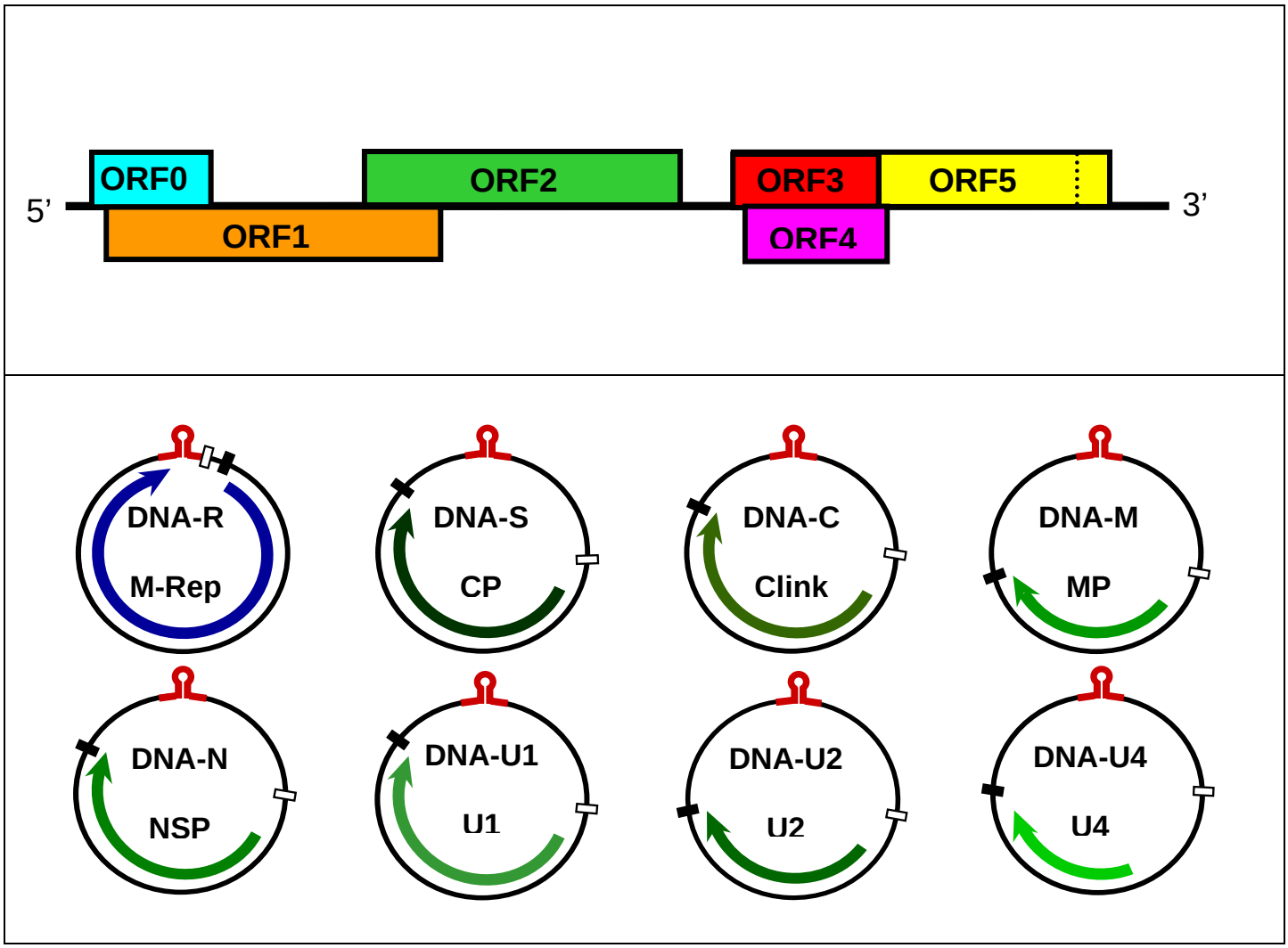
Adane Abraham

CHARACTERIZATION AND GENOME ORGANIZATION OF NEW LUTEOVIRUSES AND NANOVIRUSES INFECTING COOL SEASON FOOD LEGUMES



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Characterization and Genome Organization of New Luteoviruses and Nanoviruses Infecting Cool Season Food Legumes

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CHAPTER 1

General Introduction

Viruses and virus diseases of cool season food legumes

Legume crops play a major role worldwide as source of human food, feed and also in crop rotation. Faba bean (*Vicia faba* L.), field pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medik.), chickpea (*Cicer arietinum* L.), and grasspea (*Lathyrus sativus* L.), collectively referred to as cool season food legumes (Summerfield et al. 1988) are of particular importance in developing countries of Asia, North and Northeast Africa where they provide a cheap source of seed protein for the predominantly poor population. Diseases including those caused by viruses are among the main constraints reducing their yield. Bos et al. (1988) listed some 44 viruses as naturally infecting faba bean, chickpea, field pea and lentil worldwide. Since then, a number of new viruses were described from these crops including *Faba bean necrotic yellows virus* (FBNYV) (Katul et al. 1993) and *Chickpea chlorotic dwarf virus* (CpCDV) (Horn et al. 1993), which are widespread and economically important. Most of the viruses of cool season food legumes are known to naturally infect more than one host within this group of crops (Bos et al. 1988, Brunt et al. 1996 and Makkouk et al. 2003a).

Virus symptoms in cool season food legumes vary depending on the virus or its strain, host species or cultivar and the prevailing environmental conditions. For practical purposes however, the symptoms are roughly categorized into two broad groups (Makkouk et al. 1994). Mosaic/mottle symptoms mainly caused by mechanically transmitted viruses invading parenchyma tissues represent one group, and yellowing, stunting, leaf roll and necrosis symptoms mostly caused by phloem-limited viruses namely luteoviruses, nanoviruses and a geminivirus form the second one/group. Viruses causing mosaic/mottle symptoms in their host belong to different families including *Potyviridae* (e.g. *Pea seed-borne mosaic virus* and *Bean yellow mosaic virus*), *Comoviridae* (e.g. *Broad bean stain virus* and *Broad bean true mosaic virus*) and *Bromoviridae* (e.g. *Alfalafa mosaic virus*, *Cucumber mosaic virus* and *Broad bean mottle virus*) (Bos et al. 1988, Brunt et al. 1996 and Makkouk et al. 2003a). Most of these viruses are non-persistently transmitted by aphids, may be seed-borne depending on the host (Bos et al. 1988), and are therefore of global concern due to international seed movement as primary source of infection in the field.

Yellowing, stunting and leaf roll diseases primarily caused by luteoviruses are considered to be the most destructive viral diseases of cool season food legumes worldwide (Bos et al. 1988). So far five luteoviruses have been reported to infect cool season food legumes from different parts of the world. These are *Bean leaf roll virus* (BLRV) (Ashby et al. 1984), *Beet western yellows virus* (BWYV) (Bosque-Perez and Buddenhagen, 1990, Fortass et al. 1997), *Soybean dwarf virus* (SbDV) (Tamada and Kojima, 1977), reported first as a synonym Subterranean clover red leaf virus (SCRLV) (Wilson and Close, 1973), *Chickpea stunt disease-associated virus* (CpSDaV) (Naidu et al, 1997) and *Pea enation mosaic virus-1* (PEMV-1) (Demler et al 1995). Pea leafroll virus (PeLRV), Legume yellows virus (LYV) and Michigan alfalfa virus (MAV) which were reported from legumes are considered to be synonymous to BLRV (Francki et al. 1991).

In the 1990's, it became evident that some viruses genetically unrelated to luteoviruses are involved in the etiology of yellowing and stunting diseases of some legumes. These viruses some of which were once considered as luteoviruses (Chu et al. 1995) are later named nanoviruses. These include *Subterranean clover stunt virus* (SCSV), *Milk vetch dwarf virus* (MDV) and FBNYV. In addition, a leafhopper-transmitted *Chickpea chlorotic dwarf virus* (CpCDV, *Mastrevirus, Geminiviridae*) (Horn et al. 1993) was reported to cause similar symptoms in legumes such as chickpea and faba bean, further indicating the diversity of viruses associated with this group of diseases. Despite their taxonomic affinity however, luteoviruses and nanoviruses infecting legumes share important ecological properties since they are phloem-limited and thus cause similar symptoms, share overlapping host range and aphid vectors. Consequently, members of these groups of viruses often occur in mixed infections in the field posing the need for an integrated approach for their study and control.

Taxonomy of luteoviruses

The International Committee of Taxonomy of Viruses (ICTV) recognized luteoviruses as a separate virus group in 1975 (Fenner, 1976), as a genus *Luteovirus* in 1995 (Randles and Rathjen, 1995) and later as a separate family *Luteoviridae* (D'Arcy et al 2000). The family *Luteoviridae* is divided into three genera namely *Luteovirus, Polerovirus* and *Enamovirus,* depending on genome organization, sequence similarity and methods of gene expression (D'Arcy et al. 2000). As a group, luteoviruses possess icosahedral particles about 25 nm in diameter consisting of a major ~22 kDa coat protein and a minor component of 52 kDa. They

are naturally transmitted only persitently by aphids and not mechanically, circulate but do not replicate in their aphid vectors and are confined to the phloem tissues of the infected plant.

The genome of luteoviruses is ~5.7 kb single stranded positive sense RNA the 3' end of which is not polyadenylated and without tRNA-like structure (D'Arcy and Domier, 2004). In general, the 5' half of the *Luteovirus* genome is phylogenetically related to that of members of *Tombusviridae* family whereas that of members of the genera *Polerovirus* and *Enamovirus* is closer to the genus *Sobemovirus* (D'Arcy et al. 2000). Three luteoviruses namely BLRV, SbDV and Sugarcane yellow leaf virus (ScYLV) that are suggested to have their genome evolved from recombination between ancestral luteoviruses and poleroviruses (Domier et al. 2002, Rathjen et al. 1994, Smith et al. 2000) are recently assigned to genera based primarily on the phylogenetic affinity of their polymerase gene. Accordingly, BLRV and SbDV are classified as members of genus *Luteovirus* whereas as ScYLV is assigned as a member of genus *Polerovirus* (D'Arcy and Domier, 2004). At present, there are 26 species in the family *Luteoviridae* of which 15 are assigned to one of the genera (D'Arcy and Domier, 2004) while the others are not yet assigned mainly due to lack of sufficient sequence information.

Members of family *Luteoviridae* share five to six major open reading frames (ORFs) designated ORF0 through 5, the same number corresponding to those ORFs coding for proteins of similar arrangement and possible functions (Fig. 1). These ORFs are separated by an intergenic region of ca. 100 nt in members of *Luteovirus* and ca. 200 *in Polerovirus* and *Enamovirus* into a gene cluster divergent among the genera (ORF 0, 1 and 2) at 5' half and conserved (ORF 3, 4 and 5) at 3' half of the genome. Members of genera *Polerovirus* and *Enamovirus* have genome-linked protein (VPg) and ORF0 which is absent in members of the genus *Luteovirus* (Fig 1). PEMV-1, the sole member of genus *Enamovirus* does not possess ORF4 (Fig. 1). Some luteoviruses such as *Barley yellow dwarf virus-PAV* and *Potato leafroll virus* (PLRV) are shown to have minor ORFs named ORF6 and/or ORF7 in the 3' half of their genome (Miller et al. 1995, Ashoub et al. 1998). The genome organization of type members of the three luteovirus genera and the corresponding major ORFs are presented in Fig. 1.

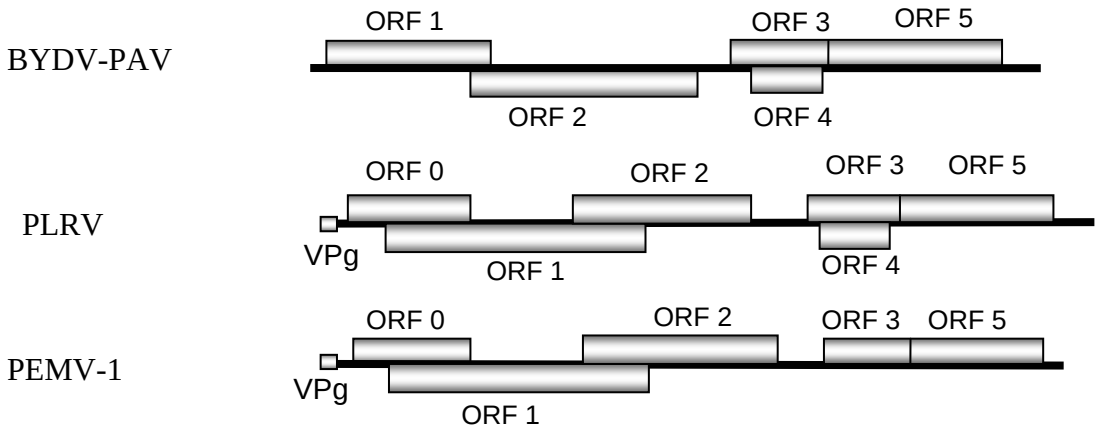

Fig. 1. Genome organization of the three genera of the family *Luteoviridae*

Taxonomy of nanoviruses

Nanoviruses have multipartite ssDNAs encapsidated within small isometric particles (18-20 nm) and are persistently transmitted by aphids. They were formerly referred to as non-geminated ssDNA plant viruses and later assigned as members of family *Circoviridae* (Chu et al. 1995) and then reclassified to a floating genus *Nanovirus* (Randles et al. 2000). Recently, the differences in genome size and sequence homology, number of components and biological properties (host range and vectors) led to their classification to the family *Nanoviridae* consisting of two genera: *Nanovirus* and *Babuvirus* (Vetten et al. 2004). Genus *Nanovirus* presently consists of three members, SCSV, MDV and FBNYV all of which are mainly limited naturally to legume hosts and transmitted naturally mainly by *Aphis craccivora. Banana bunchy top virus* (BBTV) is currently the only member of *Babuvirus* recognized by ICTV.

The genomic information of members of *Nanoviridae* is distributed in over at least 6-8 molecules of circular ssDNA (Burns et al. 1995, Katul et al. 1998, Sano et al. 1998, Vetten et al. 2004) (Fig. 2 and Table 1). Each of these ssDNA components is approximately 1 kb in size and appear to be structurally similar (Fig. 2) in having a positive sense DNA containing a conserved stem-loop structure (and other conserved domains) in the non-coding region and a coding region individually encapsidates as virus particles. Each of the DNAs of members of *Nanoviridae* encode only a single protein with the exception of a second virion sense ORF nested within the master rep encoding protein of BBTV (Vetten et al. 2004). A further peculiarity of nanoviruses is the occurrence of additional autonomously replicating DNAs in addition to the putative genomic DNAs which are considered to be satellites (Vetten et al. 2004).

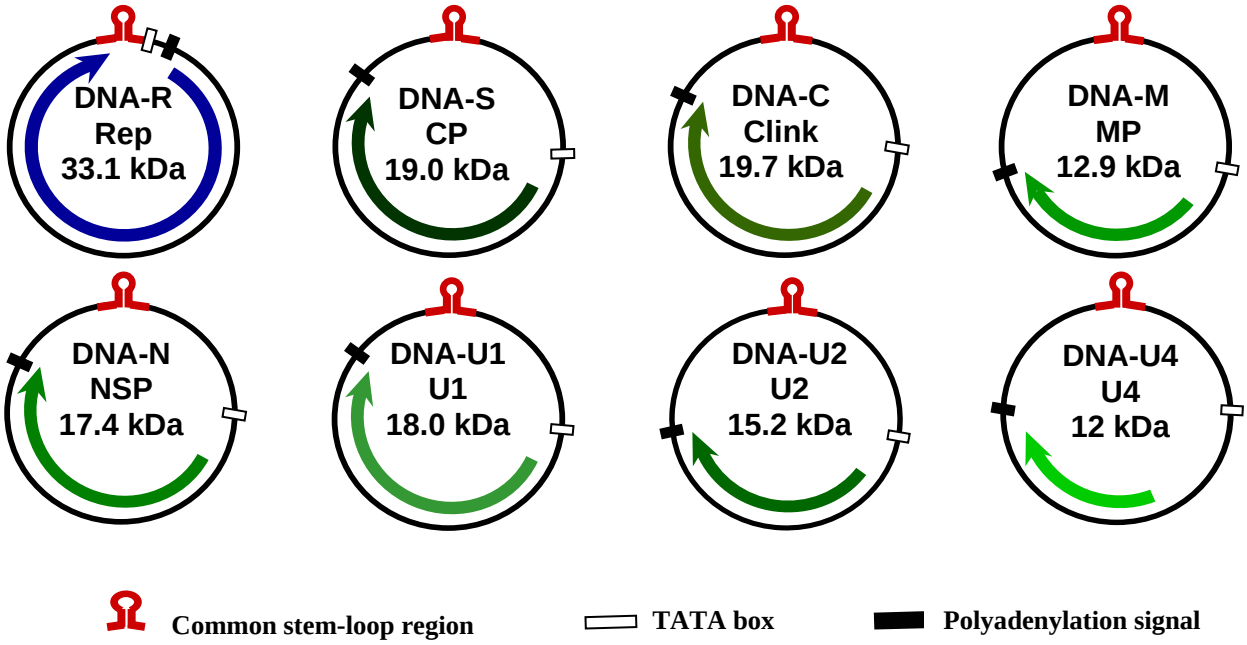

Fig. 2. Diagram illustrating the putative genomic organization of *Faba bean necrotic yellows virus* (an Egyptian isolate). Each of the DNAs has a size of about 1000 nt. Arrows refer to the approximate size of encoded protein (also given in kDa) and direction of transcription. *Milk vetch dwarf virus* (MDV) and *Subterranean clover stunt virus* (SCSV) have similar structure although DNA-U4 has not been identified in both viruses and U2 has not been is not identified in SCSV (see also Table 1) (modified from Vetten et al. 2004).

Until recently, the naming of nanovirus DNAs has led to confusion as different authors independently gave names to the different DNA molecules when they were first identified. To solve this problem, ICTV has recently adopted a standard nomenclature of the genomic DNAs of members of *Nanoviridae*. The DNAs were named in such a way that their designation should reflect the function of the protein they encode and those encoding similar putative proteins be given the same name in individual viruses within the family (Vetten et al. 2004, Table 1). DNAs encoding genes with unknown function are named tentatively as U1 through U5 (U derived from the term Unknown).

Table 1. Designation, size and functions of the proteins encoded by the various DNA components of members of family *Nanoviridae.*

Protein*	Protein Size (kDa)	Encoding DNA component	Identified from				Protein function(s)§
			FBNYV	MDV	SCSV	BBTV	
M-Rep	33.1-33-6	DNA-M	+ (2)	+ (11)	+ (8)	+ (1)	**R**eplication initiator
CP	18.7-19.3	DNA.S	+ (5)	+ (9)	+ (5)	+ (3)	**S**tructural (capsid)
Clink	19.0-19.8	DNA-C	+ (10)	+ (4)	+ (3)	+ (5)	**C**ell cycle link
MP	12.7-13.7	DNA-M	+ (4)	+ (8)	+ (1)	+ (4)	**M**ovement
NSP	17.3-17.7	DNA-N	+ (8)	+ (6)	+ (4)	+ (6)	**N**uclear shuttle
U1	16.9-18.0	DNA-U1	+ (3)	+ (5)	+ (7)	-	**U**nknown
U2	14.2-15.4	DNA-U2	+ (6)	+ (7)	-	-	**U**nknown
U3	10.3	DNA-U3	-	-	-	+ (2)	**U**nknown
U4	10 or 12.5	DNA-U4	+ (12)	-	-	-	**U**nknown
U5	5.0	DNA-R	-	-	-	+ (1)	**U**nknown

Legend: *Master replication initiator protein (M-rep), coat protein (CP), cell cycle link protein (Clink), movement protein (MP), and nuclear shuttle protein (NSP). U1 to U5 are temporary designations until the protein function has been determined. A + (yes) or – (no) indicates whether a protein has been described from the virus species or not. The former designation number of the encoding DNA is given in parantheses. § The underlined and bold letters indicate how the DNA component designation is derived and may help as a memory guide (after Vetten et al. 2004)

Objectives and scope of the study

Food legumes such as faba bean, chickpea and lentil are affected by yellowing and stunting diseases mostly caused by viruses (and nanoviruses) singly or in mixed infection. These diseases result in significant yield losses in countries like Ethiopia, Sudan, Egypt and Morocco where these crops are economically very important. However, the knowledge on the exact identity and genetic diversity of the viruses is limited since virus identification has been based mostly on mere serological diagnosis of field samples with no information on other virus properties. In most previous serological surveys, a significant portion of samples showing typical virus-like symptoms gave no serological reaction with the available antibodies and thus the causal agents remained unidentified (e.g. Abraham et al. 2000, Fortass and Bos, 1991, Makkouk et al. 2003b, Tadesse et al. 1999). This was due to the fact that some of them belonged to yet unrecognized viruses for which specific antibodies were not available. Although nucleotide sequence information is the most reliable means of identifying and charac-

terizing virus isolates, no such data is available in the database for any legume luteovirus species from countries of Northeast and North Africa and West Asia. In the case of nanoviruses for which reliable serological and sequence information is available, the number of samples used in previous studies is often too low and not representative in terms of distribution of virus distribution within a country or a region. Therefore, the main objectives of this work as presented in this dissertation were:

- To identify and characterize the major virus(es) associated with yellowing and stunting diseases of cool season food legumes at biological, serological and molecular level and to develop reliable diagnostic tools.
- To generate partial and if possible complete nucleotide sequence data for representative luteovirus and nanovirus isolates in order to assess their genetic diversity and taxonomic status.

The results of the different aspects of this study are presented in this dissertation as independent but related manuscripts (Chapters 2-7) followed by general discussion and conclusion (Chapter 8).

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CHAPTER 2

Characterization of Chickpea chlorotic stunt virus, a new luteovirus from Ethiopia [1]

A. D. Abraham, W. Menzel, D.-E. Lesemann, M. Varrelmann and H. J. Vetten

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ABSTRACT

A. D. Abraham, W. Menzel, D.-E. Lesemann, M. Varrelmann and H. J. Vetten. 2005. Characterization of Chickpea chlorotic stunt virus, a new luteovirus from Ethiopia. Phytopathology 95:

In attempts to identify the causal agent of yellowing and stunting symptoms in chickpea and faba bean plantings near Ambo, Ethiopia, serological analysis indicated the occurrence of an unknown or uncommon luteovirus. Therefore, degenerate primers were used for RT-PCR amplification of the viral coat protein (CP) encoding region from both chickpea and faba bean samples. Cloning and sequencing of the amplicons obtained yielded nearly identical (96%) nucleotide sequences of a previously unrecognized luteovirus. Its CP amino acid sequence was most closely related (identity of ~78%) to that of *Groundnut rosette assistor virus*. Its virion morphology and capsid protein sizes were also characteristic of a luteovirus. Of the four aphid species tested, only *Aphis craccivora* transmitted the virus in a persistent manner. The host range of the virus was confined to a few species of the Fabaceae. A rabbit antiserum raised against virion preparations cross-reacted unexpectedly with Beet western yellows virus-like viruses. This necessitated the production of murine monoclonal antibodies, which in combination with the FBV antiserum permitted both sensitive and specific detection of the virus in field samples by triple antibody sandwich ELISA. Because of the characteristic field and greenhouse symptoms in chickpea, the name Chickpea chlorotic stunt virus (CpCSV) is proposed for this new luteovirus.

Additional keywords: aphid transmission, cross reaction, decoration titer, electron microscopy, host range, monoclonal antibodies, polerovirus, sequence analysis, *Turnip yellows virus*

[1] This paper was submitted for publication in *Phytopathology* on March 25, 2005.

INTRODUCTION

With production in 2003 estimated to be over 1 million metric tons produced on nearly 1.2 million hectares (15), Ethiopia is a primary producer of food legumes, the majority (87%) of which consists of cool-season food legumes, such as chickpea, faba bean and lentil. Virus diseases are among the important biotic constraints for the production of these crops. About ten viruses have been identified by serological means from these crops in the country (1,2,50). While *Pea seed-borne mosaic virus* chiefly infects lentil, luteoviruses such as *Soybean dwarf virus* (SbDV) and *Beet western yellows virus* (BWYV) appear to be very common in both chickpea and lentil (1,50). In faba bean, the nanovirus *Faba bean necrotic yellows virus* (FBNYV) and, in particular, a number of unidentified luteoviruses seem to be widespread in Ethiopia (2). These luteoviruses appear to be the predominant cause of yellowing and stunting diseases of cool-season food legume crops in Ethiopia. This also includes chickpea stunt which is considered the second most important chickpea disease after fungal wilt and root rot disease (26).

The family *Luteoviridae* currently consists of 15 virus species in three genera, *Luteovirus*, *Polerovirus*, and *Enamovirus*, and there are 11 unassigned members in the family (11). These viruses are genetically diverse but collectively share several features clearly distinct from other plant viruses. These features comprise phloem restriction, transmission by aphids in a persistent circulative manner, lack of mechanical transmission, a high degree of vector specificity, host range restriction often to a single plant family, and serological relationships between most members of the family (39). For simplicity, we refer to all viruses of this family here as luteoviruses.

Five luteoviruses have so far been reported to infect faba bean and/or chickpea in different parts of the world, namely *Bean leaf roll virus* (BLRV) (6), BWYV (8,18), SbDV (34,52), *Pea enation mosaic virus-1* (PEMV-1) (13), and *Chickpea stunt disease-associated virus* (CpSDaV), which has been reported from India and is closely related to, but distinct from, BWYV (43,46). It should be noted that virus isolates previously referred to as BWYV have been recently reclassified as four distinct virus species namely BWYV, *Beet chlorosis virus*, *Beet mild yellowing virus* (BMYV) and *Turnip yellows virus* (TuYV) on the basis of differences in host range and ORF0 sequences (12,22,23). However, these viruses, which are referred to here as BWYV subgroup, are serologically closely related and cannot be distinguished readily by serological means (23,48,49). The only available sequence information (ORF0 and ORF3) for a legume (faba bean) isolate of the BWYV subgroup (Acc. no.

AF167478) indicates that it is more closely related to TuYV than to the other species of the BWYV subgroup (23). Although not known to infect faba bean and chickpea, two other luteoviruses reported from legume crops are *Indonesian soybean dwarf viru*s (ISDV) from soybean (27) and *Groundnut rosette assistor virus* (GRAV) from groundnut (25,42).

Despite the high prevalence of luteoviruses in faba bean and chickpea in countries like Ethiopia and Morocco, often their accurate identification was not possible mainly due to the lack of specific antibodies or molecular detection tools. For the same reason some authors referred to the causal viruses only as a group (e.g. 2,16,17,36). Consequently, the exact identity of some of the luteovirus(es) associated with legumes in the region has remained unknown.

In this work, a previously unrecognized luteovirus was isolated from symptomatic chickpea and faba bean plants in Ethiopia in 2002 and characterized in greater detail. We determined some of its biological, serological and molecular properties and describe the production of poly- and monoclonal antibodies that contributed to the serological characterization and permitted routine detection of this new luteovirus species. Since field observation and greenhouse studies suggest that this virus causes most pronounced symptoms in chickpea, we propose the name Chickpea chlorotic stunt virus (CpCSV).

MATERIALS AND METHODS

Origin, initial serological analysis and maintenance of virus isolates. Faba bean and chickpea plants showing yellowing and stunting symptoms were collected from fields near Ambo, central Ethiopia. All samples were serologically analyzed in double (DAS) and triple antibody sandwich (TAS) ELISA as described by Clark and Adams (10) and Franz *et al*. (19), respectively, using high-binding polystyrene plates (Greiner Bio-One GmbH, Germany) and the buffers described by Clark and Adams (10). In initial tests, samples were tested for the presence of luteoviruses, nanoviruses, and the mastrevirus CpCDV using the antibodies listed in Tables 1 and 2. For detection of the possible occurrence of known luteoviruses in the field samples from Ambo, specific antisera to PEMV-1 and SbDV were used in DAS-ELISA whereas TAS-ELISA detection of BLRV and viruses of the BWYV subgroup was attempted using the monoclonal antibody (Mab) B-4-6G4 and the anti-TuYV MAbs T-4D3, -1E1 and -2G5, respectively (Tables 1 and 2).

Table 1. Antisera used for immunoelectron microscopy (IEM), DAS- and/or TAS-ELISA

Antiserum to	Source and/or reference	Antiserum used in		
		IEM	DAS-ELISA	TAS-ELISA [1]
Bean leaf roll virus (BLRV)	Stock of BBA (28)	yes	no	as trapping antibody (Mab B-4-6G4) *
Beet mild yellowing virus (BMYV)	F. Rabenstein, Aschersleben, Germany	yes	no	no
an unidentified species of the Beet western yellows virus subgroup ('BWYV')	DSMZ AS-0049	yes	yes	as trapping antibody (Mabs B-2-5G4 and 510H)
Barley yellow dwarf virus-MAV (BYDV-MAV)	W.F. Rochow, Ithaca, NY, USA	yes	no	no
Barley yellow dwarf virus-PAV (BYDV-PAV)	Stock of BBA (H.L. Paul)	yes	no	no
Carrot red leaf virus (CtRLV)	Stock of BBA (H.J. Vetten)	yes	no	no
Chickpea chlorotic dwarf virus (CpCDV)	Stock of BBA (H.J. Vetten)	no	yes	no
Cereal yellow dwarf virus-RPV (CYDV-RPV)	Stock of BBA (W. Huth)	yes	no	no
Faba bean necrotic yellows virus (FBNYV)	Stock of BBA (28)	no	no	as trapping antibody (FBNYV Mab mix)
Groundnut rosette assistor virus (GRAV)	A.F. Murant, Dundee, UK	yes	no	no
Pea enation mosaic virus-1 (PEMV-1)	DSMZ AS-0017	yes	yes	no
Potato leaf roll virus (PLRV)	DSMZ AS-0741	yes	yes	no
Soybean dwarf virus (SbDV)	K.M. Makkouk, Aleppo, Syria	yes	yes	no
Turnip yellows virus (TuYV)	F. Rabenstein, Aschersleben, Germany	yes	yes	as trapping antibody (Mabs T-1E1, T-2G5, and T-4E3)

* Mabs used as detecting antibodies in TAS-ELISA (see Table 2) are given in parentheses.

Table 2. Source and specificity of the monoclonal antibodies (Mab) used in TAS-ELISA

Mab designation	Specificity	Source and/or reference
Mab B-2-5G4	raised against BLRV but reacting with several luteoviruses	Stock of BBA (28)
Mab 510H	raised against a beet isolate of the BWYV subgroup (14); identical to ATCC PVAS-647	Dr. R.R. Martin, Corvallis, Or, USA
Mab B-4-6G4	raised against, and specific to, BLRV	Stock of BBA (28)
Mab T-1E1	raised against TuYV but reacting with most species of the BWYV subgroup	F. Rabenstein, Aschersleben, Germany
Mab T-2G5		
Mab T-4E3		
Mix of FBNYV Mabs 1-1F2, 2-1A1, and 3-4F2	raised against FBNYV but reacting with many nanovirus species	Stock of BBA (19)

To establish luteovirus isolates in faba bean seedlings under greenhouse conditions for further characterization, the aphid species *Aphis craccivora* Koch was used for vector transmission from one fresh sample each from faba bean and chickpea as described below. This was only successful with the faba bean sample which, for the purpose of this paper, is referred to as FBV. At intervals of about 6 weeks, FBV was maintained in faba bean by aphid transmission. BMYV, PLRV, and TuYV were maintained in beet, *Physalis floridana*, and oilseed rape, respectively, using *Myzus persicae* for vector transmission. Desiccated leaf tissues containing BLRV, CpCDV and SbDV were from the stock of BBA and served as controls for TAS- and/or DAS-ELISA experiments.

Total RNA extraction and reverse transcriptase (RT)-PCR. Total RNA was extracted from dried and/or fresh leaf tissues of infected faba bean and chickpea using Nucleospin®Plant kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. For RT-PCR amplification, a pair of degenerate primers was derived from conserved luteovirus coat protein (CP) gene sequences available from the database. The sense (5'-GCTCTAGAATTGTTAATGARTACGGTCG-3') and antisense (5'-CACGCGTCIACCTATTTIGGRTTITG-3'; I stands for inosine) primers include the start and stop codons, respectively, of the coat protein gene. A one-step RT-PCR was carried out in a 50

µl reaction volume containing 5 µl of 10x PCR buffer, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of 100 mM dNTPs, 1 µl of each primer (10 mM), 1 µl *Taq* polymerase (Gibco, 5 U/µl) and 0.05 µl AMV reverse transcriptase (Promega, 300 U/µl) at a primer annealing temperature of 55 °C.

Cloning and sequence analysis. PCR products were purified from a 1% agarose gel using the Nucleospin® Extract kit (Macherey-Nagel, Düren, Germany), ligated into a pGEM®-T vector (Promega) with T4 DNA ligase and transformed by heat shock into DH5α® competent *E. coli* cells essentially following the Promega protocol derived from Sambrook et al. (47). Extraction of plasmid DNA was done using the Nucleospin® plasmid kits (Macherey-Nagel, Düren, Germany). DNA sequencing was carried out by a commercial company (MWG Biotech, Ebersberg, Germany). Sequence assembly, multiple alignment and identity analyses of nucleotide and amino acid sequences were carried out using the computer software DNAMAN (Lynnon Biosoft, Canada). The sequences were compared with available sequences in the databases using the basic local alignment search tool (BLAST) program (5). Phylogenetic trees were reconstructed by the aid of the ClustalX program (54) after multiple alignment of sequences and using neighbor joining algorithms and visualized using the Treeview program (45).

Vector transmission and host range studies. For vector transmission studies of FBV, colonies of four common legume aphid species, namely *Aphis craccivora, A. fabae* Scopoli and *Acyrthosiphon pisum* Harris each reared on faba bean and *Myzus persicae* (Sulzer) maintained on radish, were used. Several hundred individuals of each species at different larval stages were given an acquisition access feeding period of 48 h on FBV-infected faba bean plants, followed by an inoculation access feeding period of 48 h on young faba bean seedlings using about 30 individuals per plant. After spraying with an insecticide, the plants were kept in an insect-proof glasshouse and observed for symptom development. All inoculated plants were tested for virus infection by TAS-ELISA using Mab B-2-5G4 as described above. A similar experimental approach was used for determining the host range of FBV. However, only *A. craccivora* was used for inoculating about 10 plants of each legume and non-legume species. Both vector transmission and host range experiments were repeated three times.

Virus purification. FBV was propagated in a local faba bean cv. following inoculation of 1-week-old seedlings with apterous *A. craccivora* that had been given an acquisition access feeding of 48 h on infected plants. Since FBV induced mild symptoms in faba bean under greenhouse conditions, all plants were serologically analysed using Mab B-2-5G4 in TAS-ELISA at 4-6 weeks after inoculation. The above-ground parts of all ELISA-positive plants were harvested, ground to a fine powder in liquid nitrogen using a Waring Blendor, and kept frozen at ─20 or

–80 °C until use. The amount of faba bean tissue typically used for virion purification was 300-500 g. The purification protocol used was essentially based on the method of Takanami and Kubo (51) but omitted the use of macerating enzymes for virus extraction. The procedure comprised clarification of the initial homogenate by chloroform and butanol, concentration of virions by precipitation with polyethylene glycol 6000, and further purification by differential centrifugation and sucrose gradient centrifugation. A Zeiss EM906 electron microscope was used at a magnification of ca. 36,000x to examine the purity and to determine concentration of virion preparations. Concentrations of FBV virions were also estimated by spectrophotometry assuming an extinction coefficient of $A_{260\,nm}^{0.1\%}$ =8.6 [based on the value calculated for luteoviruses (51)].

Production and characterization of poly- and monoclonal antibodies. A rabbit was given three intramuscular injections each of c. 50 µg of FBV virions at two-week intervals. For the first injection, the virion preparation was emulsified with an equal volume of Freund's complete adjuvant (Sigma), whereas Freund's incomplete adjuvant was used for the 2nd and 3rd injections. Starting 10 days after the last injection, the rabbit was bled biweekly from the lateral ear vein. IgG isolation, labelling of IgG with alkaline phosphatase (Roche, Germany), and DAS-ELISA were conducted as described (10).

For Mab production, each of three female Balb/c mice (2-3 month old) received a subcutaneous injection of 100 µl containing c. 20 µg of FBV virions (and 50% Freund's incomplete adjuvant). Booster injections, fusion experiments, selection of hybridomas and cloning were essentially conducted as described (3,19). However, IgG to FBV were used as coating antibody in TAS-ELISA for the trapping of antigen from FBV-infected and non-inoculated faba bean. Isotyping of Mabs was done using the Hbt Mouse Monoclonal Antibody Isotyping kit (HyCult Biotechnology, the Netherlands) essentially following the manufacturer's instructions. Serial dilutions of culture supernatants ranging from 1:10 to 1:256,000 were tested in TAS-ELISA for determining Mab titers.

I**mmuno-electron microscopy**. Immunosorbent electron microscopy (ISEM) and immuno-electron microscopy (IEM) decoration titer experiments with purified FBV virions or extracts from luteovirus-infected leaves were done as described (40,41). To reveal weak decoration reactions of Mabs with FBV virions, colloidal gold (∅ 5 nm) coated with rabbit anti-mouse IgG (British Biocell International) was used essentially as described (3).

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. The coat protein size of FBV virions was determined by SDS-PAGE analysis of a purified virion preparation using a 4% stacking gel on a 12% resolving gel and the buffer system of Laemmli and Favre (30).

Proteins were stained with 0.2% (wt/vol) Coomassie brilliant blue. Purified virions as well as extracts from FBV-infected and non-infected faba bean plants were used for testing the ability of each Mab to react with FBV antigen in Western blots (55).

RESULTS

First serological indication for an unusual luteovirus. When symptomatic chickpea and faba bean samples from Ambo, Ethiopia, were serologically analyzed, many of them reacted only with Mab B-2-5G4, a broad-spectrum luteovirus-specific monoclonal antibody but not with poly- and monoclonal antibodies to CpCDV and FBNYV in DAS- and TAS-ELISA, respectively. In attempts to identify the luteovirus, specific antibodies to BLRV, PEMV, SbDV and viruses of the BWYV subgroup also failed to react with the samples (data not shown).

Table 3. Percent identities resulting from pairwise comparisons of the deduced coat protein aa sequence of CpCSV with that of other luteoviruses

Virus	Accession number	Identity (%)
Groundnut rosette assistor virus (GRAV)	AF195828	77.8
Cucurbit aphid-borne yellows virus (CABYV)	NC_003688	71.7
Turnip yellows virus (TuYV)	X13063	71.0
Beet western yellows virus (BWYV)	NC_004756	68.5
Chickpea stunt disease-associated virus (CpSDaV)	Y11530	66.5
Potato leaf roll virus (PLRV)	NC_001747	65.1
Cereal yellow dwarf virus-RPV (CYDV-RPV)	NC_004751	59.8
Bean leaf roll virus (BLRV)	NC_003369	59.0
Soybean dwarf virus (SbDV)	NC_003056	56.8
Barley yellow dwarf virus-PAV (BYDV-PAV)	AJ007929	43.7
Sugarcane yellow leaf virus (ScYLV)	NC_000874	41.8

Molecular evidence for a new luteovirus. Since our serological data suggested the possible occurrence of an unknown or uncommon member of the family *Luteoviridae*, the degenerate primers were used for RT-PCR amplification of the CP sequence of this virus. RT-PCR yielded an amplicon of the expected size (~600 bp) from several ELISA (B-2-5G4)-positive samples. Cloning and sequencing of the amplicon from a chickpea and a faba bean sample indicated that the two isolates share CP nucleotide sequence identities of 96%. Also, the alignment of the deduced CP aa sequences of the two isolates revealed an identity of 97.5%, indicating they are isolates of the same virus species that differ only in three of the 200 aa residues that form the CP of this virus (Fig. 1). BLASTP search using the deduced aa sequence revealed striking similarities with luteovirus CP sequences. Based on CP sequences this new virus appeared to be most closely related (78%) to GRAV (Fig. 1) followed by *Cucurbit aphid-borne yellows virus*

(CABYV) (72%) and TuYV (71%) (Table 3). Both pairwise comparisons of CP sequences (Table 3) and phylogenetic analysis of the CP aa sequences (Fig. 2) provided strong evidence that the new virus represents a distinct member of the family *Luteoviridae*.

```
Chickpea     MNTVVVRNNGRRRRNRRTVQRARRRNPVVVVEAPRQSQRGRRRRRNRRRASGRSTAGRRGSSETFVFS
Faba bean    ------------------------------------P-------------------------------
GRAV         -------RP-NG-A---RNR-TP--------QT---PNS----------SN.-GSRN-G--G------

Chickpea     KDNLAGSSSGSITFGPSLSDCPAFSSGILRAYHEYKITMVKLEFISEAASTSSGSIAYELDPHCKSTS
Faba bean    ------------------------------------S-------------------------------
GRAV         ----T-----------------------K-------S---V--V---S----------------S-

Chickpea     LGSYINKFGITSNGQRTFAARLINGIEWHSSDEDQFRILYKGNGGSAIAGSFRVTIKCQTQNPK  200
Faba bean    ----------------------------------------------------I----------  200
GRAV         -Q--V------R----SWMG-Y---V---DAT-----F------S--------F-----V----  199
```

Figure 1. Alignment of the coat protein aa sequences of the chickpea and faba bean isolates of CpCSV with that of its closest relative, *Groundnut rosette assistor virus* (GRAV; AF195828) using the standard run parameters of DNAMAN (Lynnon Biosoft). Amino acid residues identical to those of the chickpea isolate are indicated by a dash. A single dot denotes a gap. The nucleotide sequences reported here are available under the accession numbers AY956384 and AY956385.

Vector transmission and specificity. In three transmission experiments, only plants that were inoculated using *Aphis craccivora* that had been given prior access to FBV-infected faba bean plants developed virus symptoms 2-3 weeks after inoculation and reacted with Mab B-2-5G4. None of the plants inoculated with *Acyrthosiphon pisum*, *Aphis fabae* or *Myzus persicae* fed on FBV-infected plants developed symptoms or became ELISA-positive. This indicated that, of the four aphid species tested, only *A. craccivora* is a vector of the new luteovirus.

Experimental host range. Of the 21 different plant species inoculated with the isolate FBV in host range studies using *A. craccivora* as vector, only four legume species, *Vicia faba* L., *Cicer arietinum* L., *Lens culinaris* Medik. and *Pisum sativum* L. were infected. Despite repeated inoculation attempts (three independent experiments), the virus was not detected in all other legume species tested, such as *Arachis hypogaea* L., *Glycine max* (L.) Merr., *Medicago sativa* L., *Phaseolus lunatus* L., *P. radiata* L., *P. vulgaris* L., *Vigna subterranea* (L.) Verdc, *and V. unguiculata* (L.) Walp, suggesting that they are not susceptible. All the non-legume species tested, namely *Beta vulgaris* L., *Coriandrum sativum* L., *Chenopodium amaranticolor* Coste & Reyn, *C. quinoa* Willd, *Lactuca sativa* L., *Nicotiana benthamiana* Domin., *N. clevelandii* Gray, *Physalis floridana* Rydb., and *Raphanus sativus* L. var. *niger* (Mill.) S. Kerner were not infected.

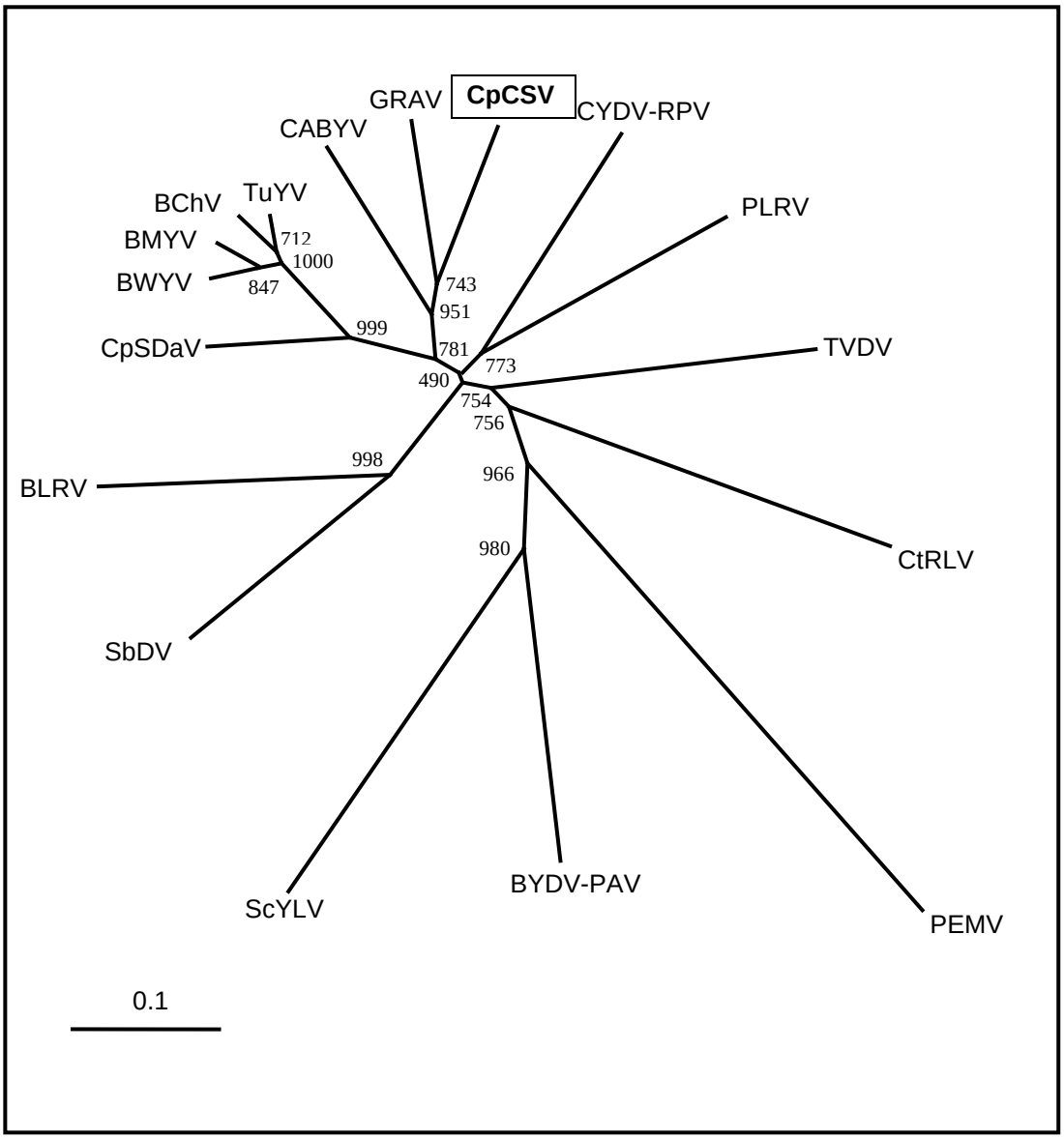

Fig. 2. Unrooted tree showing the phylogenetic relationships between the predicted aa sequences of the coat protein gene of CpCSV and that of other luteoviruses [*Beet chlorosis virus* (BChV; NC_002766), *Beet mild yellowing virus* (BMYV; X83110), *Carrot red leaf virus* (CtRLV; NC_006265), *Pea enation mosaic virus* (PEMV; L4573), and *Tobacco vein distorting virus* (TVDV; AJ704819); for abbreviations and accession numbers of the other luteoviruses see Table 3]. The sequences were aligned and neighbor-joining trees were constructed using the CLUSTAL X program. Boot strap values were calculated from 1000 replicates and are indicated at each node. The scale bar indicates 0.1% substitution per aa site.

Symptomatology. Symptoms in faba bean and chickpea plants included a slight stunting with yellowing and veinal chlorosis most pronounced at the margins of older leaves. Leaves produced after the establishment of systemic infection exhibited a reduction in leaf size and shape but only

slight yellowing or leathery texture. In general, symptoms in chickpea and, particularly, in faba bean were less severe under glasshouse conditions than those observed in the original plants under field conditions in Ethiopia probably due to difference in environmental conditions or plant cultivar. Infected lentil plants showed a striking yellowing, reddening and stunting and had smaller and distorted leaves. In pea plants infections appeared to be latent.

Morphology and physicochemical properties of purified virions. Purified FBV virions sedimented as a single component in sucrose density gradients when gradient fractions were analyzed using an absorbance monitor (ISCO model UA-5, ISCO, Lincoln, Nebraska, USA). Electron microscopy revealed numerous isometric particles measuring about 28 nm and having a smooth surface and a slightly hexagonal outline (Fig. 3). The yield of purified virions from infected faba bean plants ranged from 150-250 µg per kg tissue. SDS-PAGE analysis of purified virions gave a strong and a faint protein band, with Mr values of c. 20 x 10^3 and c. 50 x 10^3, corresponding to the major CP and the presumably proteolytically degraded (20) read-through protein, respectively.

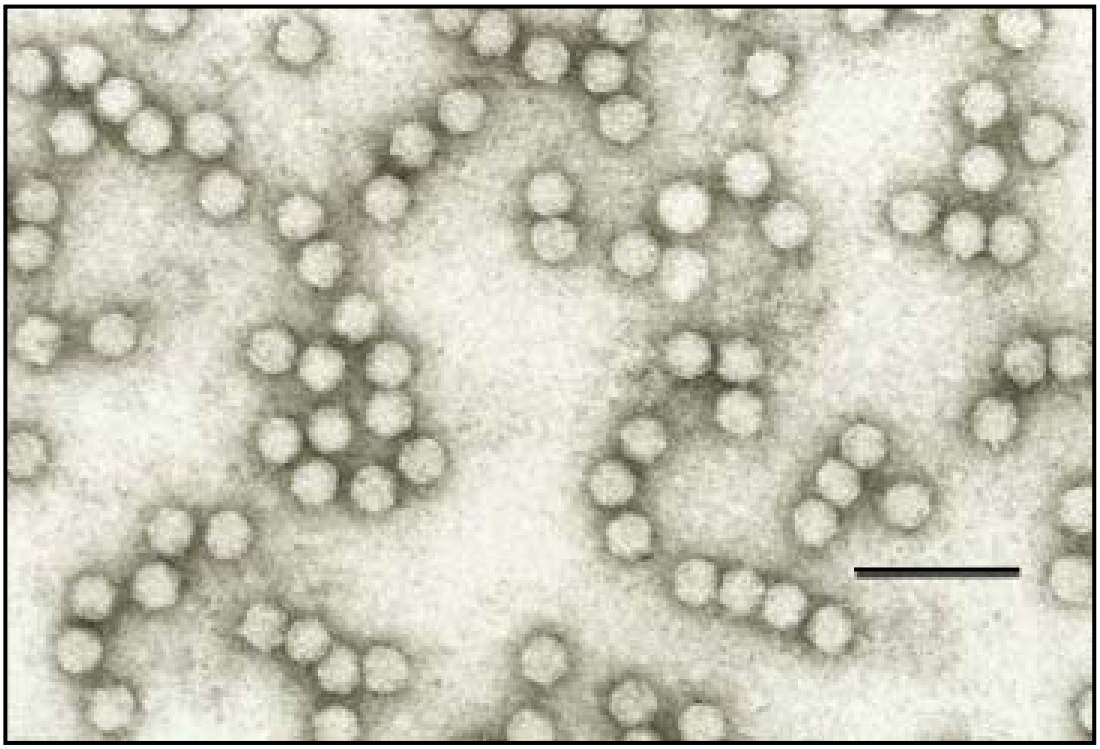

Fig. 3. Electron micrograph of a purified preparation of FBV virions showing isometric particles measuring 28 nm in diameter. Bar represents 100 nm

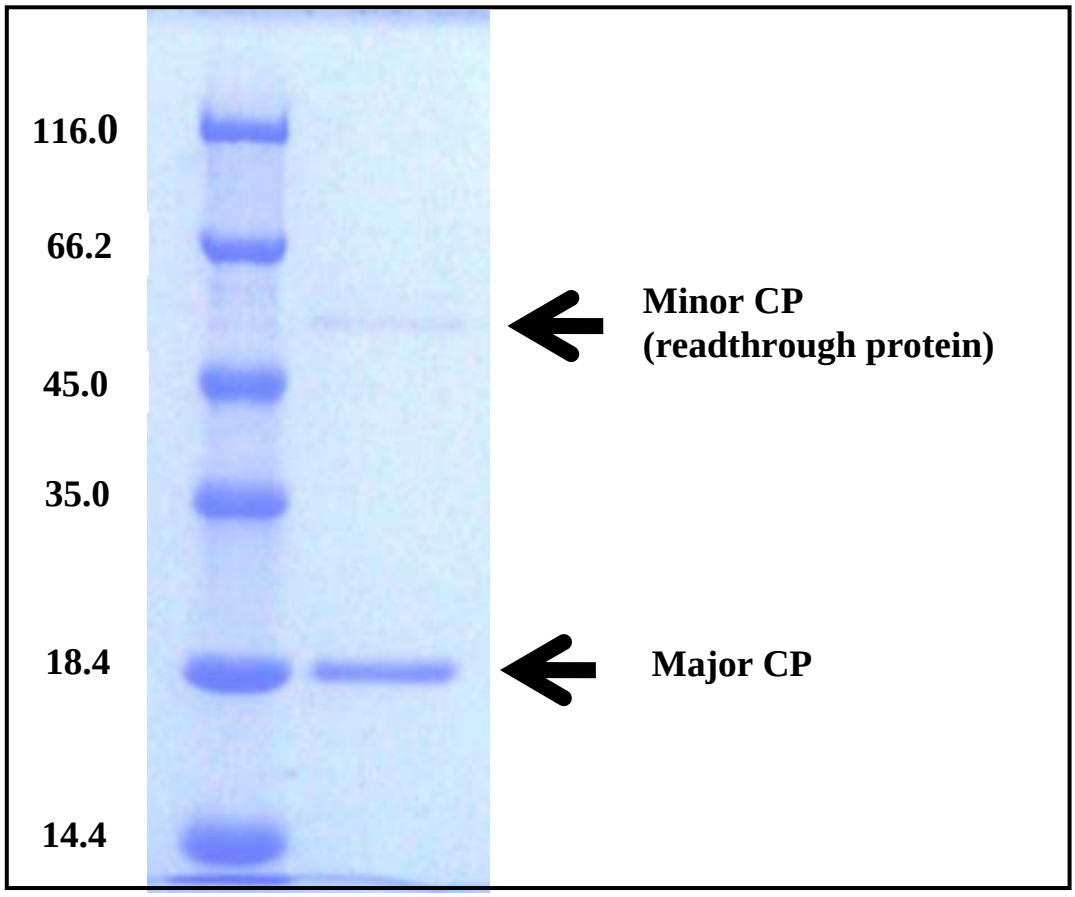

Fig. 4. Sizes of the two structural proteins of FBV. A purified virion preparation of FBV was analysed by 12 % SDS PAGE (right lane), yielding a major band of c. 20 and a minor band of c. 50 kDa. The migration of the marker proteins with their molecular masses (in kDa) is shown in the left lane.

Serological relationships. When the antiserum to isolate FBV was used in DAS-ELISA, strong homologous reactions were observed using trapping IgG and enzyme-labeled antibodies at concentrations of 1 µg/ml and 1:1000, respectively. Under these conditions no DAS-ELISA reactions were observed with extracts from BLRV-, PEMV-, PLRV-, and SbDV-infected plants. However, extracts from BMYV- and TuYV-infected plants reacted weakly with FBV antiserum (Table 4). In reciprocal DAS-ELISA experiments, antisera to 'BWYV' and TuYV gave weak and intermediate reactions, respectively, with FBV, whereas antisera to BLRV, PEMV, SbDV, and PLRV produced strong homologous reactions but failed to react with FBV and in any other heterologous combinations (Tables 1 and 4). Similarly in TAS-ELISA, the Mabs T-1E1 -2G5, and -4D3 raised against TuYV reacted only with TuYV and BMYV, but not with FBV and any of the other luteoviruses tested. In contrast, Mab 510H raised against an isolate of the BWYV sub-group gave not only very strong reactions with BMYV and TuYV but also an intermediate reaction with FBV (Tables 1, 2, and 4).

Table 4. Homologous and heterologous DAS-ELISA reactions of antisera to FBV and some other luteoviruses as well as TAS-ELISA reactions of selected monoclonal antibodies with the luteoviruses for which antisera were available

Virus species	DAS-ELISA with polyclonal IgG to							TAS-ELISA with monoclonal antibody **						
	FBV	'BWYV'	TuYV	BLRV	PEMV	SbDV	PLRV	510H	T-1E1	T-2G5	T-4D3	1-1G5	1-3H4	1-4B12
FBV	+++ *	+	++	0	0	0	0	++	0	0	0	+++	+++	+++
BMYV	+	++	++	0	0	0	0	+++	+	++	+++	0	0	0
TuYV	+	+++	+++	0	0	0	0	+++	+++	+++	+++	0	0	0
BLRV	0	0	0	+++	0	0	0	0	0	0	0	0	0	0
PEMV	0	0	0	0	+++	0	0	0	0	0	0	0	0	0
SbDV	0	0	0	0	0	+++	0	0	0	0	0	0	0	0
PLRV	0	0	0	0	0	0	+++	0	0	0	0	0	0	0

* Extinction values ($A_{405\ nm}$) for 10-fold diluted leaf extracts and following a substrate incubation period of 1 h are shown. $A_{405\ nm}$ values were classed as +++ (> 1), ++ (0.2 to 1.0), + (< 0.2), and 0 (less than two times the $A_{405\ nm}$ value for the non-infected control).

** Note that IgG to 'BWYV' served as capture antibody for Mab 510H, TuYV IgG was used for trapping of luteovirus antigen for the Mabs T-1E1, -2G5, and -4D3, and IgG to FBV were used as coating antibodies for the Mabs 1-1G5, -3H4, and -4B12.

Table 5. 'Homologous' and heterologous decoration titers observed in immunoelectron microscopy of FBV and TuYV virions following incubation with 1:50 to 1:12,800 dilutions of the antisera to the isolate FBV, 'BWYV', BMYV and TuYV

Virions of *	Antiserum to			
	FBV	'BWYV'	BMYV	TuYV
FBV	**1:6,400 ****	1:800	1:800	1:3,200
TuYV	1:1,600	1:6,400	1:3,200	**1:6,400**

* A mixture of the 'BWYV' and FBV antisera was used for overnight trapping (ISEM) of virions from FBV- and TuYV-infected faba bean and oilseed rape plants, respectively.

** Highest dilution of antiserum that gave a visible decoration for the indicated combinations. Homologous combinations are in bold.

To substantiate the ELISA data, IEM experiments were conducted. The FBV antiserum strongly decorated FBV particles in a purified virion preparation of FBV. When assessing the serological relationship of FBV to other luteoviruses in IEM, purified FBV virions were not decorated when antisera to *Barley yellow dwarf virus* (BYDV)-*MAV*, BYDV-PAV, BLRV, *Carrot red leaf virus*, PEMV, and SbDV were used at 1:50 dilutions. Whereas antisera to *Cereal yellow dwarf virus-RPV*, GRAV, and *Potato leaf roll virus* only decorated FBV virions slightly, a strong decoration was observed with the antiserum to 'BWYV', BMYV, and TuYV (data not shown). To study the apparent close serological relationship between FBV and virus species of the BWYV sub-group, antisera to FBV, 'BWYV', BMYV, and TuYV were used for IEM decoration titer experiments in various combinations. Following overnight trapping (ISEM) of virions from FBV- and TuYV-infected faba bean and oilseed rape plants, respectively, all antisera had similar 'homologous' titers (1:3,200 or 1:6,400). However, the 'homologous' and heterologous titers of the antisera tested differed by only 1-3 dilution steps (Table 5).

Properties of monoclonal antibodies. Since the FBV antiserum cross-reacted with virus species of the BWYV subgroup and thus appeared unsuitable for CpCSV identification, attempts were made to raise CpCSV-specific Mabs. From two fusion experiments, 10 stable hybridoma lines secreting antibodies specific to the isolate FBV were obtained. Five, four, and one of the Mabs were unequivocally typed as IgG1, IgG2a and IgG2b (Table 6), confirming the monoclonality of the antibodies. The Mabs gave intermediate to strong reactions with FBV (Table 6). To

confirm the FBV specificity of the Mabs, they were used for IEM decoration and Western blot experiments. However, a visible decoration reaction with purified FBV virions was not readily produced with any of the Mabs. When immunogold labeling was used for revealing weak decoration reactions of Mabs with FBV virions, only Mabs 1-3D5 and 2-3D4 were shown to bind to FBV virions (>50% of virions had 2-3 gold spheres). Immunogold labeling of the FBV particles with the other 8 Mabs either produced insignificant numbers (<25%) of gold-labeled particles or none at all (Table 6). In contrast to the polyclonal antibodies used as a control, none of the Mabs gave a Western blot reaction following SDS-PAGE analysis of purified FBV virions (results not shown).

Table 6. Properties of monoclonal antibodies (Mab) to the luteovirus isolate FBV

Mab code	IgG sub-type	Reciprocal titer of culture super-natant	TAS-ELISA reaction strength [1]	IEM decora-tion[2]	Western blot reaction[3]
1-1G7	IgG1	>256,000	+++	-	-
1-2H4	IgG1	>256,000	+++	-	-
1-3D5	IgG1	>256,000	+++	+	-
1-3H4	IgG2b	>256,000	+++	-	-
1-4B12	IgG2a	>256,000	+++	-	-
2-1B4	IgG2a	256,000	++	-	-
2-2C1	IgG2a	1,600	++	-	-
2-2C9	IgG1	800	++	-	-
2-3D4	IgG1	>256,000	++	+	-
2-6B10	IgG2a	32,000	++	-	-

[1] ELISA reactions were classed as indicated in Table 4.

[2] '+' and '-' indicate whether a significant immunogold labeling of FBV virions was observed or not, respectively.

[3] '-' indicates that no Western blot reaction was obtained following SDS-PAGE analysis of purified FBV virions.

There were striking differences in the titers of the Mab-containing culture supernatants (Table 6). For routine use in TAS-ELISA, culture supernatant dilutions of 1:50 to 1:250 were found to give the best signal to noise ratio. All the Mabs reacted only with the homologous antigen but not with any of the taxonomically related viruses namely BLRV, BMYV, PEMV, PLRV, SbDV, and TuYV in TAS-ELISA. When the 10 Mabs were individually tested for their ability to react with CPCSV in legume samples from various production areas of central Ethiopia, CpCSV was detected not only in 22 faba bean, 24 chickpea, and 2 lentil samples but also in 2 grasspea (*Lathyrus sativum* L.) and 2 fenugreek (*Trigonella foenum-graecum* L.) samples by each of the 10

Mabs. Of the 10 Mabs, 1-1G5, 1-3H4 and 1-4B12 which gave stronger reactions consistently in various tests were selected for routine use as detecting antibodies in TAS-ELISA (Table 4). Use of the polyclonal IgG to isolate FBV as capture antibody and the Mabs as detecting antibodies permitted more sensitive detection of FBV antigen in TAS-ELISA than the use of the polyclonal antibodies alone in DAS-ELISA (data not shown).

DISCUSSION

Virus surveys of legume crops in several countries of West Asia and North Africa (WANA) have provided serological evidence for the occurrence of a range of viruses (7,33,38). Whereas some of these viruses could be readily identified, the identity of others often remained obscure due to the lack of specific diagnostic tools (2,16,17,24,36,37). This problem has been particularly critical in studies on the etiology of the yellowing and stunting diseases of legume crops, which are generally thought to be caused by phloem-restricted viruses, such as mastre-, nano- and luteoviruses, in WANA countries, including Ethiopia (38).

In attempts to identify the causal agent(s) of yellowing and stunting symptoms in faba bean and chickpea plantings near Ambo, Ethiopia, samples were collected and analyzed by serological means. The use of the 'BWYV' antiserum as capture antibody and the broad-spectrum luteovirus-specific Mab B-2-5G4 as detecting antibody was a serendipitous combination, as only this combination gave strong TAS-ELISA reaction with the unknown luteovirus and, thus, provided serological evidence for a luteovirus infection in the samples studied. However, the use of specific antibodies to some luteoviruses in ELISA gave no indications for the presence of BLRV, PEMV, SbDV, and BWYV-like viruses in these samples, although these viruses (especially the latter) have been reported to occur frequently in legume crops in WANA countries (38).

Cloning and sequencing of RT-PCR products from Mab B-2-5G4-positive chickpea and faba bean samples led to the determination of the CP gene sequence of a hitherto unrecognized luteovirus. Fig. 2 shows that this virus differs clearly in CP aa sequences not only from GRAV, its closest relative, but also from CpSDaV and other legume-infecting luteoviruses. Based on the current criteria for species demarcation in the family *Luteoviridae* (11), this virus can be considered a distinct luteovirus species as it differs from other luteoviruses by more than 10% in its CP aa sequence and possesses distinctive serological and biological properties. Not only the CP sequence data presented here, but also the available sequence information for the 5' part of the CpCSV genome (our unpublished data, Chapter 4) suggests that it is a member of the genus *Polerovirus*. Together with the biological, physicochemical and serological properties deter-

mined here for the FBV isolate, we provide several lines of evidence for the occurrence of a new luteovirus infecting cool-season food legumes in Ethiopia. We proposed the name CpCSV for this virus because of the striking field symptoms of Mab B-2-5G4-positive chickpea plants from which CpCSV CP sequences were obtained and because of the characteristic symptoms induced by FBV in chickpea under glasshouse conditions.

Table 7. Comparison of major host plants and principal vector species of FBV and other dicot-infecting luteoviruses

Host/vector	Luteoviruses primarily infecting legume crops					Luteoviruses primarily infecting non-legume crops		
	FBV	BLRV[1]	CpSDaV[2]	GRAV[3]	SbDV[1]	BWYV subgroup[1,5]	CABYV[4]	PLRV[1]
Host species								
Arachis hypogaea	–	+	+	+	–	+	–	–
Cicer arietinum	+	+	+	–	+	+	–	–
Cucumis melo	·[6]	–	–	·	–	–	+	–
Glycine max	–	–	–	–	+	+	–	–
Lactuca sativa	–	–	·	·	–	+	+	·
Lens culinaris	+	+	+	–	+	+	–	–
Phaseolus vulgaris	–	+	–	–	+	–	–	–
Pisum sativum	+	+	+	+	+	–	–	+
Solanum tuberosum	·	–	–	–	–	–	–	+
Vicia faba	+	+	+	–	+	+	–	+
Vigna unguiculata	–	+	–	–	–	+	–	–
Vector species								
Acyrthosiphon pisum	–	+	–	–	+	+	–	–
Aphis craccivora	+	–	+	+	–	+	–	–
A. fabae	–	–	–	–	–	–	–	–
Myzus persicae	–	+	–	–	–	+	+	+

[1] Data collated after Brunt et al. (9); [2] data of Reddy and Kumar (46); [3] data of Hull and Adams, (25); [4] data from Lecoq et al. (31); [5] data for the individual species of the BWYV subgroup are not available. [6] no data available (·)

Our attempts to biologically characterize FBV concentrated on identifying the aphid vector(s) of FBV and on determining its experimental and natural host range. Table 7 lists some biological properties of FBV and compares them with those of GRAV and CABYV, its closest relatives, and some legume-and non-legume infecting luteoviruses. Of the four aphid species tested, only *A. craccivora* transmitted FBV, supporting the notion that luteoviruses have a high level of vector specificity (20,56). Since *A. craccivora* has been reported frequently from legume crops in Ethiopia (4), this aphid species also appears capable of acting as efficient vector of CpCSV un-

der field conditions**.** The only other luteoviruses primarily vectored by *A. craccivora* are GRAV and CpSDaV, which have been reported from sub-Saharan Africa (42) and India (43,46), respectively. In addition to sharing the same vector species and being most closely related to each other in CP aa sequences, GRAV and CpCSV may also have overlapping geographical distributions in Africa. Naidu et al. (42) suggested that the groundnut rosette disease agents including GRAV have evolved with other plants in Africa and later infected groundnut when it was introduced in the 16th century. Therefore, it would be conceivable that the two viruses have a common ancestral virus in Africa from which they have evolved to adapt to the different legume hosts. *A. craccivora* which is thought to have originated in the Mediterranean basin (21) may have played an important role in the evolution of both GRAV and CpCSV.

Since only four of the 12 legume species and none of the nine non-legume species tested became infected with FBV under experimental conditions, this virus appears to have a narrow host range confined to the Fabaceae. Our serological analysis of field samples using CpCSV-specific Mabs not only confirmed that chickpea and faba bean are natural hosts of CpCSV but also indicated that lentil, grasspea and fenugreek, three further important legume crops in Ethiopia (53), are also naturally infected with CpCSV. The latter observation was corroborated by sequence analysis of RT-PCR products obtained from lentil, grasspea and fenugreek samples (our unpublished data; Chapter 4). With the exception of groundnut, the natural and experimental host range of FBV was indistinguishable from that of CpSDaV, with which FBV also shares the same aphid vector species (Table 7). Unlike GRAV whose only known natural host is groundnut (42), FBV did not infect groundnut but only a limited number of cool-season food legumes which, with the exception of *Pisum sativum*, are not hosts of GRAV (25) (Table 7). In contrast, CABYV, the other close CpCSV relative from the Mediterranean basin, is vectored primarily by *M. persicae* and predominantly infects cucurbits (Table 7) (31).

Since the differences in CP aa sequences between CpCSV and other luteoviruses range from 22 to 58% (Table 3), it was not surprising that nearly all antisera to other luteoviruses did not decorate or only weakly decorated FBV virions in IEM and the majority of the mono- and polyclonal antibodies raised against other luteoviruses did not cross-react with FBV in ELISA. On the other hand, the obvious cross-reactions between FBV and viruses of the BWYV subgroup in both DAS-ELISA and IEM decoration experiments were most striking (Table 4 and 5). These observations were confirmed by TAS-ELISA reactions of Mab 510H with FBV and with other CpCSV isolates (our unpublished observation; Chapter 3). The reaction pattern of Mab 510H was similar to that of the antisera to viruses of the BWYV subgroup, suggesting that the latter share at least one immunodominant epitope with CpCSV. In this respect, Mab 510H appears to

resemble Mab PAV-IL1 raised against BYDV-PAV and used for the discrimination of BWYV-like viruses (12,23,48,49). Our observation that Mab 510H cross-reacts with CpCSV may have important implications as this Mab has been commercially available as PVAS-647 from ATCC and often used for the serological identification of BWYV in legume crops in several countries, such as Ethiopia (50), Iran (37), Iraq (35), Pakistan (32), Sudan (36), Syria (29) and Tunisia (44). Ellis & Wieczorek (14) had already shown that Mab 510H reacts not only with BWYV subgroup viruses but also with CYDV-RPV (formerly referred to as BYDV-RPV and considered a BWYV strain). Our studies confirm that Mab PVAS-647 is not specific for BWYV. Hence, virus isolates identified in previous reports as BWYV based on their reaction with PVAS-647 could in fact be CpCSV or a closely related virus, but not necessarily a virus of the BWYV subgroup.

Apart from the aforementioned unconfirmed reports on the serological identification of BWYV in legume crops, there is, however, molecular evidence for the occurrence of BWYV subgroup viruses in legumes in Morocco (18), India (43) and Europe (23). Because of the cross-reactivity of the FBV antiserum with viruses of the BWYV subgroup (Table 4) a more specific serological tool for routine detection of CpCSV in field samples was required. Therefore, Mabs were raised against FBV virions and shown to react only with CpCSV. In addition, each of the ten Mabs reacted with all the CpCSV-infected samples from chickpea, faba bean, lentil, grasspea, and fenugreek, indicating that the epitopes recognized by the available Mabs are conserved among Ethiopian isolates of CpCSV. Since the poly- and monoclonal antibodies produced here permitted both sensitive and specific TAS-ELISA detection of CpCSV, they appear to be useful for CpCSV detection and its identification in legume crops in Ethiopia. Our observations that all Mabs failed to give a Western blot reaction with FBV CP and only two of the 10 Mabs weakly decorated FBV particles, suggest that all Mabs recognize conformation-sensitive epitopes that appear to be predominantly internal. Moreover, we do not know as to whether they react with the major or minor (readthrough) CP. While the majority of Mabs to luteoviruses have apparently not been tested for their capability of producing Western-blot reactions with luteovirus coat proteins, there are numerous examples from other plant virus groups (e.g. 19), supporting our observation that virus-specific antibodies do not necessarily produce Western-blot reactions.

The availability of the antiserum and Mabs to CpCSV as well as sequence data described here will be of vital importance for CpCSV detection and identification, for determining its diversity/variability, and for assessment of its relative importance. All this information will be crucial for resistance breeding and for developing strategies of CpCSV control.

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CHAPTER 3

Coat protein variability among Chickpea chlorotic stunt virus isolates from five countries [2]

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ABSTRACT

Chickpea chlorotic stunt virus (CpCSV), a hitherto unrecognized luteovirus, has recently been reported to infect legume crops in Ethiopia. Using degenerate primers for RT-PCR amplification of the coat protein (CP) encoding region from various legume samples suspected to be infected with an unknown luteovirus, first evidence was obtained for the occurrence of CpCSV also in Egypt, Morocco, Sudan, and Syria. Sequence comparison and phylogenetic analysis of the CP sequences of a total of 18 isolates originating from five countries revealed two distinct groups of CpCSV isolates. Group I included isolates from Ethiopia and Sudan while group II comprised those from Egypt, Morocco and Syria, suggesting a geographically associated variation. Based on pairwise comparisons of CP amino acid sequences, the intergroup identities ranged from 90.0 to 92.2% whereas the intragroup identities were in the range of 95-100%. Owing to the differences in CP nucleotide sequences, a simple RFLP test using *Hind*III and/or *Pvu*II for cleavage of CP gene-derived PCR products was developed for distinguishing the two groups of CpCSV isolates. Isolates of both groups reacted with an antiserum to a group I isolate (from Ethiopia) in ELISA and immunoelectron microscopy. However, only group I isolates reacted with ten monoclonal antibodies (Mabs) raised against a group I isolate. This indicated that all the Mabs recognize group I-specific epitopes, thereby permitting serological discrimination of the two groups. Inoculation of four legume species using *Aphis craccivora* for vector transmission of an Ethiopian (group I) and Syrian (group II) isolate showed that the latter causes more severe symptom in faba bean than the Ethiopian isolate. Our data indicates the existence of two distinct CpCSV strain groups that differ in molecular, serological and biological properties as well as in geographical distribution. We suggest that group I isolates originating from Ethiopia and Sudan (Northeast Africa) be named CpCSV-NE and that group II isolates originating from West Asia and North Africa are referred to as CpCSV-WN.

[2] This paper will be submitted for publication in an international journal, such as *Archives of Virology*.

INTRODUCTION

Faba bean (*Vicia faba* L), chickpea (*Cicer arietinum* L), lentil *(Lens culinaris* Medik.), field pea (*Pisum sativum* L) and grasspea (*Lathyrus sativus* L), collectively called cool season food legumes (Summerfield, 1988), constitute an important part of the human diet worldwide. They are staple food for hundreds of millions of people particularly in Asia, Northeast and North Africa. Virus diseases are among the major biotic constraints to the production of cool season food legumes, which are most severely affected by yellowing and stunting diseases caused predominantly by luteoviruses (Bos et al. 1988). Several viruses belonging to various taxa are known to be associated with yellowing and stunting diseases in countries of Northeast and North Africa and West Asia. These include the nanovirus *Faba bean necrotic yellows virus* (FBNYV), the mastrevirus *Chickpea chlorotic dwarf virus* (CpCDV), and luteoviruses such as *Bean leaf roll virus* (BLRV), *Beet western yellows virus* (BWYV) and *Soybean dwarf virus* (SbDV) (Abraham et al. 2000, Fortass et al. 1997, Katul et al. 1993, Makkouk et al. 1994, 1995, 1997, Tadesse et al. 1999). Recently, a new luteovirus named Chickpea chlorotic stunt virus (CpCSV) has been shown to infect a range of cool season food legumes in Ethiopia and to cause yellowing and stunting symptoms in these legume crops (Abraham et al. submitted, 2005). The virus is transmitted by *Aphis craccivora* in the persistent manner, and its experimental host range is apparently limited to a few cool season food legume species. CpCSV shares a coat protein (CP) gene sequence identity of ~78% with *Groundnut rosette assistor virus* (GRAV) which thus appears to be its closest relative (Abraham et al. submitted, 2005).

Despite the high prevalence of luteoviruses in cool season food legumes in countries like Ethiopia and Morocco, their accurate identification has often been impossible mainly due to the lack of specific antibodies or molecular detection tools. For the same reason some authors referred to the causal viruses only as a group (e.g. Abraham et al. 2000, Fortass and Bos 1991, Fortass et al. 1996, Horn et al. 1995, Makkouk et al. 2003). Consequently, the exact identity of some of the luteovirus(es) associated with cool season food legumes in the region has remained unknown. In this paper, we present serological and molecular evidence for the occurrence of CpCSV in cool season food legumes of five countries. Moreover, we demonstrate that the CpCSV isolates can be separated into two geographic strain groups which differ in molecular, serological and biological properties.

MATERIALS AND METHODS

Origin and serological analysis of samples

Samples were collected from faba bean, chickpea, lentil, grasspea and fenugreek (*Trigonella foenum-graecum*) showing yellowing and stunting symptoms in the major growing areas of Ethiopia in 2002 and preserved over $CaCl_2$ (Table 1). Luteovirus-infected faba bean samples from Egypt and Morocco and chickpea samples from Sudan were selected from a collection of preserved samples that had formerly been analysed by serological means at BBA, Braunschweig. From luteovirus-infected faba bean plants from Ambo, Ethiopia, a CpCSV isolate, referred to as FBV (Abraham et al. submitted, 2005), was established in faba bean seedlings by vector transmission using *Aphis craccivora* and maintained in the greenhouse at BBA, Germany. A faba bean sample containing an unidentified luteovirus isolate (SV-1-03) from Syria was kindly provided by K.M. Makkouk and S.G. Kumari, Aleppo, Syria. SV-1-03 was reactivated from dried infected-faba bean tissues obtained from Syria by purifying the virus from the tissue as described by Abraham et al. submitted (2005) and allowing *Aphis craccivora* nymphs an acquisition access feeding for 48 h on purified virions using a membrane feeding system as described by Katul et al. (1993).

Table 1. Sources of CpCSV isolates, hosts and collection year for which sequences information of the coat protein gene is obtained.

	Isolate	Host plant	Location, country	Year of collection
1	Et-fb-swa	Faba bean	South Wello, Ethiopia	2002
2	Et-fb-sg	Faba bean	South Gonder, Ethiopia	2002
3	Et-fb-swb	Faba bean	South Wello, Ethiopia	2002
4	Et-fb-am*	Faba bean	Ambo, Ethiopia	2002
5	Et-fb-ho	Faba bean	Holetta, Ethiopia	1996
6	Et-cp-bd	Chickpea	Bahr Dar, Ethiopia	2002
7	Et-cp-am*	Chickpea	Ambo, Ethiopia	2002
8	Et-gp-am	Grasspea	Ambo, Ethiopia	2002
9	Et-fg-am	Fenugreek	Ambo, Ethiopia	2002
10	Et-fg-sw	Fenugreek	South Wello, Ethiopia	2002
11	Su-31	Chickpea	Sudan	1997
12	Mo-4	Faba bean	Settat, Morocco	2001
13	Mo-fb-6	Faba bean	El Jadida, Morocco	2001
14	Mo-19	Faba bean	Ben Mellal, Morocco	2001
15	Mo-23b	Faba bean	Ben Mellal, Morocco	2001
16	MV 175-94	Faba bean	Fes, Morocco	1994
17	Eg- p6-93	Faba bean	Fayoum, Egypt	1994
18	SV-1-03	Faba bean	Syria	2003

* These two samples are identical to those studied by Abraham et al. submitted (2005).

All samples were tested by triple antibody sandwich (TAS)-ELISA as described by Franz et al. (1996) using the broad-spectrum monoclonal antibody (Mab) B-2-5G4 that reacts with many luteoviruses (Katul et al. 1992), Mab B-4-6G4 that is specific to *Bean leaf roll virus* (BLRV) (Katul et al. 1992), Mab 510H that had been considered specific to BWYV (Abraham et al. submitted, 2005), and three Mabs (T-4E3, -1E1 and -2G5) raised against *Turnip yellows virus* but reacting specifically with viruses of the BWYV subgroup. Polyclonal IgG to BWYV was used as trapping antibody in all cases.

Serological differentiation of isolates

To find out whether the two groups of isolates differing in CP sequence also differ in serological properties, an Ethiopian (group I) and Syrian isolate (group II) were serologically analyzed in detail using polyclonal and monoclonal antibodies produced against an Ethiopian isolate of CpCSV (Abraham et al. submitted, 2005) in DAS ELISA (Clark and Adams, 1977) and TAS ELISA (Franz et al.1996), respectively. In addition, immunoelectron microscopy experiments was carried out as described by Milne and Lesemann (1984) using purified virus preparations of both isolates and the polyclonal antibodies to the Ethiopian isolate. Virus purification was carried out as described by Abraham et al. submitted (2005). Similarly, a range of luteovirus antisera available at BBA collection, namely those to GRAV, BWYV, BLRV, SbDV, *Pea enation mosaic virus-1* (PEMV), *Potato leafroll virus* (PLRV*), Cereal yellow dwarf virus* (CYDV)-RPV, *Carrot red leaf virus* (CtRLV), *Barley yellow dwarf virus-PAV* (BYDV-PAV) and BYDV-MAV were used in the decoration experiments on purified preparations of the two isolates.

Total RNA extraction, RT-PCR, cloning, sequence and RFLP analysis

A total of 30 luteovirus-positive samples (i.e. those reacting with Mab B-2-5G4) originating from five countries were used for amplification of CP sequences by reverse transcriptase (RT)-PCR. Further description of the samples from which CP gene sequences of CpCSV were amplified is presented in Table 1. Total RNA was extracted from $CaCl_2$-preserved leaf samples using Nucleospin® Plant kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instruction, except that the desiccated leaf tissue was ground to a fine powder without liquid nitrogen using a pestle and mortar. A one-step RT-PCR was carried out essentially as described by Abraham et al. submitted (2005) using the degenerate primer pair (sense primer 5'-GCTCTAGAATTGTTAATGARTACGGTCG-3' and antisense primer, 5'-

CACGCGTCIACCTATTTIGGRTTITG-3') derived from conserved terminal domains of the CP gene. Purification of PCR products, cloning and sequencing were done following standard procedures (Sambrook et al. 1989). Sequence assembly, multiple alignment and pairwise identity analyses of nucleotide and amino acid sequences were carried out using DNAMAN (Lynnon, Biosoft, Canada). Phylogenetic trees were constructed using Clustal_X program following alignment of sequences by neighbour joining algorithms (Thompson et al. 1997) and visualized using the Treeview program (Page, 1996).

To develop a restriction fragment length polymorphism (RFLP) method for distinguishing the two groups of CpCSV isolates that differ in CP gene sequence, the PCR products obtained from the CP genes of the isolates with known CP sequences were digested using the restriction enzymes *Hind*III and *Pvu*II (Fermentas). The latter were selected on the basis of the analysis of unique restriction sites in the CP nucleotide sequences of each strain group of CpCSV. Restriction digestion was carried out for each enzyme separately in a reaction volume of 20 µl consisting of 5 µl template (PCR product), 2 µl of 10x restriction enzyme buffer (Fermentas), 0.3 µl of enzyme and 12.7 µl of H_2O followed by incubation at 37°C for 2 h using the specific buffers provided by the manufacturer.

Biological studies

Possible differences in symptomatology between the two groups of isolates were studied by inoculating the Ethiopian (FBV) (Abraham et al. submitted, 2005) and Syrian (SV-1-03) isolates separately onto faba bean, chickpea, lentil and field pea plants. Plants were inoculated as young seedlings by giving *Aphis craccivora* an acquisition access period of two days on infected plants followed by an inoculation access period of two days on young faba bean, chickpea, lentil and field pea plants. Plants were kept in the glasshouse and later inspected for virus symptom and serologically analyzed using virus-specific antibodies.

RESULTS

The occurrence and variability of CpCSV in samples from five countries

Serological tests showed that many samples reacted with Mab B-2-5G4, a broad-spectrum Mab to luteoviruses, but not with virus-specific antibodies to BLRV, BWYV or SbDV, suggesting that the samples are either infected with CpCSV, a virus recently reported from Ethiopia (Abraham et al. submitted, 2005) or other unrecognized luteoviruses. Therefore, the degenerate prim-

ers were used for RT-PCR amplification of CP gene sequences from the samples. Out of 30 luteovirus-infected samples analyzed by RT-PCR, CP gene sequences of CpCSV were amplified

Table 2. Number of luteovirus (ELISA)-positive samples tested by RT-PCR and number of samples originating from each of the five countries and from which CP sequences of CpCSV were obtained

Country	No. of ELISA-positive samples	No. of CpSCV-infected samples *
Egypt	4	1
Ethiopia	12	10
Morocco	8	5
Sudan	5	1
Syria	1	1
Total	30	18

* From the remaining samples either other luteovirus sequences were amplified or no PCR product was obtained

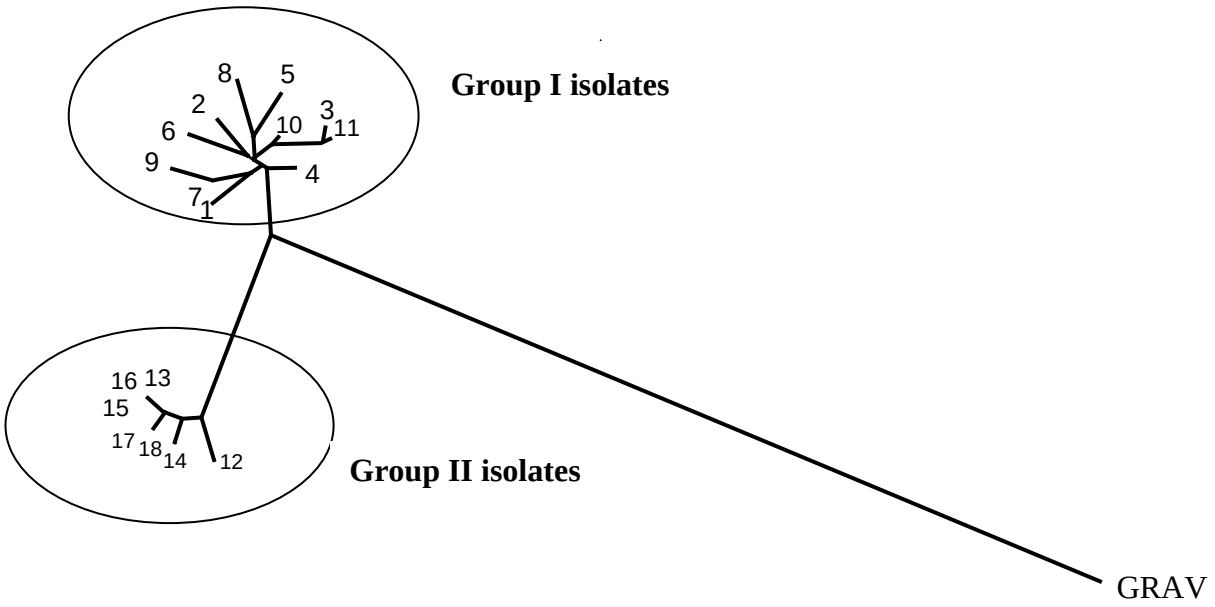

Fig 1. Unrooted tree showing the phylogenetic relationship among the deduced coat protein amino acid sequences of 18 CpCSV isolates from five countries. The numbers refer to the serial number of each isolate as listed in Table 1. *Groundnut rosette assistor virus* (GRAV) sequence (Acc. No. AF195828) was used as an outgroup. The sequences were aligned and neighbour-joining trees were constructed with CLUSTAL_X program and viewed in TreeView.

from 18 samples originating from Ethiopia, Egypt, Morocco, Sudan, and Syria (Table 2). With respect to crop host, CpCSV sequences were obtained from 12 faba bean, three chickpea, one

grasspea and two fenugreek (*Trigonella foenum-graecum*) samples. In addition, a luteovirus-positive lentil sample from Ethiopia gave a PCR product of the expected size and reacted with CpCSV antiserum in ELISA.

Table 3. Percentage of nucleotide (above the diagonal) and amino acid (below the diagonal) sequence identity of the coat protein gene among the studied CPCSV isolates. Figures showing intergroup identity are highlighted in bold.

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Et-fb-sw1		96.5	98.2	96.2	96.8	97.0	97.2	96.8	96.7	98.3	97.7	**91.9**	**91.9**	**91.7**	**91.7**	**91.9**	**91.5**	**91.9**
2. Et-fb-sg	97.0		96.7	96.7	96.5	97.8	96.7	96.5	97.5	97.2	96.5	**92.4**	**92.7**	**92.5**	**92.9**	**92.4**	**93.0**	**93.0**
3. Et-fb-sw2	97.0	96.0		96.0	96.0	96.5	96.4	96.0	96.2	97.5	99.5	**91.9**	**91.9**	**91.7**	**91.7**	**91.9**	**91.9**	**91.9**
4. Et-fb-am1	98.0	98.0	97.0		95.5	97.2	95.7	95.2	96.8	96.2	95.5	**92.2**	**92.2**	**92.0**	**92.4**	**92.2**	**91.4**	**91.7**
5. Et-fb-ho	97.0	97.0	96.0	97.0		97.8	98.2	98.7	97.5	97.5	96.2	**91.2**	**91.5**	**91.4**	**91.7**	**91.2**	**92.2**	**91.9**
6. Et-cp-am	98.0	98.3	96.0	98.0	97.5		97.5	97.2	99.7	97.4	96.4	**92.2**	**92.5**	**92.4**	**92.7**	**92.2**	**92.9**	**92.9**
7. Et-cp-bd	97.5	96.5	96.5	97.5	96.5	96.5		98.2	97.2	97.5	95.8	**92.0**	**92.0**	**91.9**	**92.2**	**92.0**	**92.4**	**92.4**
8. Et-gp-am	96.5	96.5	95.5	96.5	97.5	97.0	97.0		96.8	97.5	96.2	**91.2**	**91.5**	**91.4**	**91.7**	**91.2**	**92.2**	**91.9**
9. Et-fg-am	97.0	97.0	95.0	97.0	96.5	99.0	95.5	96.0		97.0	96.0	**91.9**	**92.2**	**92.0**	**92.4**	**91.9**	**92.2**	**92.5**
10. Et-fg-sw	98.0	98.0	98.0	98.0	98.0	98.0	97.5	97.5	97.0		97.4	**91.9**	**92.2**	**92.0**	**92.4**	**91.9**	**92.2**	**92.5**
11. Su-cp-31	96.5	96.5	99.0	96.5	96.5	96.5	96.0	96.0	95.5	98.5		**91.0**	**91.5**	**91.0**	**91.0**	**91.5**	**91.5**	**91.7**
12. Mo-fb-4	**91.0**	**92.0**	**91.5**	**92.0**	**90.0**	**91.0**	**91.5**	**90.5**	**90.0**	**91.0**	**91.4**		99.3	99.2	98.8	100	97.7	98.0
13. Mo-fb-6	**90.5**	**92.5**	**91.0**	**91.5**	**90.5**	**91.5**	**91.0**	**91.0**	**90.5**	**91.5**	**91.7**	98.5		99.8	99.5	99.3	98.3	98.7
14. Mo-fb-19	**90.0**	**92.0**	**90.5**	**91.0**	**90.0**	**91.0**	**90.5**	**90.5**	**90.0**	**91.0**	**91.5**	98.0	99.5		99.3	99.2	98.2	98.5
15. Mo-fb-23‘	**90.0**	**92.0**	**90.5**	**91.0**	**90.0**	**91.0**	**90.5**	**90.5**	**90.0**	**91.0**	**91.5**	98.0	99.5	99.0		98.8	98.2	98.5
16. Mo-fb-175	**90.5**	**92.5**	**91.0**	**91.5**	**90.5**	**91.5**	**91.0**	**91.0**	**90.5**	**91.5**	**91.4**	98.5	100	99.5	99.5		97.7	98.0
17. Eg-fb-p6-93	**90.5**	**92.5**	**91.0**	**91.5**	**90.5**	**91.5**	**91.0**	**91.0**	**90.5**	**91.5**	**92.0**	97.5	99.0	98.5	98.5	99.0		99.0
18. SV-1-03	**90.5**	**92.5**	**91.0**	**91.5**	**90.5**	**91.5**	**91.0**	**91.0**	**90.5**	**91.5**	**91.5**	97.5	99.5	98.5	98.5	99.0	100	

Fig. 1 shows the phylogenetic relationships among the CpCSV isolates from the five countries based on CP gene amino acid sequences. Using the CP sequence of GRAV, the closest relative of CpCSV, as an outgroup sequence, the tree reveals that isolates from Ethiopia and Sudan form a distinct cluster (group I) that is clearly different from those from Egypt, Morocco and Syria which constitute the second cluster (group II). In pairwise comparisons of CP nucleotide and amino acid sequences (Table 3), the intergroup identities for nucleotide and amino acid sequences ranged from 91.2 to 93.0% and from 90 to 92.2%, respectively. The intragroup identities ranged from 95 to 100% for both nucleotide and amino acid sequences of the CP gene. On the other hand, there were no indications for host plant-specific variation in CP gene sequences when the sequences of the five faba bean, two chickpea, two fenugreek and one grasspea samples from Ethiopia were compared (Fig. 1). Similarly, there was also a high degree of intragroup sequence identity among isolates regardless of their geographic origin from Ethiopia and Sudan, ranging from 95.5-99.75% for nucleotide and 95.0-99.5% for amino acid sequences. Likewise, the CP nucleotide and amino acid sequences of the group II isolates from Egypt, Morocco and Syria were also similar (97.5-100%) although there is a considerable geographic distance between the countries and samples were collected over a time period of nine years (Tables 1 and 3).

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FBV     MNTVVVRNNGRRRRNRRTVQRARRRNPVVVVEAPRQPQRGRRRRRNRRRASGRSTAGRRGSSETFVFS
SV-1-03 ---------------K------Q----------------R----------A---AV-G-------I--
GRAV    -------RP-NG-A--RNR-TP--------QT----NS---------SN.-GSRN-G--G-------

FBV     KDNLAGSSSGSITFGPSLSDCPAFSSGILRAYHEYKISMVKLEFISEAASTSSGSIAYELDPHCKSTS
SV-1-03 --------T----------------------------T----------S------------------
GRAV    ----T---------------------K-------------V--V---S----------------S-

FBV     LGSYINKFGITSNGQRTFAARLINGIEWHSSDEDQFRILYKGNGGSAIAGSFRITIKCQTQNPK  200
SV-1-03 -----------K------T-K------------------------ST---------------  200
GRAV    -Q--V------R----SWMG-Y---V---DAT-----F------S-----F-----V----  199
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Fig. 2. Alignment of the deduced coat protein amino acid sequences of CpCSV isolates from Ethiopian (FBV) and Syria (SV-1-03) with that of *Groundnut rosette assistor virus* (GRAV). Note that the Ethiopian isolates shares more identical amino acid residues (boxed) with GRAV than the Syrian isolate.

When the CP amino acid sequence of an Ethiopian (group I) and a Syrian isolate (group II) of CpCSV were aligned with that of GRAV, a close examination of the CP sequences revealed that there are nine identical amino acid positions shared by both the Ethiopian isolate and GRAV but not by the Syrian isolate whereas there is only one amino acid position identical in both the Syrian and GRAV CPs but not in the Ethiopian CP (Fig. 2). Consequently, the CP

amino acid sequence identity (78%) between the Ethiopian isolate and GRAV is higher than that (74%) between the Syrian isolate and GRAV. This difference suggests that relationship between GRAV and the Ethiopian isolate, which have an overlapping geographical distribution in sub-Saharan Africa (this study; Naidu et al. 1999), appear to be closer than that between the Syrian isolate and GRAV, which appear to be also more distinct (distant) geographically. This difference is also reflected in the phylogenetic tree (Fig. 1) where the branch length between group I isolates is much shorter that that between group II isolates and GRAV.

RFLP test for discrimination of CpCSV isolates

RFLP analysis of the PCR products showed that all the tested isolates of group I were digested with *Pvu*II *g*iving product sizes of about 430 bp and 170 bp while those of group II were not digested with this enzyme (Fig. 3). Conversely, all group II isolates were digested with *Hind*III giving product sizes of 470 bp and 130 bp whereas those of group were not digested with this enzyme. Hence, either or both of these two enzymes can be used to differentiate the two groups using PCR products.

Serological properties of CpCSV isolates

In serological tests, both FBV and SV-1-03 reacted in DAS-ELISA with a rabbit antiserum raised against FBV virions. In immunoelectron microscopy, virions of both isolates were decorated strongly with the polyclonal antibodies and weakly with antibodies to BWYV, GRAV, PLRV and CYDV-RPV. No decoration was observed with antibodies against BLRV, SbDV, CtLRV, PEMV, BYDV-PAV and BYDV-MAV. No striking differences in the reactions of FBV and SV-1-03 with the range of antisera tested were observed. On the contrary, SV-1-03 and the other group II isolates failed to give TAS-ELISA reactions with the 10 Mabs raised against a goup I isolate (FBV) whereas FBV and the other group I isolates gave strong TAS-ELISA reactions with these Mabs. This suggests that all the Mabs to FBV react with epitopes unique to group I isolates and hence can be used for discriminating isolates of the two groups.

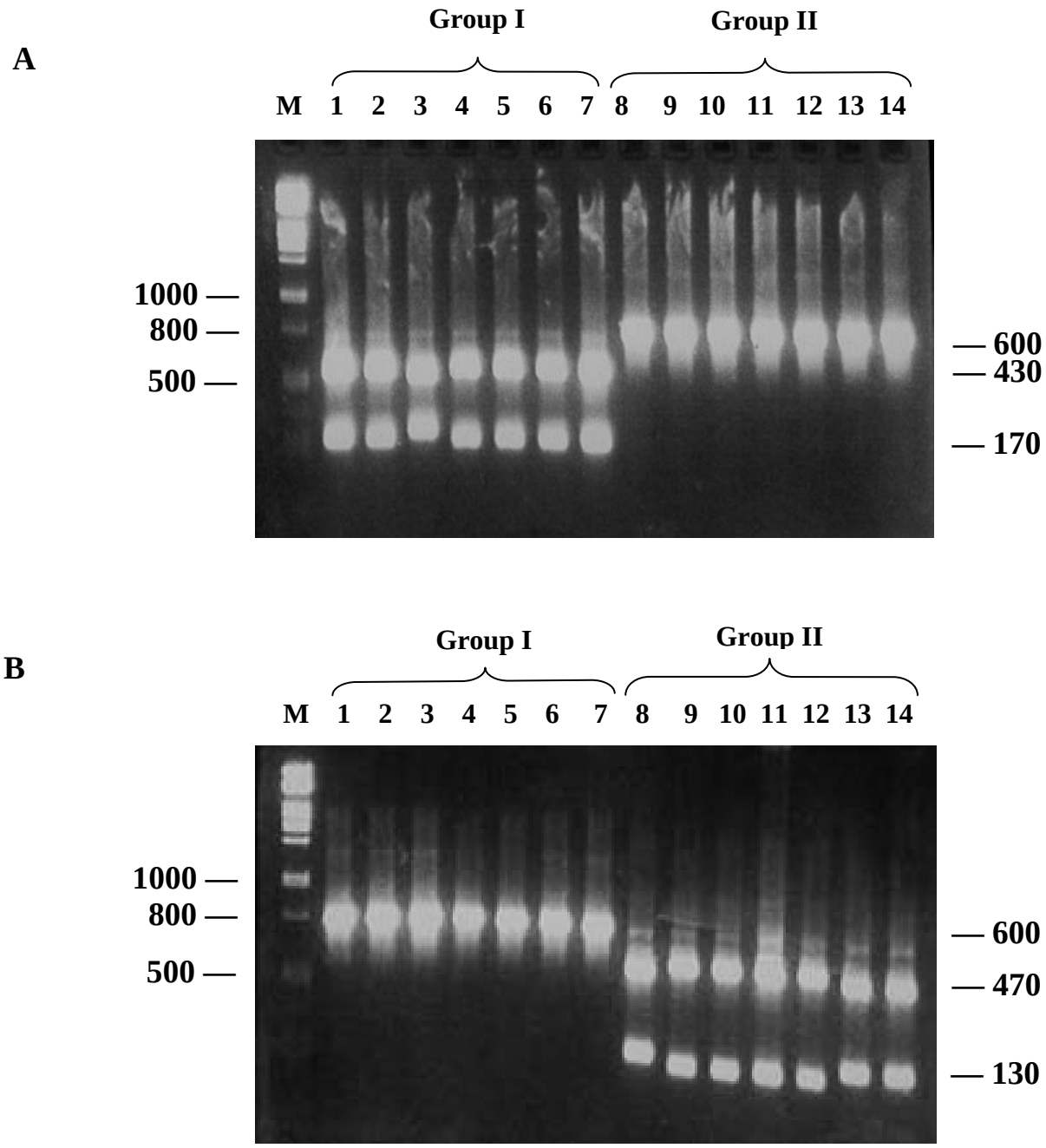

Fig. 3. Restriction fragment length polymorphisms of RT-PCR products of selected group I and group II isolates of CpCSV from five countries digested with enzymes *Pvu*II *(A)* and *Hind*III *(B)*. Lane M shows the size markers, and the other lanes contain the digested PCR products from isolates from Ethiopia (1-6), Sudan (7), Morocco (8-12), Egypt (13) and Syria (14). The approximate sizes of the marker bands and the digested products are indicated in at the left and right side of of the figure, respectively..

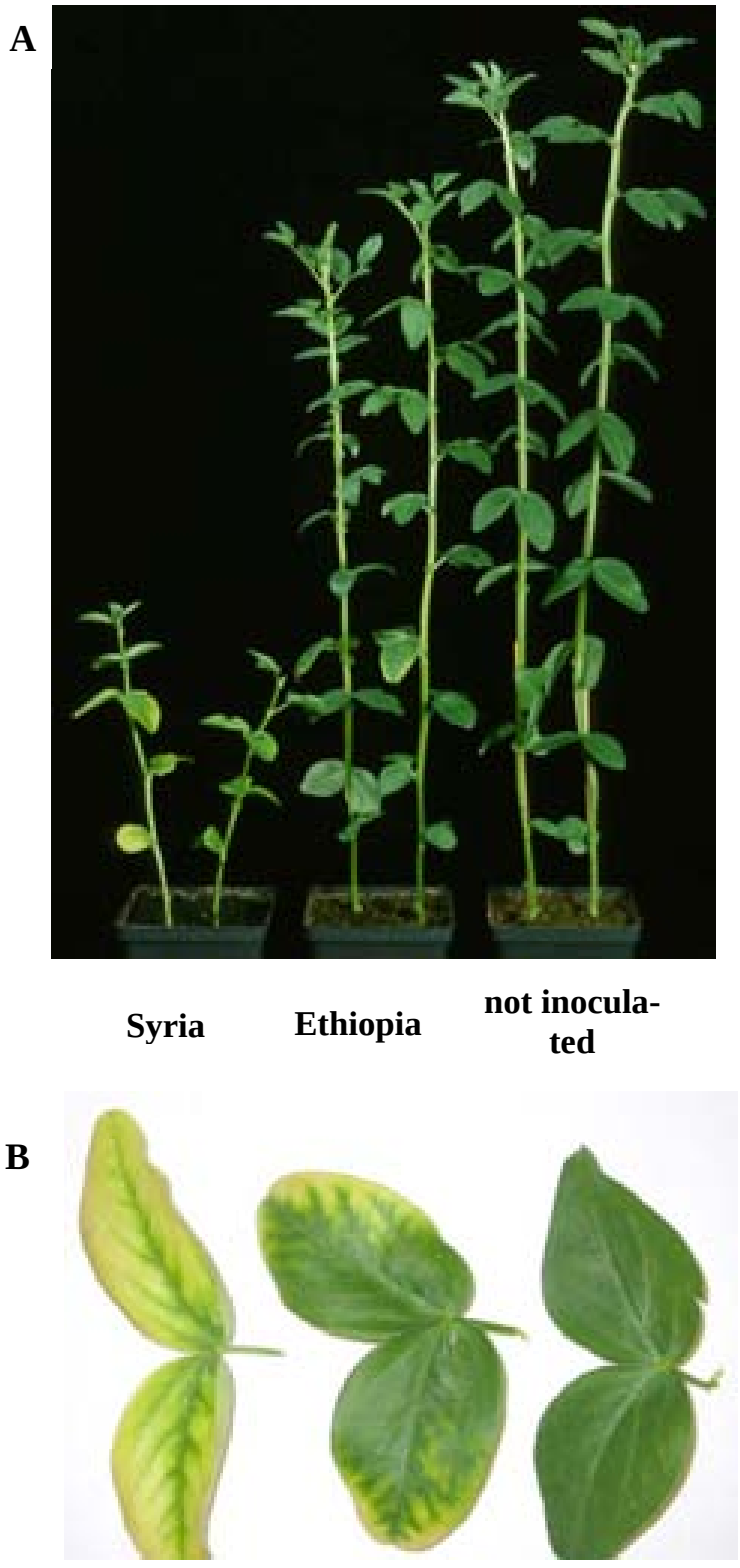

Fig. 4. Variation in plant stunting (A) and leaf yellowing (B) caused by the Syrian isolate SV-1-03 (left) and the Ethiopian isolate FBV (middle) of CpCSV in the faba bean cultivar Condor under greenhouse conditions. An uninoculated control plant and leaf is shown on the right. The photograph was taken ~ 6 week after inoculation.

Variation in symptom severity between the Ethiopian and Syrian isolates

By using a membrane feeding system for allowing *Aphis craccivora* nymphs acquisition access to purified virions of the Syrian isolate (SV-1-03), we were able not only to establish a virus culture of this isolate but also to demonstrate infectivity of CpCSV particles. In subsequent inoculation experiments, both the FBV and SV-1-03 were transmitted by *Aphis craccivora* and showed indistinguishable symptoms in three legume species, yellowing and stunting in chickpea and lentil and latent infection in field pea. In faba bean, however, the symptomatology of the two isolates was strikingly distinct (Fig. 4A and 4B). FBV caused a slight growth reduction and a mild leaf yellowing that was most obvious only in older leaves. In contrast, faba bean plants infected with SV-1-03 were severely stunted and developed leaves with a conspicuous yellowing and a leathery texture in both old and young leaves, often followed by plant death.

DISCUSSION

The results provide the first evidence for the occurrence of CpCSV in faba bean and chickpea in Sudan, Egypt, Morocco and Syria and the existence of two distinct groups of CpCSV isolates that appear to be geographically separated. At the same time, using a purified virion preparation of a Syrian isolate for a membrane feeding experiment, we were also able to demonstrate the infectivity CpCSV particles, substantiating the role of CpCSV in the etiology of the yellowing and stunting diseases of cool season food legumes and fulfilling Koch's postulates.

The occurrence of two distinct groups of CpCSV isolates in geographically adjacent countries namely Ethiopia and the Sudan (group I) on the one hand and Egypt, Morocco and Syrian (group II) on the other hand suggest the existence a geographically associated variation among the isolates. These data together with differential reaction with the Mabs and a clear difference in symptom severity observed for a representative isolate of each group indicate that the two groups represent two genetically, biologically and geographically distinct strain groups of CpCSV. For these two strain groups, we propose the names CpCSV-NE for group I isolates originating from Northeast Africa (Ethiopia and Sudan) and CpCSV-WN for group II isolates originating from West Asia and North Africa (Egypt, Morocco and Syria). The information presented here on the existence and geographically associated variation of CpCSV strains will help breeders in their attempts to incorporate virus resistance to the prevalent CpCSV strains in their region or country since sources of resistance selected in one country may not necessarily be suitable for the other countries where different virus strains can occur.

Available information indicates that for most luteoviruses, there is no strict correlation between sequence variation and geographical origin of isolates as it has been shown for BYDV-PAV (Mastari and Lapierre 1999, Bencharki et al. 1999, Bisnieks et al. 2004), PLRV (Guyader and Ducray, 2002, and references therein), GRAV (Deom et al. 2000, Wangai et al. 2001). Recently however, Moonan and Mirkov (2002) reported the existence of three populations of *Sugarcane yellow leaf virus* (ScYLV) in distinct geographical areas in the Americas and designated them as group A (North Africa and Gutamela), group B (Argentina and Brazil) and group C (Colombia). Thus, our data on CpCSV appear to be an exceptional example for a strict correlation between sequence variation and geographical origin of luteovirus isolates. The selection forces governing the differential genetic adaptation of luteovirus isolates to geographical variation are unknown.

The close phylogenetic relationship between CpCSV and GRAV, their transmission by the same aphid species,) and the fact that they have an overlapping geographical distribution in Africa (e.g. Ethiopia and Sudan (this study, Abraham et al. submitted, 2005, Hull and Adams, 1968, Naidu et al. 1999) may indicate that the two viruses share a common ancestor in Africa possibly in an unknown indigenous African legume species. It is possible that CpCSV isolates have co-evolved with these legume plants and developed into different strain groups independently in geographical isolation. The apparent closer CP relatedness between the strain group I isolates and GRAV than that between the strain group II isolates and GRAV (Fig. 1) may be due to the fact that the latter have diverged more from the ancestral virus to adapt to different environmental and other selection pressures prevailing in this country.

Despite the limited number of samples used for sequence analysis, CpCSV sequences were amplified from the majority (18/30) of the luteovirus-positive samples analyzed (Table 2). This suggests that the virus is widespread occurring wherever cool season food legumes are grown, probably being more prevalent than the other luteoviruses known so far from the region such as BLRV and SbDV (Abraham et al. 2000, Makkouk et al. 1994, 2003, Fortass and Bos, 1991, Tadesse et al. 1999). The observed variability within the sequences of the two groups together with its detection in samples collected as early as 1994 from Egypt and Morocco (Table 1) indicates that the virus has not emerged only very recently. It has probably been infecting the legume plants for decades or centuries but remained undetected and/or was wrongly identified as one of other legume luteoviruses possibly due to lack of appropriate diagnostic tools or cross-reacting antibodies. Further studies on both the incidence and variability of CpCSV in a country or a region are required for determining its economic importance.

Although results of serological surveys have contributed to a growing awareness of the importance of luteoviruses in legume crops in Northeast and North African and West Asian countries, there is unfortunately no sequence information on any legume luteovirus isolate from the region in the database. This is despite frequent reports that the use of serological methods are not sufficiently reliable for the identification of luteovirus species due to the fact that different luteoviruses share a number of immunodominant epitopes (e.g Martin and D'Arcy, 1990, Fortass et al. 1997). The sequence information generated in this work, therefore, forms the basis for further research on CpCSV including design of virus or strain specific primers. In addition, the degenerate primer pair used in this study for the amplification of CP gene sequences, were useful in our hands for detection of not only CpCSV but also other poleroviruses. Therefore, they appear suitable for obtaining CP gene sequences of further unknown luteo- and poleroviruses. Finally, their use in combination with the PCR/RFLP method described in this work and/or virus- or strain-specific polyclonal and monoclonal antibodies produced previously (Abraham et al. submitted, 2005) will certainly facilitate future studies on the identification, ecology and geographical distribution of CpCSV and its strains throughout the world.

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CHAPTER 4

Complete nucleotide sequence and organization of the RNA genome of Chickpea chlorotic stunt virus, a new polerovirus infecting legume crops[3]

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ABSTRACT

The complete nucleotide sequence of the genomic RNA of Chickpea chlorotic stunt virus (CpCSV), a new luteovirus recently reported from legume crops in Ethiopia, has been determined. The genome is 5900 nucleotides and contains six major open reading frames (ORFs) arranged in a manner characteristic of poleroviruses. It shares close overall nucleotide sequence identity (60-65%) with poleroviruses such as *Cucurbit aphid-borne yellows virus* (CABYV), *Turnip yellows virus* and *Beet western yellows virus*. However, its coat protein gene is most closely related (78%) to *Groundnut rosette assistor virus*, for which no other sequence data is available. Comparison of the amino acid sequences of its encoded proteins with those of other poleroviruses indicated that ORF0 is the least conserved of all proteins. ORF1 contains a motif typical of chymotrypsin-like serine proteases and a sequence similar to that of viral genome-linked proteins (VPg). The sequence of the putative slippery heptamer (UUUAAAC) effecting a -1 frameshift to yield an ORF1-2 fusion protein is identical to that of sobemoviruses. The ORF2 product has motifs characteristic of RNA-dependent RNA polymerases of positive stranded RNA viruses. ORF4 starts within ORF3 in another frame but unlike most luteoviruses whose ORF4 is completely embedded within ORF3, it potentially extends few nucleotides downstream of ORF3. Although the ORF5 product of CpCSV is generally most closely related to that of CABYV, recombination analysis suggested that the virus has acquired a stretch of ca. 90 amino acids at the C-terminal part of its readthrough domain from an ancestral *Soybean dwarf virus* (SbDV)-like luteovirus. Despite this putative recombination event, both sequence and phylogenetic analyses of the entire genome and the encoded proteins clearly suggest that CpCSV be classified as a definitive member of the genus *Polerovirus* (family *Luteoviridae*).

[3] This paper will be submitted for publication in an international journal, such as *Archives of Virology*.

INTRODUCTION

Luteoviruses cause economically significant yellowing and stunting diseases in a wide range of agricultural and horticultural crops. They have isometric particles housing a positive sense single-stranded RNA genome and being persistently transmitted by aphids. The family *Luteoviridae* has been divided into the genera *Luteovirus, Polerovirus*, and *Enamovirus*, which differ in genome organization, sequence similarity and gene expression strategies (D'Arcy et al. 2000). In addition, there are several virus species as yet unassigned to a genus mainly due to the lack of sufficient sequence information (D'Arcy and Domier. 2004).

The luteovirus genome contains five or six major open reading frames (ORF) referred to as ORF0 to ORF5 (D'Arcy et al. 2000). While members of genus *Polerovirus* have all these ORFs, ORF0 is absent in members of the genus *Luteovirus* whereas ORF4 is absent in *Pea enation mosaic virus*, the only member of genus *Enamovirus*. Some luteovirus genomes contain one to two (additional) minor ORFs (Miller et al. 1995, Ashoub et al. 1998). In addition, members of the genera *Polerovirus* and *Enamovirus* possess a genome-linked protein (VPg) which is absent in those of genus Luteovirus. In general, the replication-associated proteins encoded by ORF1 and ORF2 of members of the genus *Luteovirus* are phylogenetically related to those of viruses in the family *Tombusviridae* whereas the ORF1 and ORF2 products of members of the genus *Polerovirus* and *Enamovirus* are more closely related to those of viruses in the genus *Sobemovirus* (D'Arcy et al., 2000). However, the 5' half of the *Bean leafroll virus* (BLRV) and *Soybean dwarf virus* (SbDV) genome resembles that of members of the genus *Luteovirus* whereas their 3' half is more similar to that of viruses in the genus *Polerovirus* (Domier et al. 2002, Rathjen et al. 1994). On the contrary, the 5' half of the *Sugarcane yellow leaf virus* (ScYLV) genome is more closely related to that of the polerovirus genome while its 3' half is more closely to that of members of the genus *Luteovirus* (Smith et al. 2000). Phylogenetic analyses of the complete genome of these three viruses suggested that they have arisen by recombination between ancestral luteoviruses and poleroviruses (Domier et al. 2002, Smith et al. 2000). Considering the affinity of the replication-associated genes as a primary criterion for luteovirus classification, BLRV and SbDV have recently been classified as members of the genus *Luteovirus* and ScYLV as a member of the genus *Polerovirus* (D'Arcy and Domier, 2004).

Five luteoviruses namely BLRV, SbDV, *Beet western yellows virus* (BWYV), *Pea enation mosaic virus-1* (PEMV-1) and *Chickpea stunt disease-associated virus* (CpSDaV) have been

reported as causing serious yellowing and stunting diseases of cool season food legumes in different parts of the world (Bos et al. 1988, Naidu et al. 1997, Makkouk et al. 2003). These viruses have been studied in greater detail and the complete nucleotide sequence for at least one (but non legume-infecting) isolate of BWYV (Veidt et al. 1988), BLRV (Domier et al. 2002), PEMV-1 (Demler and de Zoeten, 1991), and SbDV (Rathjen et al. 1994) has been determined. It should be noted, however, that polerovirus isolates previously referred to as BWYV have been recently reclassified on the basis of differences in host ranges and ORF0 products as four distinct virus species namely BWYV, *Beet chlorosis virus* (BChV), *Beet mild yellowing virus* (BMYV) and *Turnip yellows virus* (TuYV) (Hauser *et al.* 2000, 2002; D'Arcy and Domier, 2004). The only available ORF0 sequence information for a legume (faba bean) isolate of the BWYV subgroup from France (Hauser et al. 2000) indicates that it is more closely related to TuYV than to isolates presently considered as BWYV *sensu strictu*.

In a recent study Abraham et al. (submitted, 2005) have determined some biological and coat protein properties of Chickpea chlorotic stunt virus (CpCSV), a new luteovirus infecting legumes in Ethiopia. This virus is persistently transmitted by *Aphis craccivora*, appears to infect only a few species of cool season food legumes and is most closely related to *Groundnut rosette assistor virus* (GRAV) in its coat protein amino acid sequences. Since there is increasing evidence for the occurrence of chimeric genomes in luteoviruses as exemplified by BLRV (Domier et al. 2002), SbDV (Rathjen et al. 1994), and ScYLV (Smith et al. 2000), it has become necessary to either determine the entire nucleotide sequence or to obtain sequence information from a substantial part of the 5' and 3' halves of the luteovirus genome before a virus can be assigned to a genus of the family *Luteoviridae*. In order to understand the genome organization of CpCSV and define its accurate taxonomic position, the complete nucleotide sequence of its genomic RNA was determined. Sequence analysis indicated that CpCSV is a new member of the genus *Polerovirus* (family *Luteoviridae*).

MATERIALS AND METHODS

Virus isolate and extraction of total and viral RNA

The CpCSV isolate studied originated from a faba bean plant showing yellowing and stunting symptoms in a field near Ambo, central Ethiopia, in 2002. It was maintained in faba bean

seedlings in the greenhouse at BBA, Braunschweig using *Aphis craccivora* for vector transmission at intervals of 4 to 6 weeks (as described in Chapter 2). Total RNA was extracted from CpCSV-infected faba bean leaves using the NucleoSpin® RNA Plant kit (Macherey-Nagel, Germany) following manufacturer's instructions. Viral ssRNA was extracted from purified CpCSV virions (Abraham et al. submitted, 2005) using the RNAeasy Mini Cleanup Protocol (Qiagen) as described by the manufacturer.

RT-PCR and sequencing strategy

To clone the viral RNA, two approaches were used for obtaining reverse transcriptase (RT)-PCR products. At first, the sequence of the internal part of the genome was determined using a 'Genome Walking' strategy. In this step-by-step procedure, a combination of a specific primer derived from a region of known sequence and a degenerate primer targeted to the unknown region (Table 1) was used for obtaining RT-PCR products upstream or downstream of the known sequence. Specific primers were initially derived from the coat protein (CP) gene sequence described earlier (Chapter 2) and thereafter from the newly obtained sequences. Degenerate primers were designed from conserved domains obtained by multiple alignment of BWYV, *Cucurbit aphid-borne yellows virus* (CABYV) and BMYV sequences from the database. RT-PCR was carried out as a one-step method using total RNA from CpCSV-infected faba beans as a template essentially as described in Chapter 2.

In a second approach, rapid amplification of complementary DNA (cDNA) ends (RACE) was used to determine the 3' and 5' ends of the CpCSV RNA genome as described by Dieffenbach and Dveksler (1995) with minor modifications. For 3' RACE, viral RNA extracted from purified CpCSV particles was first polyadenylated to allow for amplification of the 3' end of the genome with an oligo $d(T)_{18}$ primer (Table 1). To generate the poly(A) tail, 10 µl of viral RNA and 7.25 µl of H_2O were incubated at 70°C for 5 min and cooled on ice for 2 min. Then, 5 µl of 5x poly(A) polymerase buffer (Amersham), 1.25 µl of 10 mM rATP, and 1.5 µl of yeast poly(A) polymerase (Amersham) were added to the mixture followed by an incubation period of 90 min at 30°C. Synthesis of cDNA was done using oligo $d(T)_{18,}$ poly(A)-tailed RNA as a template and M-MMLV transcriptase (Gibco) according to manufacturer's instructions. PCR was run with 1 µl of sequence-specific sense primer (S6, Table 1) and 1 µl of oligo $d(T)_{18}$ primer as described above.

Table 1. Designation, sequence and genomic position of oligonucleotide primers used for amplification of the complete nucleotide sequence of the CpCSV genome

Primer number and designation (orientation)	Sequence (5' → 3') *	nt position
1. Poly(G) (sense)	AACTGCAGAAGGGGGGGGG	-
2. Poly-S1 (sense)	ACCTTGGCACACAAAAGAAACSAG	1-14
3. PRA-AS (antisense)	GGTCCTGGTGTCCTGATAA	462-480
4. Poly-AS1 (antisense)	ACTGTAACATATTGGGCTGCTGA**A**	1130-1154
5. Poly-S2 (sense)	CGCAACATTGATTTACGMSTGCCC	271-288
6. RdRp-S1 (sense)	GGTCCCCCMCAAYTGGGAAGG	1102-1119
7. Poly-AS2 (antisense)	AACTATTGATCTCACGTGGGTTGAAA	3634-3660
9. PRC (antisense)	TTGCTTGCTCTCTCTCCTCC	1957-1971
10. Poly-S3 (sense)	CCAAGAAGACAGAAGCGCAAGGAA	1921-1950
11. P-S5 (sense)	TTCGTCAGGGAGAACCGCATAA	2601-2623
12. Polero-AS1 (antisense)	GATITTATAYTCATGGTAGGCCTTGAG	3890-3907
13. PFF-S (sense)	CTCGTGGCTCCTTGATCCAGTG	3441-3455
14. Polero-AS2 (antisense)	CGAGCTCRTAAGMGATGGARCC	4075-4088
15. Polero-S1 (sense)	GCTCTAGAATTGTTAATGARTACGGTCG	3663-3675
16. Polero-AS3 (antisense)	CACGCGTCIACCTATTTIGGRTTITG	4251-4269
17. Polero-S2 (sense)	ATCACITTCGGGCCGWSTCTATCAGA	3900-3925
18. P-AS4 (antisense)	CAGCYKTTGTAAWTCCCYACGTTCCA	4795-4816
19. P-AS5 (antisense)	GAAGGAGTKTTAARGTCTTGTCTTTGA	4944-4969
20. S4 (sense)	GGAGAATATACAGAAAATATGTGTGAA	4905-4931
21. PRB-E (antisense)	ATCTTTAGAGTGGGCGGTTGA	5731-5752
22. P-S6 (sense)	CACCTATCCCAAAGGACAGCTTGT	4861-4884
23. Oligo d(T)18 (antisense)	GCGGGATCCTTTTTTTTTTTTTTTT	-
24. PRB-AS (antisense)	GTCGTACATATGCGCCAACGA	514-534

* IUPAC degeneracy code: W = A/T, R = A/G, Y = C/T, K= G/T, S = C/G, M = A/C). I = inosine. Restriction sites in the primers are underlined.

For 5' RACE, cDNA was synthesised as described for 3' RACE by incubating 10 µl of viral RNA but using 1 µl of sequence specific antisense primer (PRB, Table 1). By following the Nucleospin®extract 2 in 1 protocol for direct purification of PCR products (Macherey-Nagel, Germany), the preparation was freed from excess primers and eluted in a (elution) buffer volume of 35 µl. For end-tailing of the cDNA with poly(C) residues, 5 µl of terminal deoxynucleotidyl transferase (TdT) buffer, 2.5 µl of 2 mM dCTP and 16 µl of cDNA were incubated at 94°C for 30 min and cooled on ice for 2 min. To the mixture, 1.5 µl of TdT (25 U/µl, Fermentas) was added followed by incubation at 37 °C for 10 min and at 65 °C for another 10 min. PCR was run using another internal sequence-specific antisense primer (PRA) and a homopolymeric poly(G) primer (Table 1) under conditions similar to that of 3' RACE.

Cloning, sequencing, phylogenetic and recombination analyses

Purification of PCR amplicons was done from 1% agarose gel using the Nucleospin® Extract kit (Macherey-Nagel, Düren, Germany). The product was ligated into a PGEM®-T vector (Promega) with T4 DNA ligase and transformed by heat shock into DH5α competent *E. coli* cells as described by Sambrook et al. (1989). Extraction of recombinant plasmid DNA from positive clones was done using Nucleospin® plasmid kits (Macherey-Nagel, Germany). DNA sequencing was carried out by automated facilities of a commercial company (MWG Biotech, Ebersberg, Germany). Sequence assembly, multiple alignment and identity analyses of nucleotide and amino acid sequences were carried out using the computer software DNAMAN (Lynnon Biosoft, Canada). The sequences were compared with available sequences in the database using the basic local alignment search tool (BLAST) program (Altschul et al. 1997). Phylogenetic trees were constructed using the ClustalX program after multiple alignment of sequences by neighbour joining algorithms (Thompson et al. 1997) and visualized using Treeview program (Page, 1996). Databank accession numbers of virus sequences used in sequence comparison and/or phylogenetic analysis are as follows: *Barley yellow dwarf virus-PAV* (BYDV-PAV; D85783), *Beet chlorosis virus* (BChV, NC_002766), BLRV (NC_003369), BMYV (X83110), BWYV (NC_004756), CABYV (NC_003688), Carrot red leaf virus (CtRLV, NC 006265), *Cereal yellow dwarf virus-RPV* (CYDV-RPV; NC_004751), *Cocksfoot mottle virus* (CfMV, Z48630), CpSDaV (Y11530), GRAV (AF195828), PEMV-1 (L04573), PLRV (D00734), SbDV (NC_003056), ScYLV (NC_000874), *Tobacco vein distorting virus* (TVDV, AJ704890) and TuYV (X13063). Possible recombination events were tested by using the default settings of the Sister scanning (Siscan) procedure (Gibbs et al. 2000) after aligning data of the entire nucleotide sequences of CpCSV as a possible recombinant virus with those of CABYV and SbDV used as parents.

RESULTS AND DISCUSSION

The genomic RNA of CpCSV is 5900 nt in length. It consists of three untranslated regions (UTR) and six major ORFs (ORF0 to ORF5) that are arranged in a manner characteristic of poleroviruses (Fig. 1).

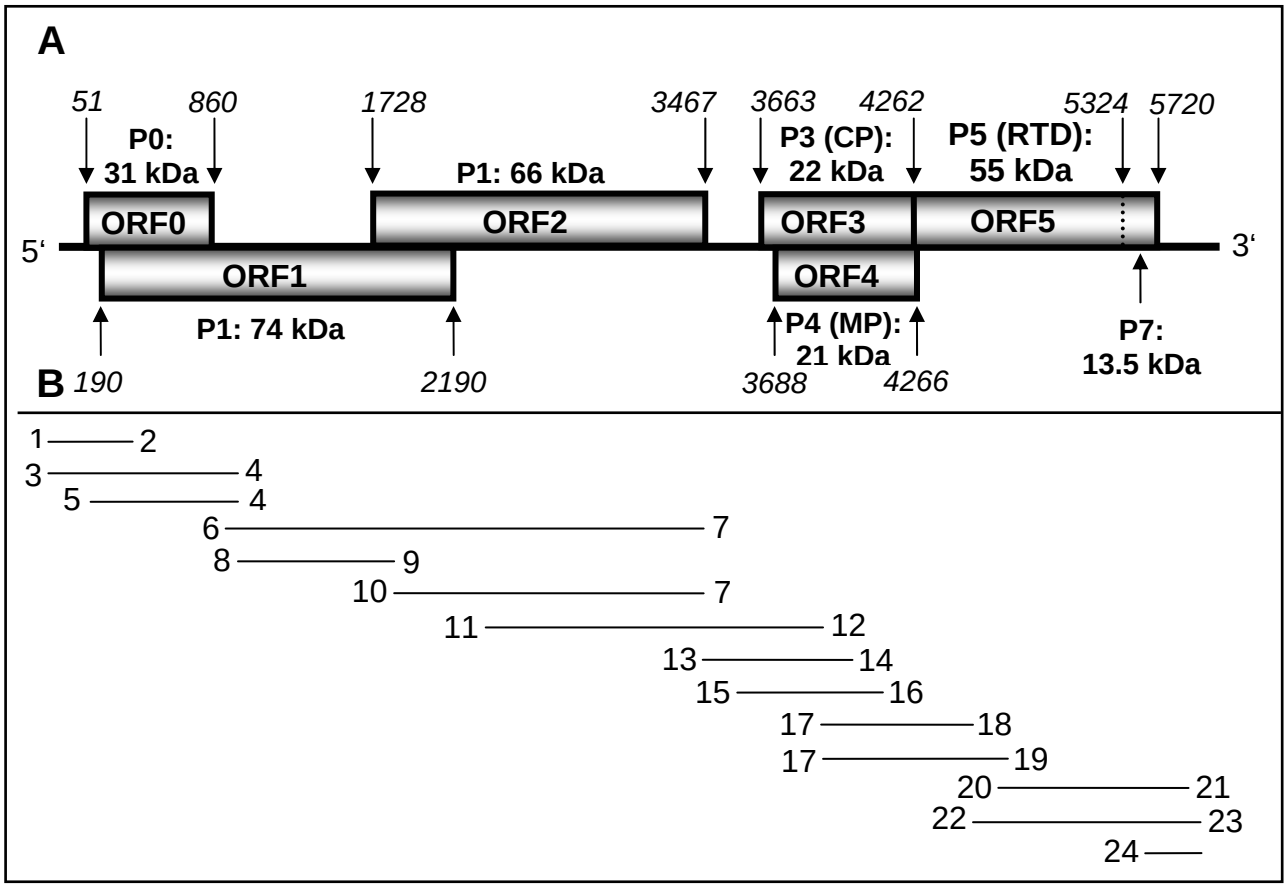

Fig. 1. (A) Schematic representation of the organization of the CpCSV genome. Grey rectangles indicate the ORFs (ORF0 to ORF7) predicted for the CpCSV RNA. The name and deduced size of the individual gene products are given above or below each ORF. The exact nucleotide position of the beginning and the end of each ORF are indicated. (B) The lines below the genome map show the approximate size and position of the various overlapping cDNA clones that were generated for determining the complete nucleotide sequence. The numbers flanking each line refer to the oligonucleotide primers listed in Table 1.

Analysis of the non-coding regions

The 5’ UTR of the CpCSV RNA consists of 50 nt with the first eight nt (ACAAAAGA) being identical to those of other poleroviruses, such as BMYV, BWYV, CABYV, and PLRV. The size of the 5’ UTR in sequenced poleroviruses ranges from 20 nt for BWYV to 175 for PLRV. The 3’ untranslated region is 177 nt in length ending with a sequence at CGGTGT which is also conserved in BMYV, BWYV, and CABYV. The intergenic region separating

ORF2 from ORF3 has 192 residues. A copy of the 5' consensus sequence ACAAAA, which has been shown to be the start site of the subgenomic RNA1 (sgRNA1) of PLRV and BWYV (Miller and Mayo, 1991, Reutenauer et al. 1993), is found in the intergenic region or immediately upstream thereof in all polerovirus genomes sequenced so far (Miller et al. 1995, Smith et al. 2000). In the CpCSV genome, an identical sequence is found a few bases upstream of the start of the intergenic region. If a mechanism of sgRNA1 transcription similar to that of PLRV and BWYV is used in CpCSV, a sgRNA1 of 2440 nt would be produced. A comparison of the 5' terminal sequence and the sequence domain encompassing the possible sgRNA1 start sites in the CpCSV genome with those in other polerovirus genomes is presented in Fig. 2. BLAST search revealed no significant similarity between any of the noncoding sequences and other viral sequences in the database.

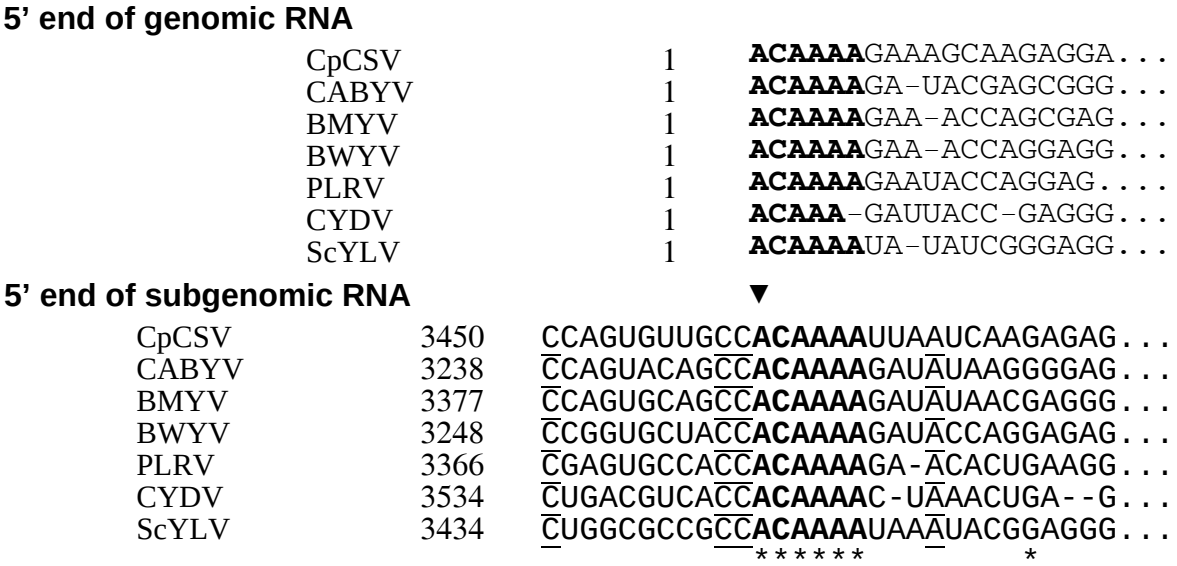

Fig. 2. Alignment of the 5'-terminal nucleotides of the RNA genome of CpCSV and other poleroviruses with those of the demonstrated or predicted start sites of their subgenomic RNA. Alignment was made with DNAMAN package and manually adjusted to obtain the best possible match. ▼ denotes the position of the 5'-terminal nucleotide of subgenomic RNA1 of PLRV and BWYV (Miller and Mayo 1991, Reutenauer et al. 1993). Residues conserved in all genomic and subgenomic RNAs at the star site are in bold. Underlined residues are conserved only in sg RNAs.

Table 2. BLAST search data (E values) ranking the amino acids sequence homologies between each of the six major CpCSV gene products and the four most closely related homologous proteins of other luteovirus species.

Relative order [1]	P0		P1		P2 (RdRp)		P3 (CP)		P4 (MP)		P5 (RTD)	
	Virus	E value[2]	Virus	E value	Virus	E value	Virus	E value	Virus	E value	Virus	E value
1	TuYV [3]	$5e^{-6}$	TuYV [3]	$2e^{-82}$	TuYV [3]	0.0	GRAV	$6e^{-66}$	CABYV	$6e^{-36}$	CABYV	$8e^{-84}$
2	BWYV	$7e^{-4}$	CABYV	$4e^{-78}$	BWYV	0.0	CABYV	$4e^{-59}$	TuYV [3]	$7e^{-25}$	PEMV-1	$9e^{-64}$
3	BMYV	0.02	BMYV	$1e^{-72}$	CABYV	0.0	TuYV [3]	$6e^{-59}$	BChV	$2e^{-24}$	SbDV	$7e^{-64}$
4	CABYV	0.40	CtRLV	$5e^{-71}$	BMYV	0.0	CpSDaV	$7e^{-59}$	BWYV	$1e^{-23}$	BLRV	$2e^{-50}$

[1] Viruses are listed in the order of the BLAST search data whereby only one isolate of each virus species is shown.

[2] Note that Expect (E) values of less than $1e^{-2}$ usually indicate homology and those of less than $1e^{-6}$ almost always indicate homology (Altschul et al. 1997).

[3] This isolate of TuYV was formerly referred to as BWYV-FL1 (Veidt et al. 1989).

Analysis of the coding regions

ORF0 of CpCSV starts at the first AUG codon (nt 51-53) and potentially encodes a protein (P0) of 30.8 kDa (270 amino acids). This makes this protein the biggest in size compared to its counterparts in other poleroviruses which range from 238 amino acids for CABYV to 256 for CYDV-RPV. P0 is the least conserved polerovirus protein which shares very low but significant homology only with the P0 of TuYV and BWYV (Table 2). It has been already noted earlier that this protein is the most divergent in poleroviruses (Miller et al. 1995, Smith et al. 2000). Pfeffer et al. (2002) demonstrated that the expression level of ORF0 in BWYV, PLRV and CABYV is regulated partly by a leaky scanning mechanism due to the unfavourable context of its initiation codon. The context of the ORF0 initiation codon of CpCSV is also poor, lacking both the A and G at –3 and +4 positions, respectively, possibly not permitting efficient translation in plants (Lütcke et al. 1987). This suggests that a similar mechanism may operate in the regulation of P0 expression in CpCSV. The P0 of BWYV and CABYV have been shown to act as a suppressor of posttranscriptional gene silencing (Pfeffer et al. 2002).

ORF1 encodes a 73.9-kDa protein (P1) homologous to the ORF1 product of other poleroviruses and PEMV-1. This protein has been shown to be a serine protease with the motif HX29-34 [D/E]X62-63TXKGYSG (Gorbalenya et al. 1989, Koonin and Dolja, 1993, Hulanicka et al. 1999) that is conserved in all poleroviruses and PEMV-1. CpCSV P1 also contains this motif and is hence likely to have an analogous protease function. A sequence similar to the N-terminal part of the virus genome-linked protein (VPg) described for PLRV (van der Wilk et al. 1997) is mapped to the amino acid positions 411 (starting with a threonine residue) in the CpCSV P1. The ORF1 product is most closely related to that of BWYV subgroup viruses and CABYV (Table 2).

ORF2 coding for a RNA-dependent RNA polymerase (P2) with a predicted molecular mass of 66.5 kDa overlaps ORF1 by 463 nucleotides. This protein is expressed as a fusion protein from ORF1 by –1 frameshift with two features, a slippery heptanucleotide site and a secondary structure needed to promote efficient frameshifting (Miller et al. 1995, Prüfer et al. 1992). The putative frameshifting slippery heptamer of CpCSV has the sequence UUUAAAC (nt 1728-1734) which conforms with the canonical sequence described by Miller et al. (1995). However, this heptanucleotide is different from that of any other luteovirus sequenced so far but identical to a shifty sequence of sobemoviruses such as *Cocksfoot mottle virus* (CfMV) (Mäkinen et al 1995) (Fig. 3), further supporting the evolutionary relationship of sobemoviruses and poleroviruses in this part of the genome. The sequence UUUAAAC has been shown to give the best frameshifting in eukaryotic systems and changing the BWYV slippery sequence from GGGAAAC to

UUUAAAC increased the frameshift rate three-fold (Garcia et al. 1993), suggesting that the later is more efficient. The slippery sequence is followed after six bases by a sequence that could potentially form a pseudoknot structure. The predicted secondary structure of the CpCSV frameshift site and comparison of its slippery site with those of other luteoviruses and CfMV is shown in Fig. 3. The core RNA polymerase motif (GXXXTXXXN(X25-40)GDD present in RNA-dependent RNA polymerases of plus-strand RNA viruses (Kamer and Argos, 1984) is mapped to amino acid position positions 450-480 of P2. According to BLAST results, P2 appears to be one of the most conserved polerovirus protein with Expect value of 0.0 for nearly all poleroviruses (Table 2).

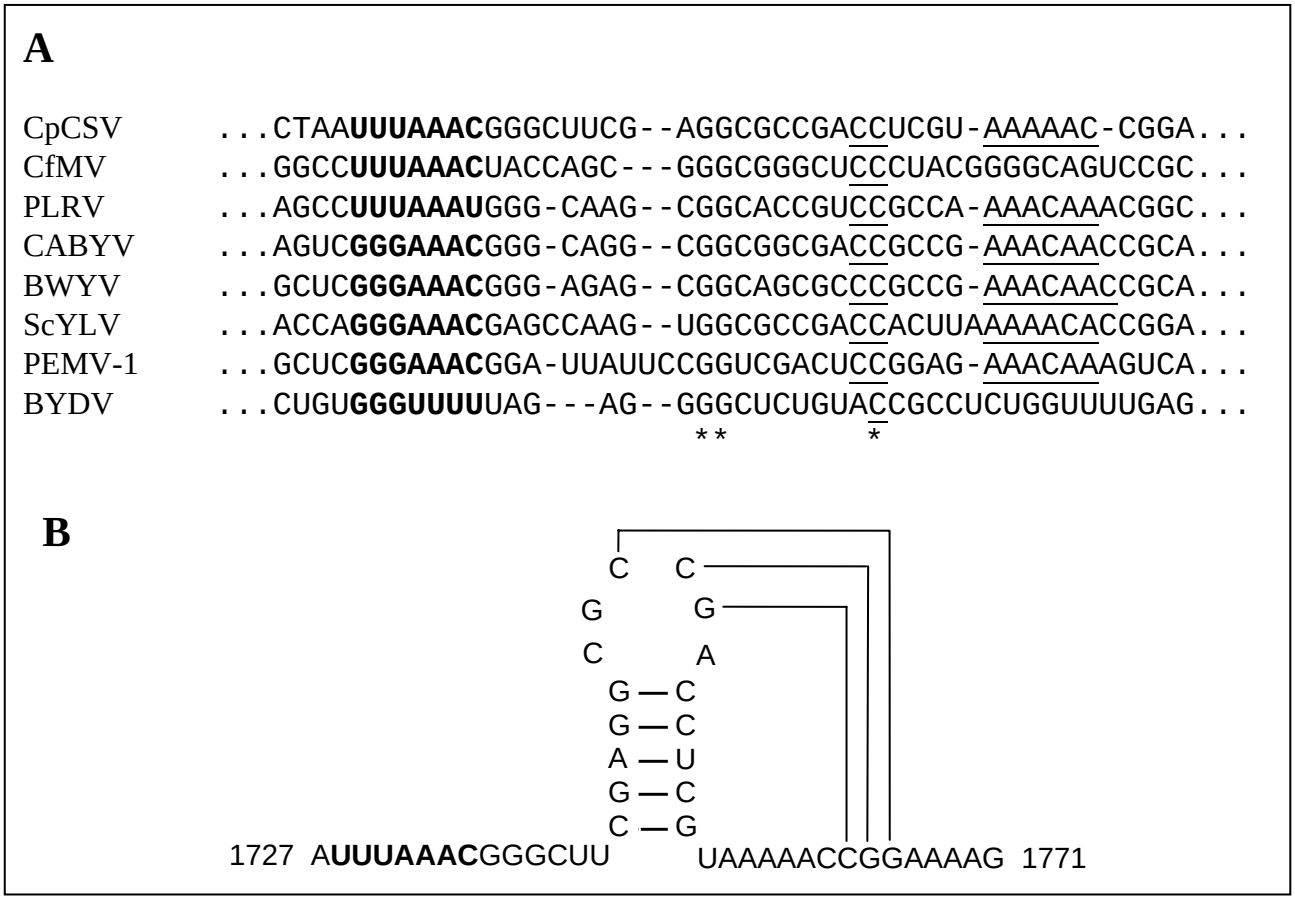

Fig. 3. (A) The alignment of shifty heptamer and adjust sequence of CpCSV with those of other luteoviruses and *Cocksfoot mottle virus* (CfMV, genus *Sobemovirus*). The slippery heptamer sequence is in bold. The nucleotides proposed to be conserved in all luteoviruses possessing a sobemovirus-like RdRp (Miller et al. 1995) are underlined. (B) Putative pseudoknot structure adjacent to shifty heptamer sequence and surrounding residues in the CpCSV genome.

ORF3 encodes the coat protein (CP) of CpCSV and is most similar to that of GRAV (78%)-followed by that of CABYV (72%), TuYV (71and CpSDaV (66.5%) (Table 2)**.** Since only the sequence of CP gene of GRAV genome is available (Scott et al. 1996), we cannot rule out the pos-

sibility that CpCSV genome is phylogenetically most closely related to GRAV. The least related CP (42%) was that of ScYLV.

```
PLRV      1  MSTVVVKGNVNGGVQQPRMRRRQSLRRRANRVQPVVMVTAPGQPRRRRRRRGG
             | ||||:   ||     | ||      || |  |||:| || || | ||||
CpCSV     1  MNTVVVRN..NG.....RRRRNRRTVQRARRRNPVVVVEAPRQPQRGRRRRN

PLRV     54  NRRSRRTGVPRGRGSSETFVFTKDNLVGNTQGSFTFGPSLSDCPAFKDGILKA
              ||    : :  ||||||||| |||| |   ||:||||||||||||  |||:|
CpCSV    47  RRRASGRSTAGRRGSSETFVFSKDNLAGSSSGSITFGPSLSDCPAFSSGILRA

              109
PLRV    107  YHEYKITSILLQFVSEASSTSSGSIAYELDPHCKVSSLQSYVNKFQITKGGAK
             ||||||  : |:|:||| |||||||||||||||  || ||:||| ||  | :
CpCSV   100  YHEYKISMVKLEFISEAASTSSGSIAYELDPHCKSTSLGSYINKFGITSNGQR
              102

                     170 173 176/7
PLRV    160  TYQARMINGVEWHDSSEDQCRILWKGNGKSSDSAGSFRVTIKVALQNPK  208
             |: ||:|||:||| | ||| |||:|||| :|  |||||:|||   ||||
CpCSV   153  TFAARLINGIEWHSSDEDQFRILYKGNG.GSAIAGSFRITIKCQTQNPK  200
                     163 166 169/70
```

Fig. 4. Alignment of the predicted coat protein (CP) amino acid sequence of CpCSV and PLRV showing amino acid residues predicted to form a trimer axis in the CP (Terradot et al. 2001). Amino acids forming 'acidic patches' in the PLRV CP and their equivalents in the CpCSV CP are highlighted and numbered. The vertical lines and colons indicate identical and similar amino acids, respectively.

Based on computer-assisted homology modeling by aligning the PLRV CP sequence with the known crystal structure of the S domain of the CP of *Rice yellow mottle virus* (genus *Sobemovirus*), Terradot et al. (2001) predicted the three-dimensional structure of the PLRV CP. In their model, five acidic amino acids (E_{109}, E_{170}, D_{173}, E_{176} and D_{177}) are predicted to be present on the surface of PLRV particles at the trimer axis. Since these residues are conserved in luteoviruses, alignment of the CpCSV CP sequence with that of PLRV (Fig. 4) showed these conserved acidic amino acid residues at coordinates E_{102}, E_{163}, $S_{166,}$ E_{169}, D_{170} . It is suggested that this patch may be important in molecular recognition and could confer specific properties of each genus and/or the family (Terradot et al. 2001). The substitution of neutral amino acid S (serine) instead of D (aspartic acid) in the position 166 is unique to CpCSV since all dicot-infecting luteoviruses have the acidic residue D at that position (and E for PEMV-1). However, detailed mutational analysis

of the BWYV CP targeting these predicted conserved surface domains (Brault et al. 2003) showed that mutation of D_{168} and E_{171} (corresponding to S_{166} and E_{169} of CpCSV, respectively) did not abolish virus accumulation *in planta* and aphid transmission, although that in D_{173} (corresponding to D_{170} in CpCSV) did, implying its importance in virus aphid interaction.

ORF4 of CpCSV starts few nucleotides downstream of the ORF3 initiation codon but in a different reading frame. The CpCSV ORF4 potentially encodes a 21 kDa protein (P4) which has been shown to act as a movement protein (Miller et al. 1995). Unlike most luteoviruses whose ORF4 is completely embedded in the CP gene sequence, the ORF4 of CpCSV extends just few bases beyond the CP gene termination codon (nt 4262-4264) with its termination codon at nt 4266-4268. Only GRAV and CABYV have a similar arrangement of ORF4 in relation to ORF3 (Guilley et al. 1994, Scott et al. 1996). Consequently, these three viruses have a slightly (about 18 amino acids larger as compared to BWYV) larger predicted P4 than the equivalent protein of other luteoviruses. It is not known whether this size variation of the ORF4 protein of luteoviruses plays a role in the life cycle of the individual viruses.

ORF5, coding for an ORF3/5 fusion (readthrough) protein of 77 kDa, is separated from ORF3 by an amber termination codon. A cytosine rich sequence downstream of the readthrough stop codon is required as a signal for efficient readthrough in BYDV-PAV (Brown et al. 1996). This 5’ end of ORF5 contains a repeated nucleotide pattern of the form [CCNNNN]7-16 (where N is any base) in the genome of previously described luteoviruses (Miller et al 1995) with a slightly different pattern recently reported for ScYLV (Smith et al. 2000). The putatively equivalent sequence in CpCSV, however, has a unique pattern described by the form CCCCC[CCNNNN]6GCA[CCNNNN]4. The amino acid sequence of the N-terminal half of the readthrough domain (P5) is conserved in all luteoviruses and the same applies to CpCSV. Guilley et al. (1994) identified in the C-terminal region a stretch of ca. 45 amino acids that is similar in the P5 of BWYV, CABYV and PLRV but absent from the P5 other polero- and luteoviruses and named it BWYV/CABYV/PLRV homology region. Such a motif is not present in CpCSV or other recently sequenced viruses namely BLRV, SbDV or ScYLV.

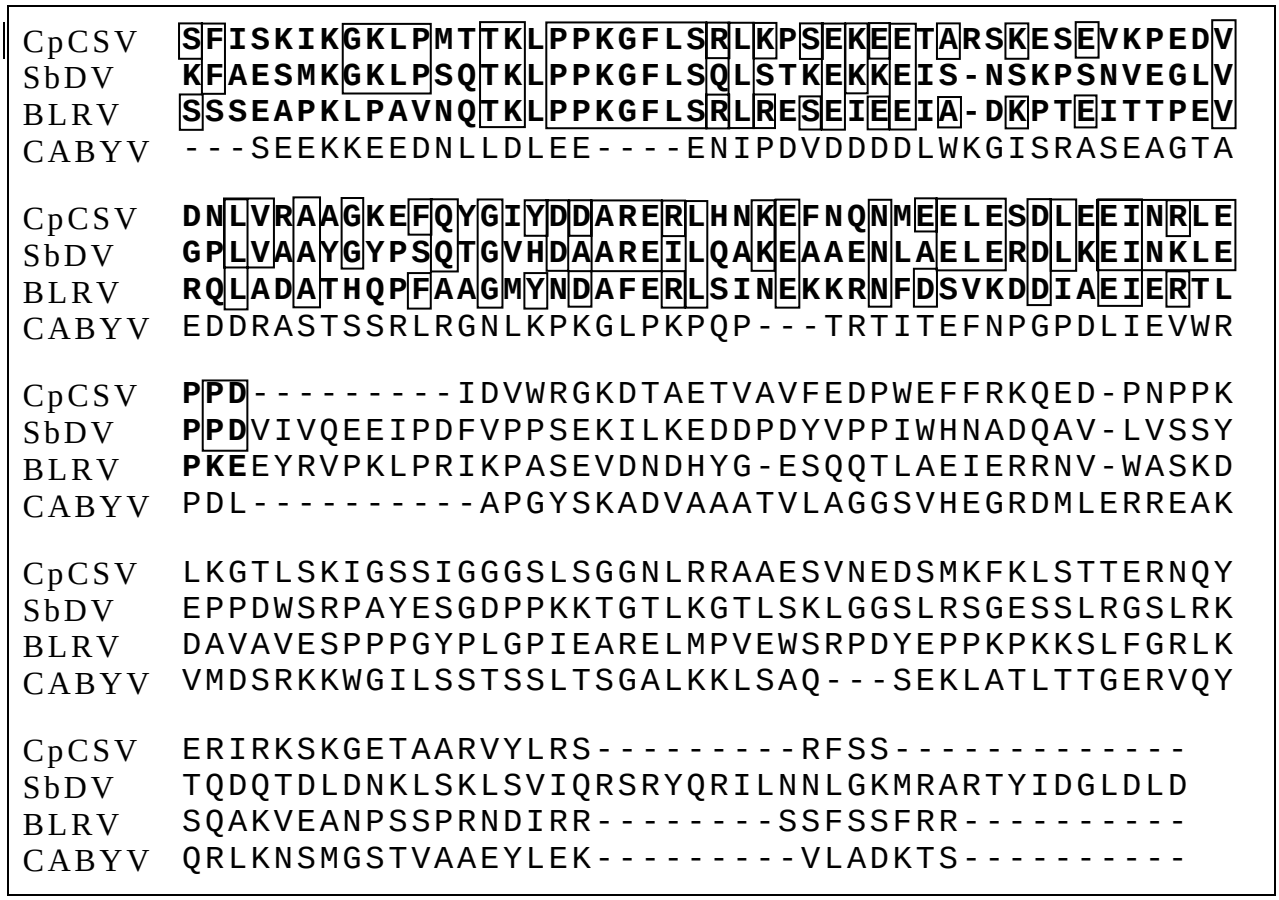

Fig. 5. Alignment of the C-terminal part of the readthrough domain (RTD) of CpCSV, SbDV, BLRV and CABYV. The RTD region where there is a striking similarity between CpCSV, SbDV and BLRV is in bold. Boxes indicate amino acid residues perfectly conserved (the amino acid substitutions F and Y, T and S, D and E, and R and K were also considered identical) between CpCSV and SbDV and/or BLRV but absent in any other luteovirus. CABYV was included in the alignment as it is the closest relative of CpCSV in the remaining part of the genome.

Although most parts of the CpCSV genome and particularly its 3' half were phylogenetically closely related to CABYV, a stretch of about 90 amino acids in the C-terminal part of its readthrough domain was strikingly different from CABYV but very similar (identity of ~50%) to the corresponding fragment of SbDV (Fig. 5). This led us to suspect a recombination event in this region. Recombination analysis using the Sister scanning program (Gibbs et al. 2000) gave a strong support for recombination (Z value >3) at this site (Fig. 6), suggesting that the CpCSV is a recombinant virus with most of its genome originating from an CABYV-like (or possibly

GRAV-like) ancestor which has acquired a P5 fragment from an SbDV-like ancestor. Interestingly, most of the conserved amino acid motifs in this region are found only in SbDV, BLRV (Fig. 5) and to a lesser extent in PEMV-1, all of which naturally infect legumes. It is therefore possible that this motif acts as a host range determinant. The effect of this stretch is also reflected in the BLAST search data for P5. This indicated that the CpCSV P5 is more closely related to the P5 of the luteoviruses SbDV and BLRV and the enamovirus PEMV-1 than to the P5 of poleroviruses other than CABYV. This is in sharp contrast to the relationships observed for other CpCSV proteins (Table 2).

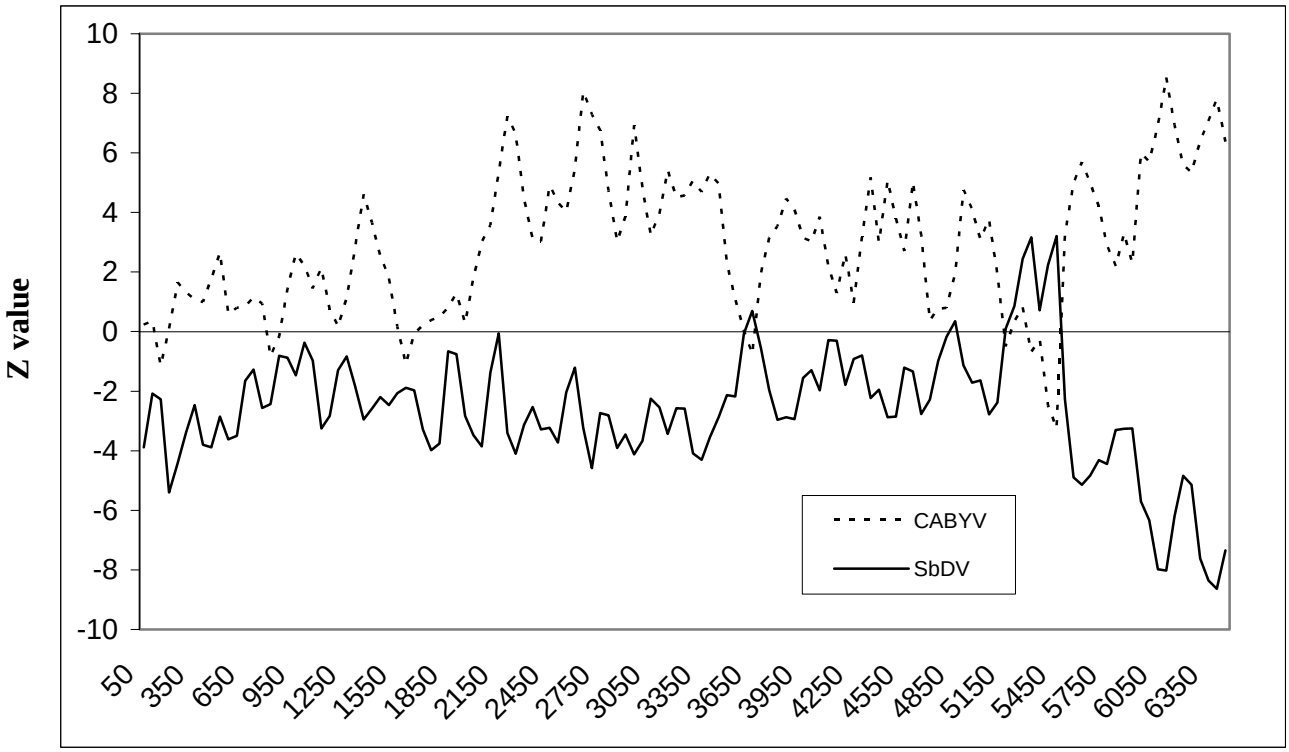

Fig. 6. Graph showing the result of the recombination analysis of aligned luteovirus genome sequences using the Sister scanning procedure. The complete nucleotide sequence of CpCSV was compared with those of CABYV (dashed line) and SbDV (solid line). Z score values greater than 3 strongly suggest a close phylogenetic relationship to a certain virus (Gibbs et al. 2000). Note that peaks at nt position 5300 and 5400 have Z score values of greater than 3.

Ashoub et al (1998) showed the presence of a second subgenomic RNA coding for P6 and P7 in PLRV and CABYV and proposed the existence of only a P7-encoding ORF7 in BWYV and

BMYV at the 3’ end of ORF5 and in the same reading frame as ORF5. In the CpCSV genome, we failed to find an ORF similar in size and arrangement to the ORF6 of PLRV or CABYV. However, a putative ORF7 (nt 5364-5716) with a favorable initiation context is predicted in the same frame with ORF5 (Fig. 1) and codes for a protein of 13.5 kDa, a size comparable to 14.5-kDa protein of PLRV ORF 7.

Phylogenetic and taxonomic relationship to other luteoviruses

Recombination events that occurred during luteovirus evolution have resulted in genome parts with different phylogenetic history. This has resulted in difficulties of classifying viruses such as BLRV, SbDV and ScYLV (Domier et al. 2002, Rathjen et al. 1994, Smith et al. 2000, Gibbs and Cooper, 1995). Available evidence suggests that most interspecies recombination events between luteoviruses involve the exchange of the whole 5’ half of the genome of one ancestral parent with the 3’ part of the other with the recombination site located at the sgRNA1 start site in the intergenic region (Miller et al. 1995, 1997)**.** These authors have proposed a model that in luteoviruses the start site of both sgRNAs (i.e. sgRNA1 and sgRNA2) acts as sites of recombination. In agreement with this model, the putative transition of the CABYV-related sequence to the SbDV-related sequence in CpCSV ORF5 coincides with the sgRNA2 transcription start site of CABYV described to be at nt position 4888 (Ashoub et al. 1998). Similar to our results, recombination analysis of the complete nucleotide sequences of BChV isolates indicated that the P5 of a European isolate has a stretch of sequence similar to that of BMYV while that of a Californian isolate has a sequence similar to that of TuYV (isolate BWYV-FL1) (Hauser et al. 2002). Although, the exact sgRNA2 start site for these poleroviruses has not yet been determined, the approximate location resembles that of CABYV. Our finding and this observation for the BChV genome support the aforementioned model and suggest that sgRNA2 start sites of some luteoviruses may be recombination hotspots just like those of sgRNA1.

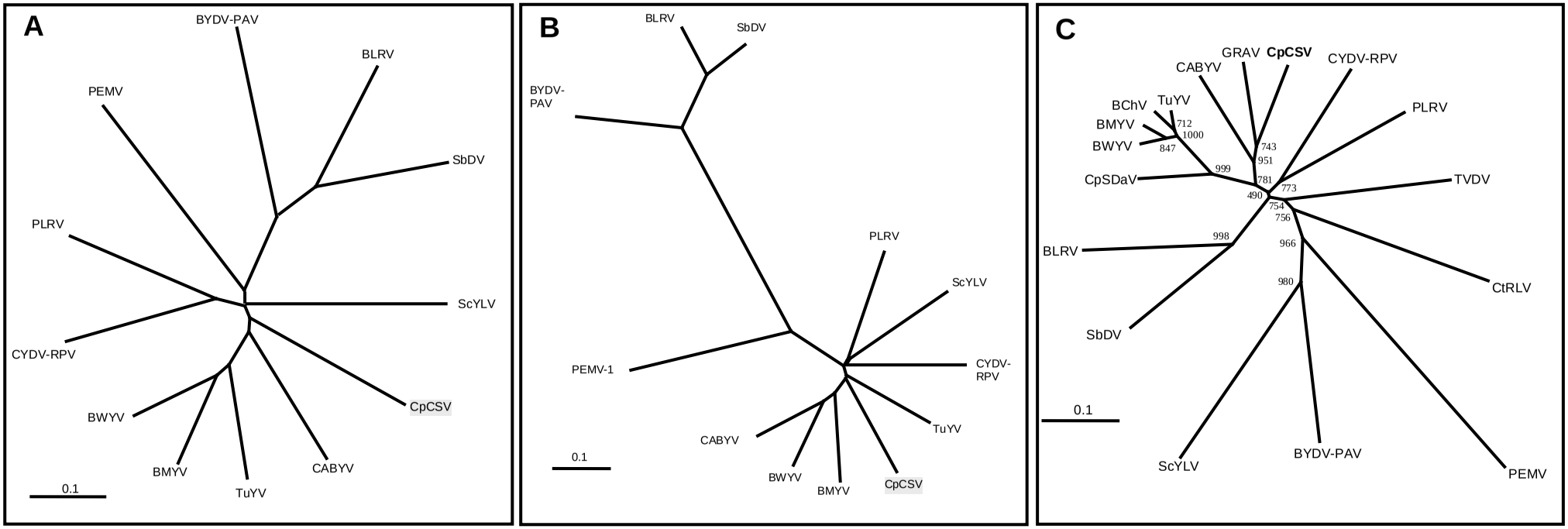

Fig. 7. Phylogenetic trees illustrating the relationship of CpCSV with other luteoviruses based upon (A) the total nucleotide sequence, (B) the amino acid sequence of the RNA-dependent RNA polymerase, and (C) the coat protein amino acid sequence. Trees were drawn using the neighbour joining method after multiple alignments with the ClustalX program.

The two genes widely used in luteovirus taxonomy are those coding for the coat protein and polymerase proteins since they are highly conserved among luteoviruses (D'Arcy and Domier, 2004). The phylogenetic relationships of the overall nucleotide, the polymerase and CP gene amino acid sequences of CpCSV are presented in Fig. 7 A-C. In all cases, CpCSV is placed in close proximity to poleroviruses such as CABYV, TuYV and BWYV. The CP phylogeny shows a closer relationship to GRAV, for which only the CP gene sequence is available. Although our result indicates that CpCSV is a possible recombinant virus in parts of its readthrough protein, it closely resembles poleroviruses in genome organization and phylogenetic relationships and presumably employs polerovirus-like replication and expression strategies. Moreover, the arrangement and nucleotide sequences of the genes in the 5' half of the CpCSV genome which are currently used as a primary criterion for assigning virus species (including recombinant viruses) to a genus in the family *Luteoviridae* (D'Arcy and Domier, 2004) resemble those of polerovirus genomes whose 5' half possibly originated from sobemovirus-like ancestors. Therefore, we propose CpCSV to be considered as a definitive member of genus *Polerovirus* of the family *Luteoviridae.*

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CHAPTER 5

Molecular evidence for the occurrence of two new and two known luteoviruses in cool season food legumes [4]

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ABSTRACT

Some faba bean, chickpea and lentil samples with yellowing and stunting symptoms that serologically reacted with broad-spectrum luteovirus antibody did not react with antibodies specific to previously known luteoviruses or the recently described Chickpea chlorotic stunt virus. This suggested the occurrence of further unrecognized luteoviruses. Amplification and sequencing of the coat protein (CP) gene from some of these samples provided evidence for the association of two previously unrecognized luteovirus sequences. Analysis of the CP sequence of a luteovirus isolate from chickpea in Sudan indicated that it is a distant relative of *Soybean dwarf virus* (SbDV) with which it shares a predicted CP amino acid sequence identity of only 66%. Since this warrants its classification as a distinct luteovirus, the name Chickpea yellows virus (CpYV) is proposed. An isolate from lentil in Ethiopia shared a CP amino acid sequence identity of 86% with viruses of the Beet western yellows virus subgroup. Due to the fact that this is less than the currently accepted threshold value of 90% in amino acid sequence identity for discriminating different luteovirus species, this isolate also appears to represent another distinct luteovirus species for which the name Lentil stunt virus (LStV) is proposed. In addition to these new luteoviruses, almost identical CP gene sequences of *Turnip yellows virus*-like virus were amplified from faba bean samples from Egypt and Morocco. CP gene sequences strikingly closely related to those of SbDV were amplified from faba bean samples from Ethiopia, Germany, Syria and China, suggesting that this virus has a much wider geographic distribution than originally thought. SbDV is reported here for the first time from Germany and Europe.

[4] The content of this paper will be published as Short Commmunications in an international journal.

INTRODUCTION

Faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L:) and lentil (*Lens culinaris* Medik) are among the major food legumes in many countries of the world. Luteoviruses namely *Bean leaf roll virus* (BLRV), *Beet western yellows virus* (BWYV) *Soybean dwarf virus* (SbDV) and *Pea enation mosaic virus-1* (PEMV-1) have been reported to infect these legume crops commonly in countries of Northeast and North Africa and West Asia *(*Makkouk et al. 1988, 1995, 2003a, Abraham et al. 2000, Tadesse et al. 1999, Fortass et al. 1997). Recently, polerovirus isolates previously considered as BWYV have been reclassified as four distinct virus species, namely BWYV, *Beet chlorosis virus* (BChV), *Beet mild yellowing virus* (BMYV) and *Turnip yellows virus* (TuYV) based primarily on variation differences in their ORF0 sequences and biological properties (Hauser et al. 2000, D'Arcy and Domier, 2004). Since many previously described BWYV-like isolates cannot be easily assigned to one of these virus species due to lack of ORF0 sequence information, for the purpose of this paper the term BWYV subgroup will be used.

In addition to the aforementioned viruses, a new polerovirus proposed to be named Chickpea chlorotic stunt virus (CpCSV) has been recently described as a cause of stunting and yellowing disease in some countries (Abraham et al submitted, 2005). However, several samples collected from different countries that show typical virus-like symptoms and reacting with broad spectrum luteovirus monoclonal antibodies 2-5G4 (Katul, 1992) did not react with antibodies specific to any of the known luteoviruses, suggesting the occurrence of other possibly unrecognized viruses. To obtain accurate information on the nature of these unidentified luteoviruses from various legume samples, attempts were made to acquire viral coat protein (CP) gene sequences from the samples. Our results provided molecular evidence for the occurrence of two new luteoviruses as well as that of TuYV and SbDV in these samples.

MATERIALS AND METHODS

Origin of samples and serological tests

The country of origin, the year of collection of representative samples from which luteovirus sequences were amplified are presented in Table 1. The leaf samples were preserved by drying over $CaCl_2$. Serological tests were conducted as TAS- (Franz et al. 1996) and DAS-ELISA (Clark and Adams, 1977) using different virus-specific antibodies including a broad

spectrum monoclonal antibody to luteovirus (2-5G4) (Katul, 1992) and known luteovirus-specific antibodies to BLRV, CpCSV, SbDV and BWYV. The source and specificity of the antibodies used has been described by Abraham et al. (submitted, 2005).

Table 1. Origin and collection year of samples from which luteovirus sequences were amplified.

Isolate	Plant host	Location, country	Year of collection	Virus sequence amplified
Et-le-3	Lentil	Ambo, Ethiopia	2002	LStV (new)
Eth26	Faba bean	Degem, Ethiopia	2003	SbDV
Eth32	Faba bean	Grar Jarso, Ethiopia	2003	SbDV
Eth57	Faba bean	Morat-Jiru, Ethiopia	2003	SbDV
Et61	Faba bean	Insaro-Wayu, Ethiopia	2003	SbDV
Et72	Faba bean	Checha, Ethiopia	2003	SbDV
Su-cp-8	Chickpea	Abu Harras, Sudan	1997	CpYV (new)
Mo-fb-21	Faba bean	Ouled Ayyad, Morocco	2001	TuYV-like
Mo-22	Faba bean	Ouled Ayyad, Morocco	2001	TuYV-like
EVp7-93	Faba bean	Fayoum, Egypt	1993	TuYV-like
EV2-94	Faba bean	Fayoum, Egypt	1994	TuYV-like
EV3-94	Faba bean	Fayoum, Egypt	1994	TuYV-like
SL1-94	Faba bean	Tel Hadya, Syria	1994	SbDV
Ger1	Faba bean	Kassel, Germany	2003	SbDV
Ger2	Faba bean	Kassel, Germany	2003	SbDV
Ch-601	Faba bean	China	2003	SbDV

Virus acronyms used are SbDV, *Soybean dwarf virus;* TuYV, *Turnip yellows virus;* LStV, Lentil stunt virus (proposed); CpYV, Chickpea yellows virus (proposed).

RT-PCR, cloning and sequence analysis of coat protein gene

Selected samples that reacted positively only with 2-5G4 but not with antibodies specific to CpCSV were used for sequencing the CP gene. Total RNA extraction, amplification of viral RNA by RT-PCR, cloning and sequencing was essentially done as described by Abraham et al. (submitted, 2005). To be able to amplify a most diverse range of luteovirus sequences, two sets of degenerate primers designed from conserved sequences of known luteovirus sequences available from the database were used. The first set included a combination of a sense primer S1 (5'GCTCTAGAATTGTTAATGARTACGGTCG3') and antisense primer AS3 (5'CACGCGTCIACCTATTTIGGRTTITG3') (I = inosine) which in previous studies (Abraham et al. submitted, 2005) was used to amplify CpCSV sequences. This primer pair is expected to amplify the complete CP gene of all known legume poleroviruses but not viruses like SbDV and BLRV since S1 does not adequately match their sequences. The second pair was a combination of the sense primer S2 (5'ATCACITTCGGGCCGWST-CTATCAGA3') with the antisense primer AS3, expected to amplify a product of ca. 340

bases from the C-terminal part of CP gene of all known legume luteoviruses including BLRV and SbDV. DNA sequencing was done by a commercial company (MWG Biotech) from two independent clones in both directions. Sequence assembly and pairwise comparison was carried out using the computer software DNAMAN (Lynnon, Biosoft, Canada). Phylogenetic analyses of nucleotide and amino acid sequences were carried out using a Clustal_X program after multiple alignment of sequences by neighbour joining algorithms (Thompson et al. 1997) and visualized using Treeview program (Page, 1996). The sequences were compared with available sequences in the database using the basic local alignment search tool (BLAST) program (Altschul et al. 1997). Databank accession numbers of luteovirus sequences used for comparison are indicated in Table 2. In addition, sequences of SbDV isolates used for comparison were those described from Japan (AB 038147-AB 038150) and USA (U51448 and L20835).

Table 2. Pairwise comparison of the percentage CP gene amino acid sequence identity of three distinct luteovirus sequences amplified from three samples with that of other luteoviruses from the database, CpCSV and to each other.

Virus/Isolate	Access. No.	Samples		
		Su-cp-8	Et-le-3	Mo-fb-21
Bean leaf roll virus (BLRV)	NC 003369	64.8	56.3	55.4
Beet mild yellowing virus (BMYV)	X83110	60.5	82.7	91.6
Beet western yellows virus (BWYV)	NC 004756	60.5	84.2	92.1
Turnip yellows virus (TuYV)	X13063	61.5	**86.1**	**94.6**
Cucurbit aphidborne yellows virus (CABYV)	NC 003688	61.3	75.1	70.6
Groundnut rosette assistor virus (GRAV)	AF195828	64.6	73.4	74.1
Potato leafroll virus (PLRV)	D 00734	61.1	66.8	65.2
Ceral yellow dwarf virus (CYDV)-RPV	NC 004751	57.3	68.0	68.3
Pea enation mosaic virus-1 (PEMV-1)	L4573	32.6	37.1	34.1
Sugarcane yellow leaf virus (ScYLV)	NC 000874	38.1	42.8	43.1
Chickpea stunt disease associated virus (CpSDaV)	Y11530	60.1	80.4	80.7
Soybean dwarf virus (SbDV)	NC 003056	**66.3**	59.1	57.4
Barley yellow dwarf virus (BYDV)-PAV	D 85873	46.4	50.5	49.0
Carrot red leaf virus (CtRLV)	NC 006265	41.8	52.5	54.5
Chickpea chlorotic stunt virus (CpCSV)	-	64.5	75.0	76.0
Eth-Le-3	-	59.2	-	87.1
Sud-cp-8	-	-	59.2	61.5
Mo-fb-21	-	61.5	87.1	-

RESULTS

Many of the samples tested reacted with both the general luteovirus antibody (2-5G4) and CpCSV polyclonal and monoclonal antibodies while others reacted only with 2-5G4. Those samples that did not react with CpCSV antibodies were used for RT-PCR amplification of

the CP-gene and subsequent sequencing. These included some faba bean samples from Egypt and Morocco that reacted specifically to polyclonal BWYV antibodies and those from Ethiopia, Syria and Germany that reacted with SbDV polyclonal antibodies. A chickpea sample from Sudan (Su-cp-8) and a lentil sample from Ethiopia (Et-le-3) reacted with 2-5G4 but did not show clear reactions with the virus specific polyclonal antibodies used. None of the samples reacted with BLRV antibodies.

Using the primer pair S1/AS3, amplicons of about 600 bases were obtained from some faba bean, chickpea and lentil samples. Cloning and sequence analysis indicated the presence of diverse luteovirus sequences that included previously unrecognized viruses. Pairwise comparison of CP amino acid sequences revealed three distinct types of sequences when compared to those of other luteoviruses from the database as shown in Table 2. A sequence from a chickpea sample from Sudan (Su-cp-8) shared closest amino acid sequence identity (only 66.3%) with SbDV followed by BLRV (64.8%) (Table 2). Phylogenetic analysis (Fig. 1) also showed that the isolate is closer to SbDV and BLRV than to other luteoviruses. However, it differs from SbDV and BLRV as much as they are distinct from each other.

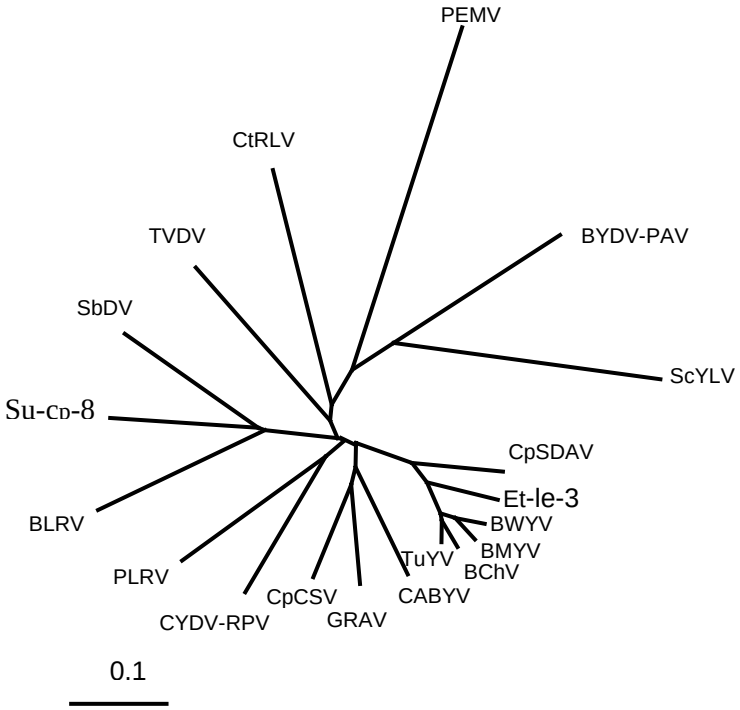

Fig. 1. Unrooted dendrogram showing the phylogenetic relationship of the predicted coat protein amino acid sequences of Su-cp-8 and Et-le-3 with those of other luteoviruses from the database. Database accession number of the luteovirus sequences and virus acronyms used are given in Table 2.

Multiple alignment of the predicted amino acid sequence encoded by Su-cp-8 with those of SbDV and BLRV is shown in Fig. 2. The difference in amino acid sequence is distributed

over the entire CP polypeptide chain although the N-terminal part of the protein is more divergent than the C-terminal part.

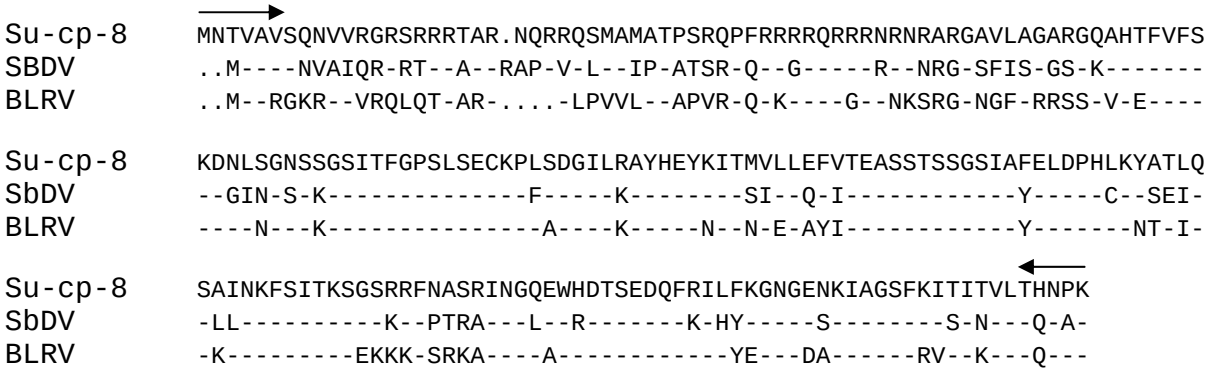

Fig. 2. Alignment of the predicted coat protein amino acid identity of Su-cp-8 with those of *Soybean dwarf virus* (SbDV) and *Bean leaf roll virus* (BLRV). The arrows indicate the primer sequences. Database accession numbers of SbDV and BLRV sequences are indicated in Table 2.

A luteovirus sequence obtained from a lentil sample from Ethiopia (Et-le-3) was also distinct from all other luteovirus sequences known so far. Pairwise comparison of the predicted CP amino acid sequences with those of other luteoviruses showed that it is most closely related to TuYV with an identity of 86% (Table 2). It is phylogenetically close to viruses of the BWYV subgroup (Fig. 1) but appears to be less related to species of this subgroup than

Fig. 3 Alignment of the predicted coat protein amino acid identity of Et-le-3 with those of *Turnip yellows virus* (TuYV), *Beet western yellows virus* (BWYV), *Beet chlorosis virus* (BChV), *Beet mild yellowing virus* (BYMV) and *Chickpea stunt disease as-*

sociated virus (CpSDaV). The arrows indicate the primer sequences. Database accession number of the luteoviruses are given in Table 2.

they are to each other. Multiple alignment of predicted amino acid sequences encoded by Et-le-3 with viruses of BWYV subgroup and CpSDaV shows (Fig. 3) that most of the amino acid differences are concentrated at N-terminal part of its CP.

Using the primer pair S1/AS3, CP sequences of luteovirus isolates very similar to TuYV were also amplified from three faba bean samples from Egypt and two from Morocco (Table 1). The predicted amino acid sequences of the coat proteins of all the five isolates sequenced showed that they were almost indistinguishable from each other (99-100% identity). A representative isolate from Morocco (Mo-fb-21) used for sequence comparison had the closest CP amino acid sequence identity (95%) with TuYV but was also highly similar (92%) to BWYV and BMYV sequences (Table 2).

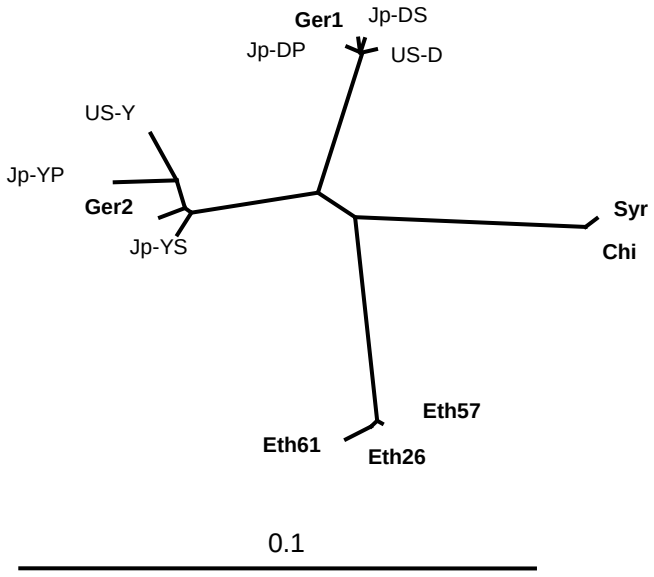

Fig. 4. Unrooted dendrogram showing phylogenetic relationship among partial coat protein nucleotide sequences of SbDV isolates. Sequenced isolates are from Ethiopia (Eth26, Eth57 and Eth61), Germany (Ger1 and Ger2), Syria (Syr) and China (Chi) while sequences from Japan (Jp-YP, -YS, -DS and –DP) and USA (US-Y, US-D) were obtained from the databank. Isolates with Y, YS, YP cause yellowing symptoms in soybean while D, DP and DS cause dwarfing symptoms.

SbDV sequences of ca. 340 nt from the C-terminal part of the CP gene were obtained from faba bean samples from Ethiopia, Germany, Syria and China using S2/AS3 primer pair. The percentage nucleotide sequence identity between the isolates ranged from 90-100%. Phylogenetic comparison of the nucleotide sequence of the isolates with those from the database showed that the grouping is roughly correlated to the geographical origin of the samples

with the exception of the German isolates. Asian (Chinese and Syrian) isolates have nearly identical sequences and formed a distinct cluster as different from isolates previously reported from Japan and USA while the Ethiopian isolates are homogeneous among themselves and formed their own cluster (Fig. 4). The two German isolates, however, fall into clusters comprising the two different strains from Japan and USA. Interestingly, one of the German isolates clustered with dwarfing isolates originating from USA and Japan while the other clustered with yellowing strains suggesting that they might belong to the respective symptom groups.

DISCUSSION

The sequence data presented indicated the association of luteoviruses with distinct CP sequences some of which are clearly different from CpCSV (Abraham et al. submitted, 2005) and other previously described luteoviruses with the samples studied. In particular, Su-cp-8 and Et-le-3 have sequences only remotely related to the previously described luteoviruses SbDV and TuYV with percentage amino acid sequence identities of 66% and 86%, respectively (Table 2). According to current luteovirus species demarcation criteria recommended by the International Committee of Taxonomy of viruses (ICTV) (D'Arcy and Domier, 2004), distinct virus species should differ by more than 10% in amino acid sequences of one of their gene products and also have distinct biological and serological properties (D'Arcy and Domier, 2004). Since the percentage identity of the CP gene amino acid sequence of both Su-cp-8 and Et-le-3 was clearly less than the identity threshold value of 90%, these isolates should be considered as two distinct and new viruses. To facilitate future studies, the name Chickpea yellows virus (CpYV) is proposed for Su-cp-8 since the virus was isolated from plants showing yellowing symptom. Similarly, for Et-le-3 which was isolated from a lentil plant with stunting symptom, a tentative name Lentil stunt virus (LStV) is suggested. However, to consider these viruses as definitive species in the family *Luteoviridae*, information on their biological and serological properties is required.

Phylogenetically CpYV is most closely related to SbDV and BLRV (Fig. 1). Tadesse et al. (1999) reported the occurrence of a virus that serologically reacted with MAb 4-3B11, an antibody known to react only with SbDV and BLRV (Katul, 1992) from chickpea samples in Ethiopia. The authors further indicated that these samples did not react with antibodies specific to SbDV or BLRV. It is possible that these samples are infected with a virus that shares a common epitope with these two viruses. Since CpYV is closer in its CP sequence to

these two viruses than any other known luteovirus, the possibility that these samples are infected with CpYV cannot be ruled out. Further virus surveys in legume growing areas of the world including Ethiopia and Sudan should be conducted to obtain information on the occurrence and geographical distribution of this new virus.

It was previously reported that BWYV is the most common virus in chickpea and lentil in Ethiopia (Tadesse et al 1999). However, in a recent study (Abraham et al. submitted, 2005) no BWYV subgroup viruses were detected serologically or by molecular means suggesting that these group of viruses are at least not widespread. At present, it is not clear whether LStV or any other virus of the BWYV group is more common in Ethiopia.

At present, the only legume (faba bean) luteovirus isolate from BWYV subgroup for which ORF0 sequence data is available is from France which on the basis of this recent reclassification should be considered a TuYV isolate (Hauser et al. 2000). However, Fortass et al (1997) reported the CP gene sequence of a chickpea isolate of BWYV from Morocco although the sequence is not available in the database. Pairwise comparison of this sequence with that of our faba bean isolate from Morocco (Mo-fb-21) and the TuYV sequence from the database indicated that the chickpea isolate from Morocco shares 95% amino acid sequence identity with each of them. These observations suggest that BWYV-like isolates from faba bean and chickpea from Morocco and Egypt are likely to be TuYV isolates. However, since sequence information of ORF0 for these isolates is not yet available, we tentatively prefer to these isolates as Turnip yellows virus-like isolates.

The occurrence of SbDV is reported here for the first time from Germany and Europe at large. It is likely that further testing of faba bean in Germany or elsewhere in Europe would provide further information on the incidence and distribution of the virus. The data also presents the first report of SbDV infecting faba bean in Ethiopia and China indicating that SbDV has wider geographical distribution than it was once thought. SbDV isolates from soybean are categorized into four groups of isolates based on the type of symptoms and vector aphids (Terauchi et al. 2001). Apart from some biological and serological data on a lentil isolate of SbDV from Syria (Makkouk et al. 1997), little information is available on isolates infecting cool season food legumes in the region. It is not clear whether the two German isolates each of which clustered with yellowing and dwarfing strains of soybean (Fig. 2) also show different symptoms in faba bean. Sequence and biological information on more isolates from different geographical area would help to obtain a better understanding of the nature of variation of SbDV isolates from different crops and geographical origin.

Since both CpYV and LStV are proposed here as new viruses based only on information on the CP gene sequence, there is a need to substantiate these results with reliable serological and biological data. The bottleneck for such future study would be the lack of serological or molecular diagnostic tools for specific detection and identification of these new viruses from field samples. In this respect, the sequence information generated here could be used for designing specific PCR primers that can be used for diagnostic purposes. In addition, until suitable virus propagation and purification protocols are developed, the CP sequence information may be used for producing virus-specific antibodies using bacterially expressed coat protein.

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CHAPTER 6

Analysis of the ssDNA genome of two serologically distinct nanovirus isolates from faba bean in Morocco [5]

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ABSTRACT

Using monoclonal antibodies raised against a *Faba bean necrotic yellows viru*s (FBNYV) isolate from Egypt and an Ethiopian nanovirus isolate proposed to be named Faba bean necrotic stunt virus (FBNSV), a considerable serological variability among nanovirus isolates from faba bean in Morocco was revealed. To obtain an enhanced understanding of this nanovirus variability in Morocco, the entire genomes of two serologically contrasting isolates referred to as Mor5 and Mor23 were sequenced. The eight circular ssDNA components, each identified from Mor5- and Mor23-infected tissues and thought to form the complete nanovirus genome, ranged in size from 952 to 1005 nt for Mor5 and from 980 to 1004 nt for Mor23 and were structurally similar to previously described nanovirus DNAs. However, Mor5 and Mor23 differed from each other in overall nucleotide and amino acid sequences by 25% and 26%, respectively. Mor23 was most closely related to typical FBNYV isolates described earlier from Egypt and Syria, with which it shared a mean amino acid sequence identity of about 94%. On the other hand, Mor5 most closely resembled a FBNSV isolate from Ethiopia, with which it shared a mean amino acid sequence identity of approximately 89%. The serological and genetic differences observed for Mor5 and Mor23 are comparable to those observed earlier for FBNYV, FBNSV, and *Milk vetch dwarf virus*. In agreement with the current criteria for nanovirus species demarcation, this suggests that Mor23 and Mor5 represent isolates of FBNYV and FBNSV, respectively. This is the first report on the occurrence and complete genome sequence of two nanovirus species in a country, thus providing evidence for faba bean crops being infected by two distinct nanovirus species in a restricted geographic area.

[5] This paper will be submitted for publication in an international journal, such as *Archives of Virology*.

INTRODUCTION

The recently established family *Nanoviridae* comprises the genera *Babuvirus* and *Nanovirus*. Whereas *Banana bunchy top virus* (BBTV) is the only species of the genus *Babuvirus*, the genus Nanovirus currently comprises three species, namely *Faba bean necrotic yellows virus* (FBNYV), *Milk vetch dwarf virus* (MDV) and *Subterranean clover stunt virus* (SCSV) (Vetten et al. 2004). Nanoviruses cause severe yield losses in a wide range of legume crops in Australia, Spain, and many countries of Africa and Asia (Makkouk et al. 2003, Chu & Vetten 2003, Vetten & Katul 2001). They are persistently transmitted by aphids such as *Aphis craccivora* and *Acyrthosiphon pisum* and have a wide host range which is, however, largely restricted to plant species of the family Fabaceae (Franz et al. 1997, 1998; Vetten & Katul 2001, Chu & Vetten 2003).

Members of the *Nanoviridae* have a multipartite single-stranded (ss) DNA genome. Each of its circular ssDNA components ranging in size from 977 to 1111 nt appears to be individually encapsidated in a small isometric particle measuring only 18 nm in diameter. Up to 12 distinct DNA components have been identified from virion preparations of different nanovirus species. Although some of the DNAs have been shown to be satellite-like DNAs encoding Rep proteins that only initiate replication of its cognate DNA but are not required for the replication of the viral DNAs (Timchenko et al. 1999; Horser et al. 2001a, 2001b), the number and types of ssDNA components constituting the integral genome parts have not been experimentally determined yet for any of the species. However, there is circumstantial evidence that the babuvirus genome consists of six distinct ssDNAs while the nanovirus genome comprises eight species of circular ssDNA. All of them seem to be structurally similar in being positive sense, transcribed in one direction, and containing a conserved stem-loop structure and other conserved domains in the noncoding region (NCR) (Vetten et al. 2004).

To standardize the naming of the various DNAs that are considered integral parts of the nanovirid genome, a new nomenclature based on functional equivalence and/or sequence homology of DNAs and their encoded proteins has been recently adopted. Accordingly, homologous DNAs coding for master Rep, structural (coat), cell-cycle link, movement and nuclear shuttle proteins are referred to as DNA-R, -S, -C, -M and –N, respectively. The remaining four non-rep DNAs for which the functions are yet unknown are named DNA-U1 to -U4 (Vetten et al. 2004).

The production of 19 monoclonal antibodies (Mabs) raised against a typical FBNYV isolate from Egypt (FBNYV-Eg) not only contributed to more sensitive detection of FBNYV in

plants and aphids but also permitted the identification of at least six distinct epitopes on particles of FBNYV-like nanovirus isolates (Franz et al. 1996). The observation that polyclonal antibodies to FBNYV-Eg gave weak and strong reaction with SCSV and MDV, respectively (Katul et al. 1993), and that one and 16 of the 19 Mabs to FBNYV-Eg cross-reacted with SCSV and MDV, respectively (Franz et al. 1996), suggested that the serological relationship of FBNYV to MDV is close and that to SCSV is only distant. On the other hand, this also indicated that the majority of the Mabs to FBNYV-Eth are unable to discriminate FBNYV not only from MDV but also from other yet unknown nanovirus species that are closely related to FBNYV. Therefore, we cannot rule out the possibility that the frequent use of these non-discriminating Mabs may have led to the erroneous serological identification of FBNYV in several Asian and African countries. The fact that several Ethiopian faba bean samples containing a FBNYV-like isolate failed to react with four of the 19 Mabs to FBNYV-Eg (Franz et al. 1996), prompted us to sequence the genomic DNAs of a representative nanovirus isolate from Ethiopia. Sequence analysis of the eight genomic DNAs of the Ethiopian isolate indicated (Katul and Vetten 1999) that its DNA sequences are notably distinct from those of typical FBNYV isolates from Egypt and Syria (Katul & Vetten, 1999). Since the DNA sequences of the Ethiopian isolate differed from those of FBNYV to the same extent (by 25%) as FBNYV differs from MDV(Katul and Vetten, 1999), the Ethiopian isolate seems to represent a distinct nanovirus species for which the name Faba bean necrotic stunt virus (FBNSV) has been proposed (Katul and Vetten, unpublished). This taxonomic assignment is in agreement with the current criteria for nanovirus species demarcation (Vetten et al. 2004). Furthermore, Mabs that react specifically with the FBNSV isolate from Ethiopia (FBNSV-Eth) but not with different FBNYV isolates have been also produced meanwhile and appear to allow for specific detection of FBNSV (Vetten, unpublished data).

Morocco is one of the major faba bean growing countries where viruses such as *Broad bean mottle virus* (BBMV) and luteoviruses are among the important production constraints (Fortass and Bos, 1991, Fortass et al. 1996). Although there is unconfirmed serological evidence for occurrence of FBNYV-like nanovirus isolates in faba bean crops in Morocco (Franz et al. 1996), the relative importance of this (these) virus(es) for faba bean production in the country is unknown. Apart from the observation (Franz et al. 1996) that nine faba bean samples from the Fez area and one faba bean sample from Meknes failed to react respectively with one and two of the 19 Mabs to FBNYV-Eg, none of the FBNYV-like isolates from Morocco has been adequately characterized.

The epitope profiles observed for few incidentally collected samples from Morocco (Franz et al. 1996) indicated that the nanovirus isolates in the country differ to some extent from typical FBNYV isolates. This prompted us to conduct a serological analysis of a limited number of geographically diverse faba bean samples from Morocco using the available panel of MAbs. Since two of the samples gave very contrasting epitope profiles, they were selected for nucleotide sequencing. Sequence analysis of the eight ssDNAs that are thought to make up the genome of the two serologically distinct nanovirus isolates from Morocco provided strong evidence for the occurrence of two nanovirus species, a largely typical FBNYV isolate and a FBNSV strain distinctly different from the type isolate of FBNSV from Ethiopia.

MATERIALS AND METHODS

Virus isolates and serological analysis

Ten leaf samples collected from faba bean plants showing yellowing, stunting and necrosis symptoms in farmers' fields in different areas of Morocco in 2001 were analyzed serologically by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) as described by Franz et al. (1996). The geographic origin of the samples is given in Table 1. While polyclonal IgG to FBNYV (Katul et al. 1993) was used for antigen trapping, the following Mabs were used individually as detecting antibodies in TAS-ELISA:

(i) a mixture of three broad spectrum Mabs (1-1F2, 2-1A1, 3-4F2) to FBNYV-like nanoviruses (Franz et al. 1996);

(ii) Mabs 1-3D8, 2-5H9, 2-3E12-D5, and 3-4A5 raised against an FBNYV isolate from Egypt (FBNYV-Eg) but discriminating some FBNYV-like viruses (Franz et al. 1996), and

(iii) the Mabs 8-2G10, -4F9, -6F8, and -8G11 specific to an Ethiopian isolate of FBNSV (FBNSV-Eth) (Vetten, unpublished data).

Table 1. Origin and TAS-ELISA reactions of faba bean samples from Morocco with broad-spectrum and discriminating monoclonal antibodies (Mabs)

Faba bean samples	Collection site	Broad-spectrum Mabs to nanoviruses	Mabs raised against FBNYV-Eg but discriminating nanovirus isolates				Mabs specific to FBNSV-Eth			
			2-3E12-D5	3-4A5	1-3D8	2-5H9	8-4F9	8-6F8	8-2G10	8-3G11
Mor1	Sidi El Aidi, Settat, Chaouia	+++ *	—	+++	+++	—	—	+	—	+
Mor4	Ouled Said, Settat, Chaouia	+++	—	+++	+++	+	—	++	—	++
Mor4'	Ouled Abbou, Settat, Chaouia	++	—	++	++	—	—	—	—	—
Mor5	Boulaouane, El Jadida, Doukkala	+++	—	—	—	+	+++	+++	+++	+++
Mor19	Souk Sebt, Ben-Mellal, Tadla	+++	—	+++	+++	+	—	+	—	++
Mor23	Fkih Ben Salah, Ben-Mellal, Tadla	+++	—	++	+++	—	—	—	—	+
Mor23'	Fkih Ben Salah, Ben-Mellal, Tadla	+++	—	+++	+++	—	—	+	—	++
FBNYV-Eg **		+++	+++	+++	+++	+++	—	—	—	—
FBNSV-Eth **		+++	—	—	—	—	+++	++	+++	+++

* * Extinction values ($A_{405\ nm}$) for 10-fold diluted leaf extracts and following a substrate incubation period of 1 h are shown. $A_{405\ nm}$ values were classed as +++ (> 0.8), ++ (0.2 to 0.8), + (< 0.2), and — (less than two times the $A_{405\ nm}$ value for the non-infected control).

** FBNYV-Eg and FBNSV-Eth against which the monoclonal antibodies had been raised were used as control antigens.

Oligonucleotide primers used and immunocaptue (IC)-PCR

For amplification of the Mor5 and Mor23 genomes, primer pair P3/20 (Timchenko et al. 1999) was used to selectively amplify a part of DNA-R. Since universal primers specific for a particular nanovirus DNA (except for DNA-R) are not available, primer pairs 2F3/44R and 75F/76R derived from conserved domains in the non-coding region of the non-*rep* DNAs of FBNYV (Katul and Vetten, unpublished) were used to randomly amplify a part of several non-*rep* DNAs. In addition, several primers specific to one or several DNA components of each of the isolates were designed and used to randomly or selectively amplify the respective DNA components or to obtain the complete sequence of an already identified component. The sequences of the primer pairs used are listed in Table 2.

Table 2. Oligonucleotide primer pairs used for amplification of Mor5 and Mor23 sequences. The letters F and R following the primer designation denote the orientation as forward and reverse, respectively.

Designation of primer pair	Forward (F) primer	Reverse (R) primer
1. 23F / 44R	CACGAATCACAGATCCTGAT	AAGCGAA(A/T/C)CTGACGGAAGA
2. 75F / 76R	TAGTATTACCCCGTCCC	GTAATACTAAGCCCCGTC
3. 3F / 20R	ATATGCTGGTGCTTTACA	AATTACAATCCTATCCTCACT
4. 50F / 55R	TTAAGTTAATAAATGACATCTATC	GCTGATACTATCCTTCCATG
5. 51F /53R	ACGAAGCAAAGCATATATTCAGTGC	TGGAAGCGTGGTTGAATTCTTCATC
6. 45F / 47R	AACTCTCCATACTCCCAWGGACCTTCAA	TATGGCCCACAAGGTGGAGAAGGG
7. M3F / M3R	TACACGCAGTTGAAGAACACA	CTGGGAATCATCATCACAGCA
8. M5F / M5R	GCCAGGATAATATGATTACTATAA	TAATTCACTAACTCTCCAGGA
9. 9C4F / 9C4R	ATCCTGTAAATCAGCCTAATACTGGAA	TGGAGTTCTGGTAAGTGTTGAAGCAA
10. 9C6F / 9C6R	GAAGAAGCCTTAGGAGAGTTTTGTA	AGGTACTCATGATATGAATTCCAGAAA
11. M12F / M12R	CAATCAATGATGATACGCAACAA	GCGAACTTCCATCTGAACTA
12. C4C6F/C4C6R	GATGGTCCCCACTCCGCACTAAC	CTGAAGCATACCGCTACGCGGAG
13. moC6F/moC6R	TAGTTCGGAAGGCTTAGCTG	ATGATACGAGCTCCAGAATC
14. moC10F/moC10R	CCTGTAGACATGGATGACCTA	GGTCTTCAGTAATCAACCGTT
15. moC8F/moC8R	CGGACTCAATCTCAGCCGTT'	AACCACGAAGCTTCCTCGG
16. moC12F/moC12R	TTATCTCTTCACGCGGTTTCG	GCCCAATAGGCCCAACATAAA
17. mopC3F/mopC3R	GGATATCATCTGTAGAAGATG	ATTAAAGTAGACCCACTTGC
18. mopC5F/moC5R	TTGGAATTGGTCAGGCAAG	CTGGTATTTATAGAGAGAGA

Amplification of viral sequences was done in a 0.5 ml Eppendorf tube using the IC-PCR procedure described by Franz et al. (1999). The PCR mixture consisted of 5 µl 10x PCR buffer, 2 mM $MgCl_2$, 0.6 mM dNTPs, 4 µM of each primer and 1 µl Taq polymerase (Gibco, 5 U/µl). PCR was performed in a MJ Research (Massachusetts, USA) thermocycler with a temperature profile of 5 min at 95°C for initial denaturation followed by 30 cycles of 1 min at 95°C for

denaturation, 1 min at 40-65°C for annealing depending on the primer composition and 1 min at 72°C for extension, and followed by a final extension of 10 min at 72°C.

Cloning and analysis of restriction fragment length polymorphisms (RFLPs)

PCR products were subjected to electrophoresis on a 1% agarose gel and purified using the Nucleospin® Extract kit (Macherey-Nagel, Düren, Germany). Purified products were ligated into a PGEM-T® vector (Promega) and transformed by heat shock to competent *E.coli* cells (DH5α) as described by Sambrook et al. (1989). Clones were examined for inserts by PCR using standard T7 and SP6 vector primers. Initially, two or three randomly selected clones were sequenced to obtain a part of one to three non-*rep* DNA components. To obtain the remaining non-*rep* DNAs, several dozens of the generated clones were individually subjected to RFLP analysis of PCR products. For this, cloned PCR products were digested using 13 restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hind*III, *Hpa*I, *Nco*I, *Pvu*II, *Sty*I, *Xba*I, *Xho*I, *Tth*111I) (Fermentas) according to the manufacturer's instructions. These enzymes were expected to recognize different restriction sites on each of the DNAs of the other known isolates. The resulting fragments resolved on 2% agarose gel were analyzed for different restriction patterns. Representative clones possessing distinct restriction patterns were selected and sequenced. Once a part of a given non-*rep* DNA had been sequenced, additional clones were generated from each sample by using primer pairs (Table 2) derived from component-specific coding sequences.

Sequencing and sequence analysis

The nucleotide sequence of each DNA component was determined from both strands of at least two independent clones. DNA sequencing was carried out by automated facilities of a commercial company (MWG Biotech, Ebersberg, Germany). Sequence assembly, multiple alignment and identity analyses of nucleotide and amino acid sequences were carried out using the DNAMAN software package (Lynnon Biosoft, Canada). The sequences were compared with the available sequences in the database using the BLAST program (Altschul et al. 1997). Phylogenetic analysis was done by multiple alignment of the sequences of homologous components of previously sequenced isolates obtained from the database using the ClustalX program (Thompson et al. 1997). The results of the multiple alignment of sequences by the neighbor joining algorithms was visualized using the Treeview program (Page, 1996). Database accession numbers of nanovirus sequences used for sequence comparison and/or phy-

logenetic analysis were as follows: FBNYV isolate from Syria (Syr) (Y11405-9, AJ005965, AJ005967, AJ749902), Egyptian (Eg) isolate of FBNYV (AJ132179-84, AJ132186, AJ749903), FBNSV-Eth (AJ749894-901, AF159704-5), MDV (AB000923-7, NC_003648), SCSV (NC_003812-13, NC_003815-17, NC_003819) and *Banana bunchy top virus* (BBTV; S56276, L41574-8).

RESULTS

Epitope profiles of the Moroccan samples

Seven of the 10 Moroccan samples reacted with the mixture of broad-spectrum Mabs, indicating the occurrence of FBNYV-like nanoviruses in Morocco. The reaction patterns of the Mabs with the Moroccan samples are shown in Table 1. Six of the seven nanovirus-positive samples gave strong reactions with two of the four discriminating Mabs raised against FBNYV-Eg and no to intermediate reactions with the Mabs to FBNSV-Eth. The epitope profile of these six samples was similar to that of FBNYV-Eg. However, they differed from FBNYV-Eg in failing to react with Mab 2-3E12-D5, in not or weakly reacting with Mab 2-5H9, and in producing weak to intermediate reactions with two of the four Mabs to FBNSV-Eth. Only one sample (Mor5) reacted strongly with all four Mabs to FBNSV-Eth but failed to react with three of the four discriminating Mabs raised against FBNYV-Eg. In this reaction pattern, Mor5 resembled FBNSV-Eth.

Detection of eight genomic DNAs from both Mor5 and Mor23

Since the epitope profiles obtained for individual samples indicated a considerable serological variation in the nanovirus(es) infecting faba bean in Morocco, Mor5 and Mor23, two isolates with contrasting epitope profiles, were selected for nucleotide sequence analysis to obtain a better understanding of this nanovirus variability. Eight distinct circular ssDNAs were identified each from Mor5 and Mor23 and completely sequenced. The individual DNAs ranged in size from 952 to 1005 nt for Mor5 and from 980 to 1004 nt for Mor23 (Table 3), amounting to a genome size of 7886 and 7936 nt, respectively. The size of individual DNAs, the properties of the encoded protein as well as the positions of important sequence elements are presented in Table 3. RFLP and sequence analysis provided no indications for the presence of DNA components other than the eight DNAs considered integral parts of the nanovirus genome (Vetten et al. 2004).

Table 3. Nucleotide size, TATA-box position and properties of the ORFs and gene product sizes of the eight DNA components of the Mor5 and Mor23 genomes

Genome segments	Isolates	DNA size (nt)	TATA-box position [a]	ORF				
				Initiation codon position [b]	Initiation context	No. of amino acids	Protein size (kDa)	Termination codon
DNA-R	Mor5	1003	75	121	AATATGG	286	33.16	TGA
	Mor23	1003	75	121	AATATGG	286	33.16	TGA
DNA-S	Mor5	993	267	305	AAAATGG	172	19.13	TAA
	Mor23	1004	285	326	AAAATGG	172	19.00	TAA
DNA-M	Mor5	984	280	298	ACGATGT	112	12.68	TAG
	Mor23	989	279	342	TCCATGG	114	12.94	TAG
DNA-C	Mor5	988	273	313	GAAATGG	169	19.83	TAA
	Mor23	992	273	314	AAGATGG	169	19.85	TGA
DNA-N	Mor5	1005	272	380	AAAATGG	153	17.54	TAA
	Mor23	990	318	365	AAAATGG	153	17.39	TAA
DNA-U1	Mor5	983	275	368	TAGATGC	144	16.89	TAA
	Mor23	992	272	360	GTTATGG	155	18.98	TGA
DNA-U2	Mor5	978	289	346	TTGATGG	121	14.44	TAA
	Mor23	989	279	356	AAGATGC	124	15.18	TAA
DNA-U4	Mor5	952	261	354	GTTATGG	106	12.18	TAA
	Mor23	980	298	375	CAGATGG	105	12.43	TAG

[a] Position of TATA box is the number of nucleotides from the first nucleotide of the stem loop to the first base of the TATA box.

[b] Position of initiation of the ORF is the number of nucleotides from the first nucleotide of the stem loop to and including the first nucleotide of the initiation codon.

Analysis of the noncoding regions of the Mor5 and Mor23 DNAs

The noncoding regions (NCR) of all eight DNAs of each isolate share a highly conserved, inverted repeat sequence predicted to form a stem-loop structure characteristic of nanovirus DNAs (Katul et al. 1998, Sano et al. 1998). An alignment of these conserved stem-loop and flanking iteron-like sequences of the Mor5 DNAs is shown in Fig 1. The stem-loop sequences (nt 1-28 or 30) of Mor5 are identical to those of FBNSV-Eth and differ from that of Mor23 and FBNYV-Eg only in the nucleotide positions 4 and 25 of DNA-C and -U1. The nonanucleotide sequence TAGTATTAC, which contains the origin of replication (Timchenko et al. 1999), is perfectly conserved in all the DNAs of both isolates. Moreover, the domains flanking the stem-loop region are largely conserved in all the DNAs. For each DNA, the position of the potential promoter (TATA box, TATATAA) sequence preceding the ORFs is indicated in Table 3. Putative polyadenylation signal sequences (AATAAA), which are required for transcription termination (Rothnie et al. 1994), are found downstream of the ORF in all DNAs.

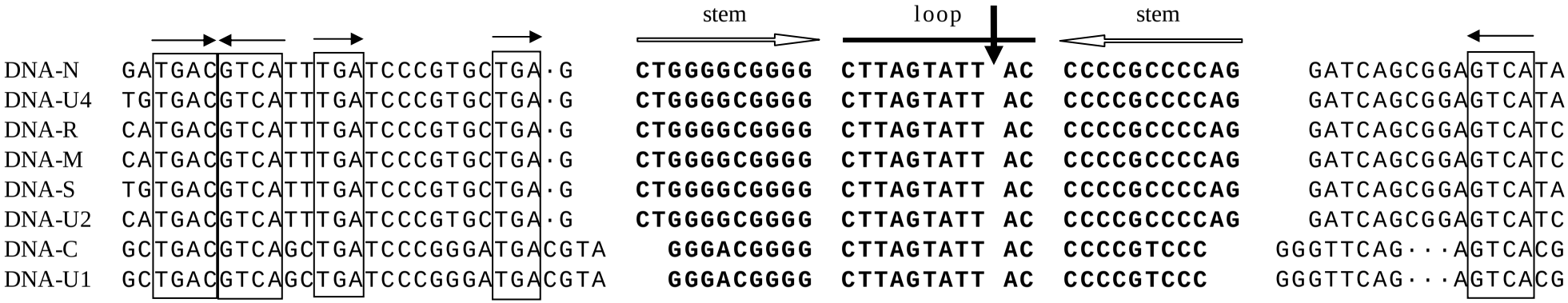


Fig. 1. Alignment of the replication origin sequences of the eight Mor5 DNAs. Inverted repeat sequences (open horizontal arrows) potentially forming a stem-loop structure are in bold. The vertical arrow indicates the position of cleavage by the master Rep protein. Conserved iteron-like sequences possibly acting as recognition or binding sites for the master Rep protein are boxed and the orientation of these repeats is indicated by horizontal solid arrows.

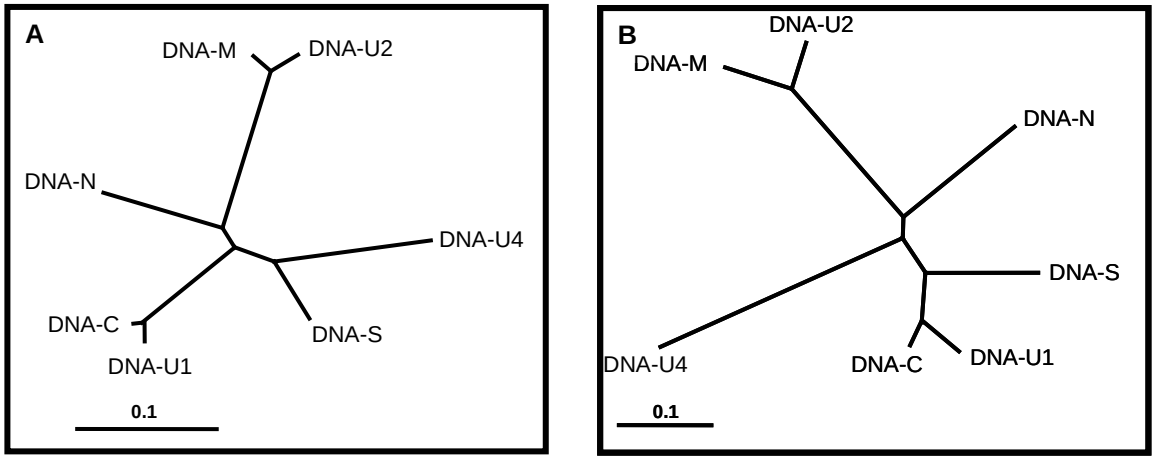


Fig. 2. Phylogenetic trees illustrating the relationship among the NCR nucleotide sequences of the seven non-*rep* DNAs of Mor5 (A) and Mor23 (B).

In addition to the conserved stem-loop sequence and adjacent regions referred to as common stem-loop sequence (CSL), the NCR of the seven non-rep DNA components of each isolate contained further conserved domains. This became evident from phylogenetic trees illustrating the relationship among the NCRs of the seven non-rep DNAs of Mor5 (Fig 2A) and Mor23 (Fig. 2B). For both isolates, the DNA-U1 and -C NCRs were most similar (96% identity) followed by the DNA-M and -U2 NCRs (93% identity). However, these two pairs appeared to be most divergent from each other. The DNA-S and -U4 NCRs branched off from the node joining the DNA-M and -U2 NCRs whereas the NCR of DNA-N formed a separate branch showing the least relationship to all the others. In general, the trees of the Mor5 and Mor23 NCRs had a similar topology with the exception of slight difference in the branching pattern of the DNA-S and -U4 NCRs. Since DNA-R has a large ORF and its NCR only contains the CSL, it was excluded from this NCR comparison.

Sequence comparison of the Mor5 DNAs with the homologues of FBNSV-Eth, its closest relative, revealed that there are some variations arising from possible deletion or insertion events in the NCR. Together with all other FBNYV isolates (Eg and Sy) including Mor23, Mor5 has a stretch of 55 nucleotides in the closely related NCRs of DNA-M (nt 819-874) and -U2 (nt 817-872), whereas this stretch is absent in the corresponding DNAs of FBNSV-Eth. Moreover, the NCR of the Mor5 DNA-N has a 21-nt insertion (nt 90-110) which is absent from the DNA-N of all the other sequenced isolates. Unlike all other nanovirus isolates sequenced so far, the NCR of the Mor5 DNA-U4 also lacks 32 bases (nt 142-173) making this the smallest nanovirus DNA in size (Table 3).

Table 4. Pairwise comparison of the total nucleotide and predicted amino acid (coding) sequence identities between DNAs of Mor5 and homologous DNAs of definitive and tentative members of the family *Nanoviridae*

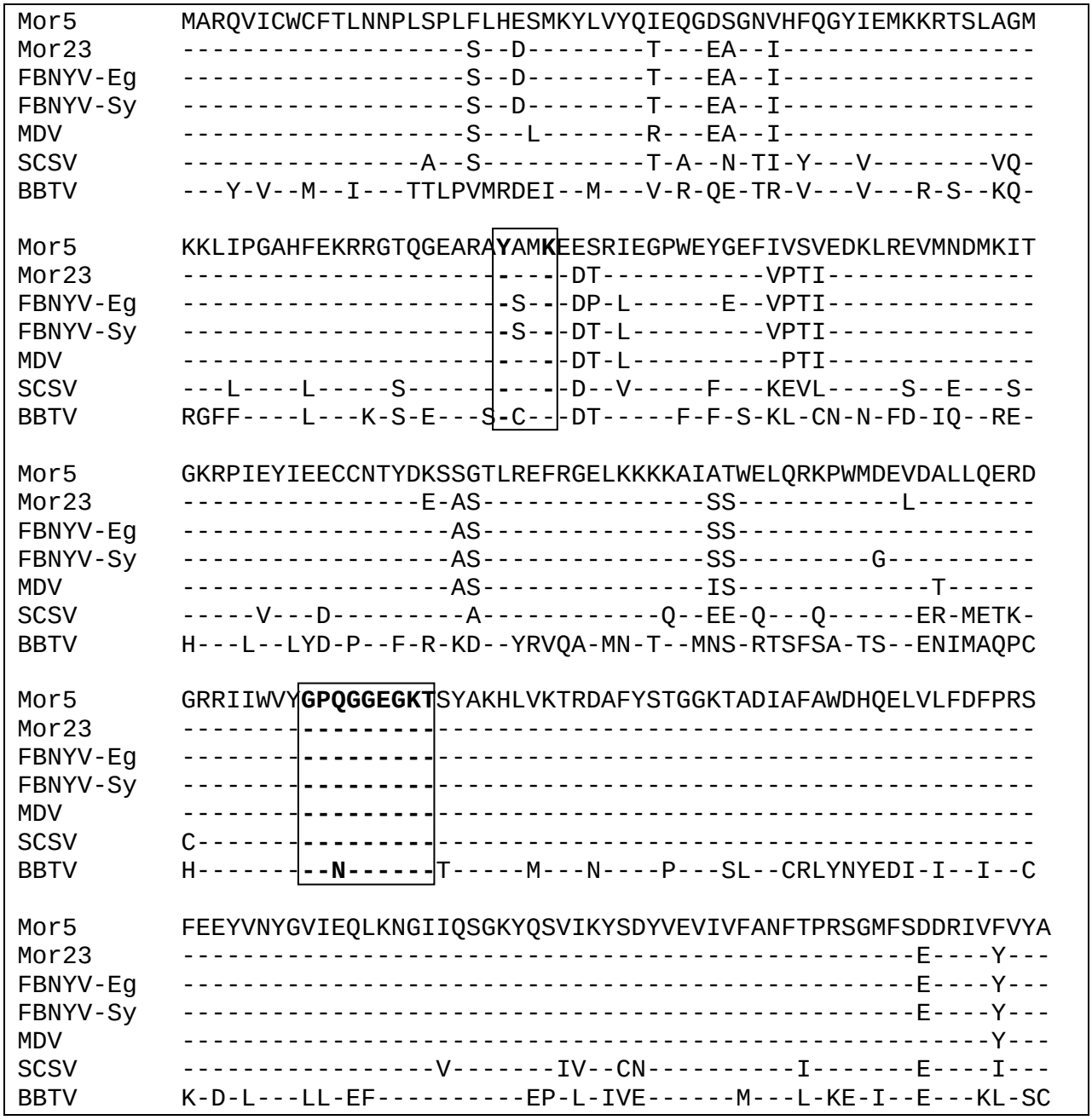
Isolate/Virus	DNA components																Mean identity	
	R		S		M		C		N		U1		U2		U4			
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Mor23	90.7	93.0	78.8	84.9	70.4	76.8	76.3	75.7	79.5	90.8	73.7	67.4	66.4	54.5	62.2	50.5	74.7	74.2
FBNSV-Eth	95.1	96.5	88.4	98.8	83.1	86.6	90.9	89.9	86.3	96.7	89.1	84.7	84.3	80.2	80.6	78.3	87.2	88.9
FBNYV-Eg	90.1	92.7	78.6	84.3	69.9	74.1	76.9	76.3	80.7	88.9	72.8	65.3	62.6	54.6	62.4	52.4	74.2	73.6
FBNYV-Sy	90.4	92.7	78.4	83.7	68.4	75.9	76.8	74.6	79.3	88.9	72.6	66.7	65.9	55.4	61.0	54.3	74.1	74.0
MDV	88.9	93.7	78.8	83.7	69.5	75.9	77.7	71.0	80.4	88.9	74.0	68.8	57.8	57.9	- *	-	75.3	77.1
SCSV	79.7	83.6	56.5	56.1	38.8	44.1	60.1	46.3	59.9	67.3	56.6	44.4	-	-	-	-	58.6	57.0
BBTV	56.0	53.5	41.9	21.8	41.5	9.1	45.1	13.0	47.3	42.8	- *	-	-	-	-	-	46.4	28.0

* Dashes indicate that comparison was not possible because a homologous DNA component has not yet been identified from this nanovirus. They were excluded from the calculation of the mean identity values (%).

Table 5. Pairwise comparisons of total nucleotide and predicted amino acid (coding) sequence identities between DNAs of Mor23 and homologous DNAs of other members of the family *Nanoviridae.*

Isolate/Virus	DNA components																Mean identity	
	R		S		M		C		N		U1		U2		U4			
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Mor5	90.7	93.0	78.8	84.9	70.4	76.8	76.3	75.7	79.5	90.8	73.7	67.4	66.4	54.5	62.2	50.5	74.7	74.2
FBNSV-Eth	89.5	93.4	79.5	84.3	71.7	76.8	76.6	75.1	78.4	92.8	74.6	67.8	65.2	56.2	64.8	49.5	75.0	74.5
FBNYV-Eg	96.8	97.9	91.1	97.7	82.3	90.4	92.4	92.9	95.3	97.4	91.6	91.6	82.4	92.7	90.6	87.6	90.3	93.5
FBNYV-Sy	97.2	98.3	91.9	97.1	85.7	91.2	93.0	94.1	95.5	98.0	92.1	95.5	91.0	96.8	91.8	87.6	92.3	94.8
MDV	92.3	96.5	80.3	83.7	69.5	78.6	78.2	71.6	78.9	90.2	76.3	74.0	61.5	53.2	- *	-	76.6	78.2
SCSV	78.9	83.2	63.4	55.6	57.7	47.3	62.2	45.1	62.4	65.3	59.7	45.5	-	-	-	-	64.0	57.0
BBTV	57.6	54.5	48.5	19.9	48.3	8.8	50.8	17.6	54.4	42.8	- *	-	-	-	-	-	51.9	28.7

* Dashes indicate that comparison was not possible because a homologous DNA component has not yet been identified from this nanovirus. They were excluded from the calculation of the mean identity values (%).



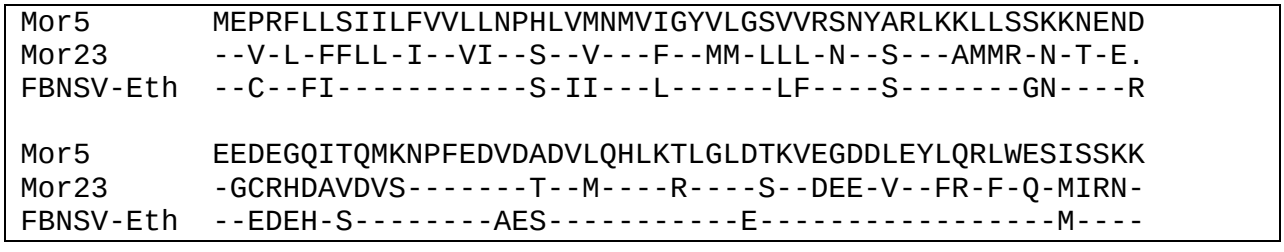


Fig. 3. Alignment of the amino acid sequences of the most conserved nanovirus gene product, the master Rep proteins (top), and the least conserved nanovirus gene product, the U4 proteins (bottom), comparing the master Rep and U4 proteins of Mor5 and Mor23 with those of other nanoviruses. Conserved active site tyrosine (YxxK) and NTP-binding motifs (GPQ/NGGEGKT) are highlighted and boxed. Amino acid residues identical to those of Mor5 are indicated by a dash; a dot denotes a gap. For databank accession numbers of the nanovirus sequences see Materials and Methods.

Analysis of the proteins potentially encoded by the Mor5 and Mor23 DNAs

Each of the eight DNAs of both Mor5 and Mor23 contains a single ORF potentially coding for a protein that is comparable in size and sequence not only to its homologue of the other isolate but also to that of the other nanoviruses. The ATG start codon of all the ORFs is in a favourable context (Table 3) that at least partially agrees with the optimal translation context of plant mRNAs (Lütcke et al. 1987). Key amino acid motifs such as the NTP-binding motifs (GPQ/NGGEGKT) and active site tyrosine (YxxK) of the Rep proteins (Timchenko et al. 1999) (Fig. 3, top) and LxCxE motifs of the Clink protein encoded by DNA-C (Aronson et al. 2000) are conserved in their respective positions in the corresponding proteins from both isolates.

Pairwise comparisons of the total nucleotide and encoded proteins of the two isolates were carried out with the available sequences of two FBNYV isolates, FBNSV-Eth, and other nanoviruses (Tables 4 and 5). In agreement with the serological data, the coat protein amino acid sequences of Mor5 and Mor23 were more closely related to those of FBNSV-Eth (identity of 99%) and FBNYV-Eg (and -Sy) (98%), respectively. Similarly, the total nucleotide sequence DNA-S of Mor5 is most similar (88%) to that of FBNSV-Eth while that of Mor23 has an identity of 91-92% with FBNYV-Eg and -Sy (Tables 4 and 5). Comparisons of the remaining seven DNAs also showed similar relationships at both the nucleotide and protein level although the degree of divergence varied considerably between the individual DNAs and their encoded protein. Of all the nanovirus proteins, the master Rep protein encoded by DNA-R is the most conserved gene product with identities ranging from 93% to 97% for FBNYV, FBNSV and MDV isolates and as low as ~83% and ~54% for SCSV and BBTV, respectively. On the other hand, DNA-U4 appears to encode the least conserved nanovirus protein with identities as low as 50% between isolates (Tables 4 and 5). Fig. 4 shows a multiple alignment of each of these two proteins by comparing the Mor5 and Mor23 amino acid sequences with those of homologous nanovirus sequences available from the database. Table 4 and 5 also show that all the proteins encoded by Mor5 and Mor23 shared significant levels of sequence similarity with the corresponding proteins of other nanoviruses with possible exception of DNA-C and DNA-M of BBTV.

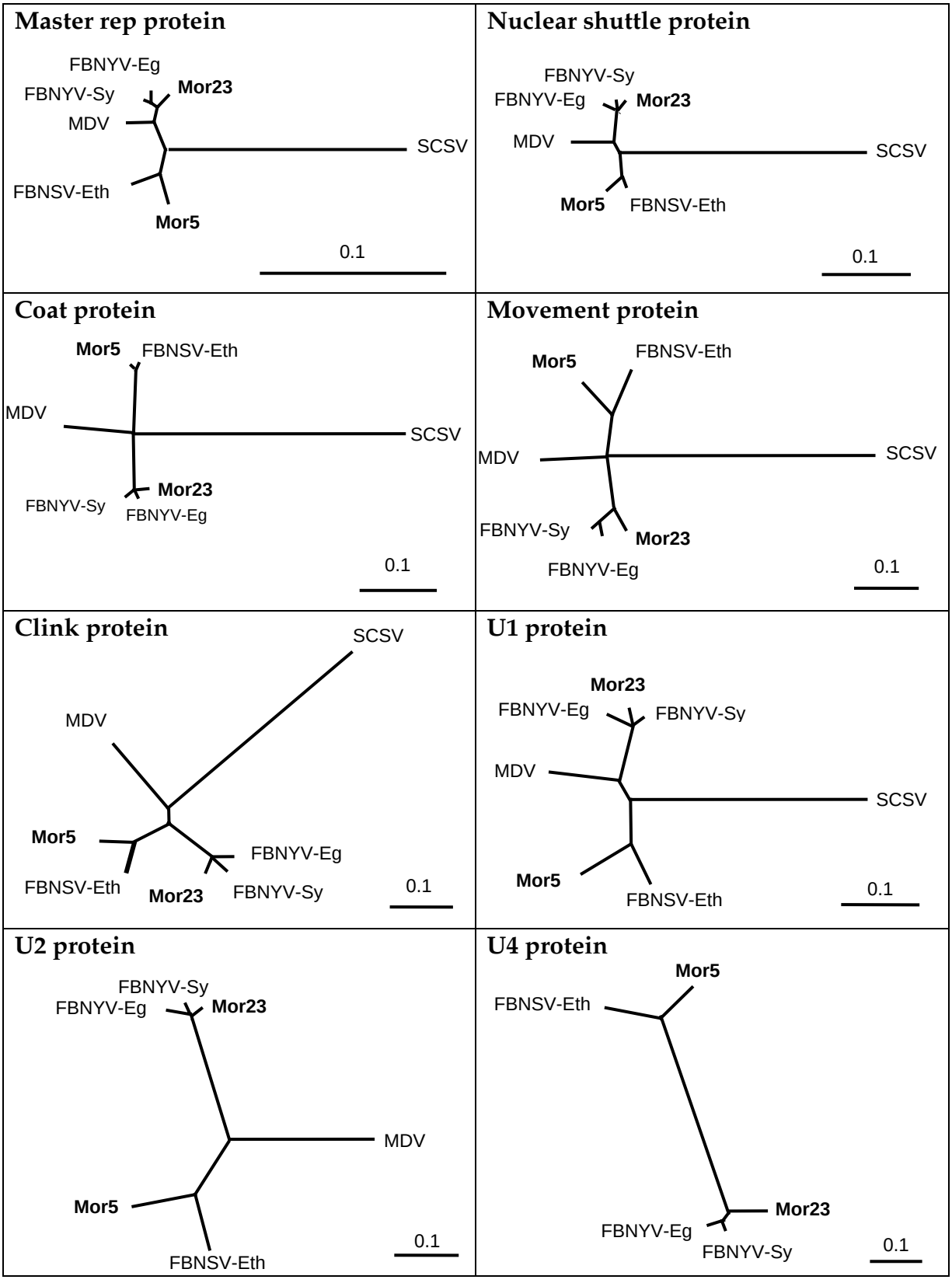


Fig. 4. Phylogenetic trees illustrating the relationship between the proteins encoded by the eight Mor5 and Mor23 DNAs and those of other members of the genus *Nanovirus*, namely *Faba bean necrotic yellows virus* (FBNYV), *Milk vetch dwarf virus* (MDV), *Subterranean clover stunt virus* (SCSV) and the Ethiopian isolate of Faba bean necrotic stunt virus (FBNSV-Eth), a tentative nanovirus species.

Sequence comparison also revealed that there are some variations stemming from possible deletion or insertion events in the coding regions. Unlike FBNSV-Eth, the DNA-M ORF of Mor5 and other FBNYV isolates had a 18-nt deletion at its 5'-terminal region. The DNA-U1 ORF of Mor5 also codes for a protein that lacks 13 amino acids at its C-terminus as compared to its closest relative FBNSV-Eth. The DNA-U2 of both Mor5 and FBNSV-Eth codes for a protein that is 3 amino acids shorter than the Mor23 or FBNYV homologues.

Phylogenetic analysis of all eight deduced protein amino acid sequences of Mor5 and Mor23 together with those of the other nanovirus isolates clearly demonstrated that Mor5 and Mor23 cluster with FBNSV-Eth and FBNYV-Eg (and -Sy), respectively. This grouping was similar for the different proteins although the relative position of individual isolates within each cluster varied for the different proteins indicating different levels of conservation.

DISCUSSION

In a previous study of the FBNSV-Eth genome, the primer pair 3F/20R and the two primer pairs 23F/44R and 75F/76R had been used successfully for amplification of DNA-R and the seven non-rep DNAs, respectively (Katul and Vetten, 1999). RFLP analyses of over one hundred PCR-generated clones obtained from Mor5 and Mor23 DNA with the primer pairs 23F/44R and 75F/76R and sequencing of dozens of these clones indicated that this experimental approach consistently permits the detection of the seven non-rep DNAs of a nanovirus. Although the primers had been derived from a highly conserved region that is shared by all non-rep DNAs, there were no indications for the presence of a DNA other than the already known non-rep DNAs. Moreover, no satellite-like *rep* DNAs were encountered. This was not unexpected as the satellite-like *rep* DNAs of nanoviruses differ from the integral parts of the nanovirus genome strikingly in the non-coding and coding sequences (Timchenko et al. 1999, 2000) and, thus, the experimental approach used here did not allow for amplification of any satellite-like DNAs. Our study on the Mor5 and Mor23 genomes supports the recent proposal by Vetten et al. (2004) that the complete genome of members of the genus *Nanovirus* consists of eight distinct DNAs. However, this notion can only be substantiated if inoculation of plants with cloned copies of all these eight DNAs leads to infections that are indistinguishable in all biological properties from those by wild-type nanoviruses. The latter aspect has not yet been demonstrated for any nanovirus due to experimental difficulties in generating large numbers of infected plants.

Highly conserved NCR domains shared by only some of the non-rep DNAs (e.g. DNA-M and -U2 or DNA-C and -U1) but not by others have been previously observed also for BBTV (Burns et al. 1995), FBNYV (Katul et al. 1997, 1998), MDV (Sano et al. 1998), and SCSV (Boevink et al. 1995). Hughes (2004) hypothesized that these phenomena might be explained by the occurrence of repeated recombination events between the NCRs of genome components encoding non-homologous nanovirus proteins. The author further suggested that components that have been homogenized by recent genetic exchange remain quite divergent at sequences upstream or downstream of the stem loop regions. Therefore, it is possible that a more recent recombination has taken place in the DNA-S and -U4 NCRs, which differ from the other two NCR groups despite some partial identity to each one of them.

The variation in the divergence rate of the different proteins encoded by the individual DNAs of Mor5 and Mor23 (Tables 4 and 5, Fig. 4) suggests that there are differential functional constraints acting on each of the proteins independently. It appears that proteins with known function such as replication, nuclear shuttle and movement activities are more conserved than those with unknown function (U1, U2 and U4). Such divergence suggests that for each isolate, various proteins evolved differently possibly due to different selection pressures. As it has been suggested for some RNA viruses (Naidu et al. 2003), it is possible that proteins involved in replication and virus movement or spread undergo strong selection pressure to maintain sequence integrity and function while the other genes are under divergent selection pressure which may be related to more specific function related to individual virus.

Phylogenetically, Mor23 forms a group with the isolates FBNYV-Eg and -Sy whereas the Mor5 and FBNSV-Eth form a second group clearly distinct from the former (Fig. 4). The difference in both total nucleotide and encoded amino acid sequences between the two groups is so large that the two groups should not be considered as isolates or strains of the same virus. According to the current nanovirus species demarcation criteria (Vetten et al. 2004), two distinct nanovirus species should have an overall nucleotide sequence identity of $\leq$ 75% at and/or a CP amino acid sequence identity of $\leq$ 85%. The two groups share a mean nucleotide sequence identity of slightly less than 75% and a CP amino acid sequence identity of $\leq$85% with a much lower mean percentage identity (c. 74%) for all the proteins. This is comparable to the difference between MDV and typical FBNYV isolates (Tables 4 and 5). Therefore, Mor5 is proposed to be an isolate of FBNSV whereas Mor23 is a typical FBNYV isolate. However, more information on the host range, symptomatology, and aphid

vector specificities of the FBNSV isolates would be desirable before FBNSV can confidently be assigned as a distinct species to the genus *Nanovirus*.

The results presented provide the first evidence for the occurrence of both FBNYV and FBNSV in the same country. While FBNYV has been reported from Egypt and Syria (Katul et al. 1993, 96, 98) and unconfirmed serological data suggests that it occurs in several African and Asian countries (Katul et al. 1993, Franz et al. 1996, Makkouk et al. 2003), FBNSV has been reported previously only from Ethiopia (Katul and Vetten, 1999 and unpublished data). Because of the serological and molecular variability observed for FBNYV-like isolates from Ethiopia and other countries, Franz et al. (1996) and Katul and Vetten (1999) explained such observations as a geographically associated variation among FBNYV-like nanoviruses from different countries. However, the results of this study provide a more complex picture of "nanovirus variability" in a country. In particular, it was demonstrated that both FBNYV and FBNSV, two distinct nanovirus species, infect faba bean in a country. On the other hand, the Moroccan FBNSV isolate Mor5 was genetically quite distinct from the Ethiopian FBNSV isolate, from which it was serologically indistinguishable, thus providing again an indication for a possible geographically associated variation among isolates of a nanovirus species. Intensive future survey in other countries are therefore likely to reveal the occurrence of more nanovirus species and further nanovirus strains as it has been already observed in Ethiopia (Abraham et al. submitted, 2005).

The only information so far available on the variability of a nanovirus is for BBTV, for which sequence information of the DNA-R, -N, and –S of isolates from different countries revealed the occurrence of two isolate groups: Asian and South Pacific isolates corresponding to their geographical location (Karan et al. 1994, 1997; Wanitchakorn et al. 2000). A more detailed study on DNA-R sequences of BBTV isolates from Vietnam (Bell et al. 2002) indicated that although all isolates fall within the Asian group, there was a marked difference between isolates from southern and northern Vietnam. Although the number of samples used in our study did not allow us to comment on the geographical distribution of FBNYV and FBNSV isolates in Morocco, our study is the first that provides genome sequence information on two nanovirus isolates infecting faba bean in a country.

The occurrence of two distinct nanoviruses infecting faba bean in a restricted geographic area may have implication for future attempts at breeding for resistance to the viruses by conventional or molecular means. In countries like Morocco where two nanovirus species are now known to occur, it is possible that resistance targeted to only one species may not

be effective against the other. More surveys are required for to determining the occurrence, geographical distribution and relative importance of these two nanovirus species not only in Morocco but also in other countries of West Asia and North Africa.

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CHAPTER 7

Analysis of the ssDNA genome of serologically distinct nanovirus isolates from Ethiopia: evidence for a new and two known nanovirus species infecting faba bean [6]

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ABSTRACT

When 299 symptomatic faba bean samples collected in Ethiopia in 2002 were serologically analysed by TAS-ELISA using broad-spectrum monoclonal antibodies (MAbs) reacting with *Faba bean necrotic yellows virus* (FBNYV) and closely related viruses, 73 (24.4%) of the samples gave ELISA-positive reactions. Further serological analysis of the nanovirus-positive samples with seven discriminating MAbs revealed contrasting epitope profiles that were categorized into at least three groups referred to as serogroups A, B and C. Serogroup A appears to be prevalent throughout the country as it was detected in 62 (85%) of the 73 ELISA-positive samples whereas serogroups B and C were found only in few samples from southern Ethiopia. Serology and sequence analysis of the coat protein (CP) and U1 genes of serogroup A, B and C isolates indicated that each of the three serogroups might represent a distinct nanovirus species. Serogroup A was similar to the previously described, atypical 'FBNYV' isolate from Ethiopia, for which the name Faba bean necrotic stunt virus (FBNSV) has been proposed. CP, M-Rep, U1 and U2 gene sequences of a serogroup B isolate resembled those of typical isolates of FBNYV from Egypt and Syria, providing first evidence for the occurrence of FBNYV (*sensu strictu*) in Ethiopia. Serogroup C isolates appeared to be serologically most distinct from typical FBNYV isolates. Therefore, all 8 genomic ssDNAs of a representative serogroup-C isolate (Eth-231) were completely sequenced. The individual DNAs of Eth-231 ranged in size from 972 to 1002 nt and had only one major ORF potentially encoding proteins of 12 to 33 kDa. Eth-231 shared overall nucleotide and amino acid sequence identities of only ≤70% and ≤74%, respectively, with other nanoviruses including FBNYV, FBNSV and *Milk vetch dwarf virus.* Moreover, DNA-C of Eth-231 is very distinct in nucleotide sequence from that of other nanoviruses and encodes a Clink protein that lacks the typical LxCxE motif required for cell-cycle regulation. Our data strongly suggests that Eth-231 represents a new nanovirus species for which the name Faba bean yellow leaf virus (FBYLV) is proposed.

[6] This paper will be submitted for publication in an international journal, such as *Archives of Virology*.

INTRODUCTION

Faba bean (*Vicia faba* L) is among the major food crops grown worldwide, particularly in countries of Northeast and North Africa, Asia, and Australia. Ethiopia is the second biggest producer of this crop after China with production in 2004 estimated to be 448,000 metric tons produced on 370,000 ha (FAO, 2004). However, the average faba bean yield in the country has been consistently low (< 1 ton ha^{-1}). Among the factors contributing to this low yield are plant pathogens especially plant viruses causing significant economic losses. About 10 different viruses have been identified to affect cool season food legumes in Ethiopia of which luteoviruses and *Faba bean necrotic yellows virus* (FBNYV) in faba bean and chickpea, and *Pea seed-borne mosaic virus* in lentil are the most prevalent (Abraham et al. 2000, Abraham and Albrechtsen, 1998, Abraham and Makkouk, 2002, Tadesse et al. 1999). It was earlier observed, however, that nanovirus isolates from Ethiopia are very distinct in serological properties from those in other countries (Franz et al. 1996). Sequence analysis of the eight genomic ssDNAs of a serologically unusual nanovirus isolate from Ethiopia substantiated its genetic distinctness from typical FBNYV isolates, from which it differed by over 25% in total nucleotide sequences (Katul and Vetten 1999). Based on this information and in line with the current criteria for nanovirus species demarcation (Vetten et al. 2004), this isolate was considered a distinct nanovirus species for which the name Faba bean necrotic stunt virus (FBNSV) has been proposed (Katul and Vetten, 1999, Katul and Vetten, unpublished data). For the sake of this paper, the term FBNYV-like viruses will be used to refer to FBNYV, FBNSV or any other related nanoviruses that can be detected with the broad-spectrum Mabs originally raised against an Egyptian isolate of FBNYV.

FBNYV has a multipartite single stranded DNA genome encapsidated separately to isometric particles of about 18 nm (Katul et al. 1993, 1998). It is transmitted persistently by aphids such as *Aphis craccivora* and *Acyrthosiphon pisum* and has a wide host range that is largely confined to members of the Fabaceae (Franz et al. 1997, 1998). Although up to 12 circular ssDNAs have been found associated with FBNYV infection, eight structurally very similar ssDNA components have been consistently identified from all sequenced isolates and thus are believed to comprise the integral components of the FBNYV genome while the others are considered to be satellite-like DNAs (Vetten et al. 2004). Together with *Subterranean clover stunt virus* (SCSV) and *Milk vetch dwarf virus* (MDV), FBNYV has been assigned to the genus *Nanovirus* of the recently established family *Nanoviridae*.

Information on virus diversity and/or variability in an area is important for developing appropriate breeding strategies encompassing the whole range of natural variants. Since typical FBNYV

isolates, which appear to be common in most other countries of the region (Franz et al. 1996, Makkouk et al. 2003), had not been encountered previously in Ethiopia, it was suggested that there is a geographically associated variation among FBNYV isolates from Ethiopia and those from other countries and vice versa (Franz et al. 1996, Katul and Vetten, 1999). However, this conclusion was based on studies using only few samples underlining the need for reassessing these reports by a countrywide serological and molecular investigation of representative isolates. In this paper, the diversity of FBNYV-like isolates was studied by analyzing a large number of samples using broad spectrum and differentiating monoclonal antibodies (Mabs) as well as by nucleotide sequence analysis of representative isolates. The data generated suggests that nanovirus isolates infecting faba bean in Ethiopia belong to three related but distinct nanovirus species. The complete nucleotide sequence of a previously unrecognized nanovirus species was determined.

MATERIALS AND METHODS

Sample collection and serological analysis

Samples were collected in a field survey conducted in the major faba bean growing areas of Ethiopia during the main growing season of 2002 (Fig. 1). On each survey route, fields were selected along the main roads and plants showing symptoms suggestive of FBNYV-like infection such as yellowing, stunting, necrosis and leaf deformation were collected and preserved by drying over $CaCl_2$. When there were no visible virus symptoms, leaves from about five apparently healthy plants were randomly picked as a composite sample for subsequent serological analysis. The samples were analyzed serologically by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) (Franz et al. 1996) using polyclonal IgG to FBNYV (Katul et al. 1993) for trapping. Following incubation of leaf extracts, plates were loaded with appropriate dilutions of a broad-spectrum Mab mix, three Mabs (1-3D8, 3-4A5, 2-3E12) raised against an Egyptian isolate of FBNYV (FBNYV-Eg) (Franz et al. 1996), and four Mabs (8-4F9, 8-6F8, 8-2G10, 8-3G11) specific to an Ethiopian isolate of FBNSV (FBNSV-Eth) (Vetten, unpublished results). The broad spectrum antibody reacts with all nanoviruses including FBNYV, FBNSV and MDV.

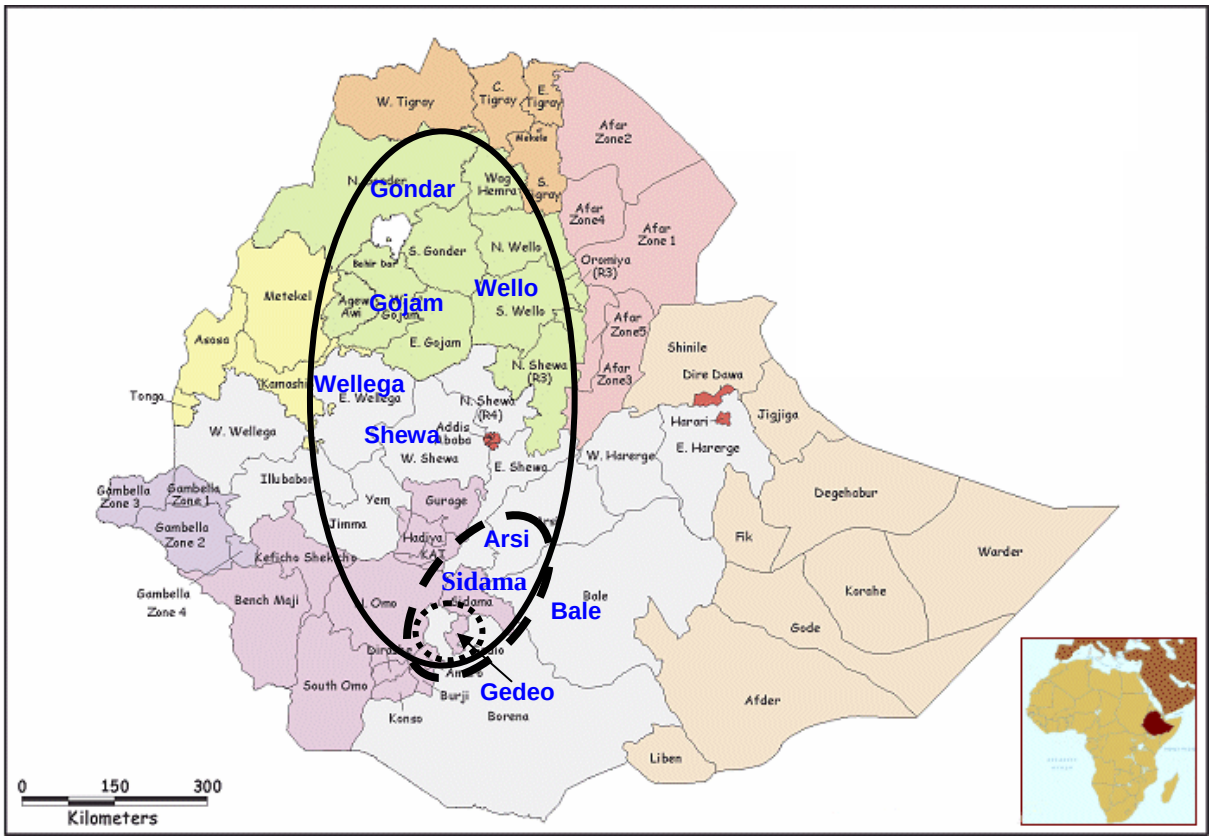

Fig. 1. Map of Ethiopia showing the areas covered in the survey for sample collection and the distribution of the different FBNYV serogroups. Areas where the different serogroups occur are encircled as with a solid line for serogroup A, a dashed line for serogroup B and a dotted line for serogroup C isolates.

Oligonucleotide primers and immunocapture (IC)-PCR of selected samples

All oligonucleotide primer pairs derived either from published sequences or sequences generated in this work and used to amplify one or more of the 8 ssDNAs of the genome of the isolates studied are listed in Table 1. To randomly amplify a part of one or more of the non-rep DNA components, the universal primer pairs 75F/76R and 23F/44R (Katul and Vetten, unpublished) were used. These primers are expected to anneal specifically to domains within and immediately downstream of the common stem loop sequence in the noncoding region of all non-rep DNAs. In addition, another general primer (PLo) derived from the same region was also used in combination with specific primers. Primer pair 3F/20R (Timchenko et al. 1999) was used to specifically amplify part of the master rep DNA (DNA-R) from all samples. Furthermore, several primers specific to one or more components were designed and used to selectively amplify one or more of the components or to obtain the complete sequence of the component already identified. In the latter case, additional clones were generated from each sample by using component-specific

primer pairs designed from coding sequences flanking the gap so that the complete circular DNA was sequenced.

Table 1. Primer pairs used for amplification of nanovirus sequences (see also Table 4).

Designation of primer pair	Forward (F) primer (5'→3')	Reverse (R) primer (5'→3')
1. 23F/44R	CACGAATCACAGATCCTGAT	AAGCGAA(A/T/C)CTGACGGAAGA
2. 75F /76R	TAGTATTACCCCGTCCC	GTAATACTAAGCCCCGTC
3. PLoF/PEt6R	TAGTATTACCCCCGCCC	AGCAAATACAATAACCTCTACA
4. PLoF/PEt4R	TAGTATTACCCCCGCCC	CCCAATTCTTTCTCTCTCA
5. PLoF/PEt3R	TAGTATTACCCCCGCCC	GTGTAAACTTTCATACGACA
6. 3F/20R	ATATGCTGGTGCTTTACA	AATTACAATCCTATCCTCACT
7. 50F/55R	TTAAGTTAATAAATGACATCTATC	GCTGATACTATCCTTCCATG
8. 51 F/53R	ACGAAGCAAAGCATATATTCAGTGC	TGGAAGCGTGGTTGAATTCTTCATC
9. 45F/47R	AACTCTCCATACTCCCAWGGACCTTCAA	TATGGCCCACAAGGTGGAGAAGGG
10. Et8F/Et8R	ATGGCAGATTGGGTTTTCTAGTC	CACTTTGATTCTGAGTGAATG
11. Et10F/Et10R	ACATATGAAGCTCTCTGCATA	CATATTTAACTCTGCAGCAGGGTA
12. Et12F/Et12R	CCCTTTGAAGATGCAGATCCA	TTTCATCTGCACAATCCACA
13. Et5F/Et5R	GATCTTGGTACTCTGCGAAGCA	CTTAGTACAATCTGTCCCAGCAA

Amplification of viral sequences by immunocapture (IC)-PCR was done as described by Franz et al. (1999) with minor modification. Purification of PCR products and cloning procedures were carried out essentially as described by Abraham et al. (submitted, 2005a). For each isolate, two or three randomly selected positive clones were initially sequenced to obtain sequence information on one to three DNA components. For the isolate Eth-231, sequence information of the non-rep DNAs not identified from these clones was obtained by PCR-RFLP analysis of several dozens of cloned PCR products using restriction enzymes as detailed in Abraham et al. (in preparation, 2005b). The resulting fragments were analyzed for different restriction patterns following electrophoresis in a 2% agarose gel. Representative clones with patterns different from those of the previously sequenced DNAs from randomly selected clones were selected and sequenced. DNA sequencing was carried out by automated facilities of a commercial company (MWG Biotech, Ebersberg, Germany).

Sequence and phylogenetic analyses

Sequence assembly and pairwise comparison were performed using DNAMAN program (Lynnon, Biosoft, Canada). Database search for related sequences was carried using the BLAST program (Altschul et al. 1997). Phylogenetic trees were constructed using the Clustal_X program (Thompson et al. 1997) after multiple alignment of sequences by neighbor joining algorithms and visualized using the Treeview program (Page, 1996). Database accession numbers of nanovi-

rus sequences used for sequence comparison and/or phylogenetic analysis were as follows: Syrian (Sy) isolate of FBNYV (FBNYV-Sy) (AJ005965, AJ005967, Y11405-Y11409, AJ749902), Egyptian (Eg) isolate of FBNYV (FBNYV-Eg) (AJ132179 - 84, AJ132186, AJ749903**),** Ethiopian (Eth) isolate of FBNSV (FBNSV-Eth) (AJ749894 - AJ749901, AF159704-5), MDV (NC_003641 - 46, NC_003648), SCSV (NC_003812 - 13, NC_003815-17, NC_003819) and Banana bunchy top virus (BBTV) (S56276, L41574 - 78).

RESULTS

Distribution and variation in epitope profile of FBNYV-like isolates from Ethiopia

A total of 299 samples collected from 112 faba bean fields throughout the country (Fig. 1) were analysed serologically. Seventy-three of the 299 samples (24.4%) were infected by FBNYV-like nanoviruses as determined by the reaction with the broad spectrum Mab (Table 2). The distribution of the nanovirus-infected samples collected and detected in different areas is presented in Table 2.

Table 2. Geographic origin of the faba bean samples from different regions of Ethiopia and their assignment to the different serogroups on the basis of their TAS-ELISA reactions with the seven monoclonal antibodies (see also Table 3)

Regions	No. of fields surveyed	No. of samples tested	No. of nanovirus-positive samples	No. samples belonging to serogroups				
				A	B	C	others*	mixed**
Arsi	11	12	6	4	2	-	-	-
Bale	10	10	2	2	-	-	-	-
Gonder	26	101	10	10	-	-	-	-
Gojam	11	44	5	5	-	-	-	-
Wellega	6	9	6	6	-	-	-	
Sidamo (Gedeo)	8	15	10	1	2	4	2	1
Wello	18	68	18	18	-	-	-	-
Shewa	19	34	14	14	-	-	-	-
Addis Ababa	3	6	2	2	-	-	-	
Total	112	299	73	62	4	4	2	1

* Samples with apparently distinct epitope profile but not confirmed by sequence analysis
** Mixed infection with isolates belonging to serogroup B and possibly serotype A.

Table 3. Nanovirus-positive samples from Ethiopia producing distinct epitope profiles and representing the three serogroups on the basis of the reactions with the seven discriminating monoclonal antibodies

Selected samples	Mab mix	Differentiating FBNYV MAbs raised against							No. of samples giving a similar epitope profile [2]	Serogroup [2]
		FBNSV-Eth				FBNYV-Eg				
		8-6F8	8-4F9	8-2G10	8-3G11	1-3D8	3-4A5	2-3E12-D5		
Eth-2	+++ [1]	+++	+++	+++	+++	-	-	-	62/73	A
Eth-183	+++	+++	+++	+++	+++	+++	+++	-	1/73	mixed, B
Eth-218	+++	-	-	-	+	++	+++	-	4/73	B
Eth-231	+++	-	-	-	-	+++	+	-	4/73	C
Eth-234	+++	-	-	-	-	-	-	-	2/73	others [3]
FBNSV-Eth [4]	+++	+++	+++	+++	+++	-	-	-	-	A
FBNYV-Eth [4]	+++	-	-	-	-	+++	++	+++	-	B

[1] Extinction values ($A_{405\ nm}$) for 20-fold diluted leaf extracts and following a substrate incubation period of 1 h. $A_{405\ nm}$ values are classed as +++ = (<0.8), ++ (0.2 to 0.8) + (< 0.2) and - (less than two times the $A_{405\ nm}$ value for the non-infected control).

[2] see Table 2.

[3] Samples producing epitope profiles apparently distinct from serogroups A, B, and C, but not confirmed by sequence analysis.

[4] FBNYV-Eg (Egypt) and FBNSV-Eth (Ethiopia) against which the monoclonal antibodies had been raised were used as control antigen.

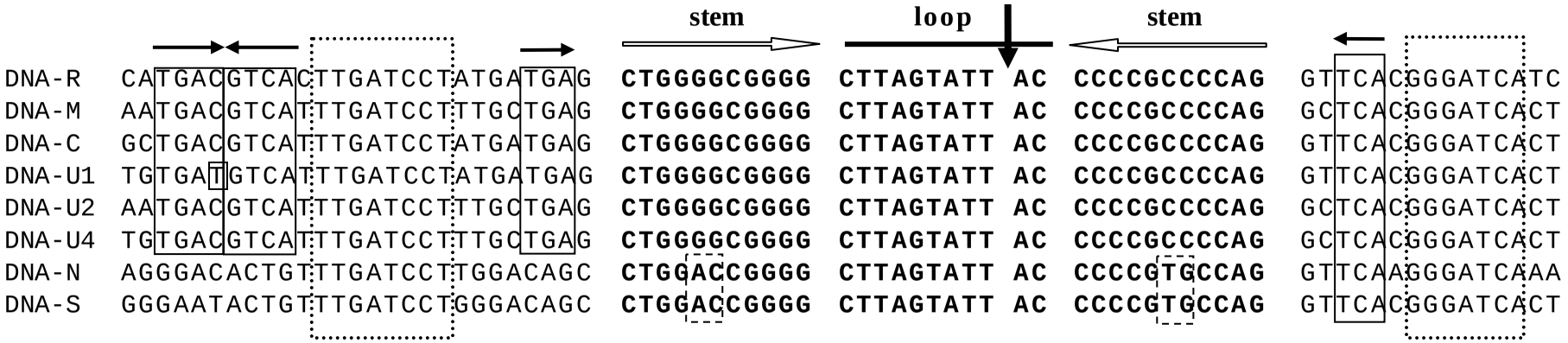

Fig. 2. Alignment of the replication origin sequences of the eight Eth-231 DNAs. Inverted repeat sequences (open horizontal arrows) potentially forming a stem-loop structure are in bold. The vertical arrow indicates the position of cleavage by the master Rep protein. Conserved iteron-like sequences possibly acting as recognition or binding sites for the master Rep protein are boxed and the orientation of these repeats is indicated by horizontal solid arrows. Other conserved domains flanking the stem loop are denoted by dotted rectangles.

Serotyping of the 73 nanovirus-positive samples from Ethiopia using seven discriminating Mabs revealed the occurrence of isolates with diverse epitope profiles that were categorized into five groups as represented by the samples Eth-2, Eth-218, Eth-231, Eth-234 and Eth-183 (Table 3). The majority (62/73 = 85%) of the infected samples (e.g. Eth-2) produced epitope profiles similar to that of FBNSV-Eth (Table 3). This group of isolates occurs in nearly all areas surveyed (Table 2, Fig. 1) and is the only type which could be found in samples from northern and central Ethiopia, the main faba bean growing regions. It is the only serogroup known so far in Ethiopia and has not been reported from another country. For convenience this group of isolates is named serogroup A (Table 3).

Some samples originating from southeastern (Tiyo area in Arsi region) and southern Ethiopia (Gedeo area in Sidamo region) (Fig 1) produced epitope profiles distinct from those of the serogroup A isolates. Some isolates represented by Eth-218 (Table 3) reacted strongly with Mabs 1-3D8 and 3-4A5 specific to typical FBNYV isolates (Franz et al. 1996) but not with the Mabs to FBNSV-Eth. Isolates with such an epitope profile that are reported here for the first time from Ethiopia are designated serogroup B. This group of isolates represents typical FBNYV isolates previously reported to occur in nearly all other countries of the region, especially in Egypt and Syria (Franz et al. 1996). A third category of isolates named serogroup C consists of some novel isolates obtained only from Gedeo area, southern Ethiopia, and represented by isolate Eth-231 (Fig. 1). This group of isolates did not react with Mabs specific to FBNSV-Eth but reacted strongly with Mab 1-3D8 and weakly with Mab 3-4A5. Such an epitope profile has never been observed for any nanovirus isolate before. The fourth group of isolates is represented by further two isolates from Gedeo zone (e.g. Eth-234) that reacted with broad spectrum antibodies but with none of the differentiating antibodies used in this study, an epitope profile also never reported previously for a nanovirus. However, since nanovirus sequences could not be amplified from these samples, no supporting evidence for the distinctness of this nanovirus isolate was obtained. Finally, one sample (Eth-183) also from Gedeo area reacted with all of the discriminating Mabs except Mab 2-3-E12-D5 which failed to react with all Ethiopian samples. Eth-183 was thought to have a mixed infection with serogroup A and B isolates. In the case of mixed infections with isolates belonging to distinct serogroups, the epitope profiles overlap each other in such a way that they can no longer be assigned to a particular serogroup.

Table 4. Types of genome components that were identified from representative nanovirus-positive samples using the indicated primer pairs and randomly selected or RFLP analyzed clones

Isolates	Location	DNAs sequenced	Primer pairs used*	Serotype**
Eth-2	Ambo, Shewa	S, U1	1	A
Eth-183	Gedeo, Sidamo	S, U1	1	Mixed, B
Eth-218	Tiyo, Arsi	R, S, U1, U2	1, 6, 7,8	B
Eth-231	Gedeo, Sidamo	All the 8 DNAs	1-4, 7-13	C

* Primer sequences are listed in Table 1.

Sequence analysis of serologically distinct nanovirus isolates

The nanovirus-positive samples Eth-2, Eth-183, Eth-218 and Eth-231 that had given contrasting epitope profiles (Table 2) were selected for sequence analysis. The types of DNAs identified and sequenced from these four isolates are indicated in Table 4. DNA-S and -U1 were identified from random clones in all the four isolates. Since DNA-S and -U1 sequences were identified from randomly selected clones of all the isolates, these two DNAs appeared to be amplified more frequently by the available primer pairs. Hence, they were used for assessing the genetic differences between the isolates. Following RFLP analysis of many other clones, two and six genomic DNAs other than DNA-S and -U1 were identified from the samples Eth-218 and Eth-231, respectively. The DNA-S and -U1 of Eth-2 and Eth-183 and the DNA-S of Eth-218 were only partially sequenced whereby the sequenced part encompassed the coding region. Complete sequences were obtained for the DNA-R (1003 nt), -U1 (990 nt) and -U2 (988 nt) of Eth-218 (Table 4). For Eth-231, all eight genomic DNAs were entirely sequenced (Tables 6).

The amino acid sequences of the proteins potentially encoded by the DNA-S and -U1 of the four isolates were used to assess the genetic diversity and phylogenetic relationship of the Ethiopian nanovirus isolates to each other and to other nanoviruses. The amino acid sequence identities obtained by pairwise comparison of the CP and U1 gene sequences of these isolates with those of previously sequenced FBNYV isolates and other nanoviruses is shown in Table 5. It can be seen that there is a close correlation between serological data and sequence identity of the isolates. For example, both the CP and U1 protein amino acid sequence identities between Eth-2 and FBNSV-Eth were very similar (~99%) (Table 5). Similarly, isolate Eth-

218 shared CP and U1 protein amino acid sequence identities of 96% and 93%, respectively, with FBNYV isolates. This is in sharp contrast to the significantly low identity obtained when Eth-2 or FBNSV-Eth is compared to Eth-218 or FBNYV-Eg which gave identity values of less than 85% for CP and 68% for U1 proteins (Table 5).

Table 5. Pairwise comparison of predicted CP (below diagonal) and U1 (above diagonal) amino acid sequences of the Ethiopian isolates with previously sequenced nanovirus isolates

Virus isolate	Ethiopian isolates				FBNSV	FBNYV		MDV	SCSV
	Eth-2	Eth-183	Eth-218	Eth-231	-Eth	-Eg	-Sy		
Eth-2	-	66.4	65.8	66.4	99.4	64.5	67.8	68.7	44.1
Eth-183	84.3	-	96.1	68.1	67.1	93.5	95.5	74.7	45.5
Eth-218	84.9	97.7	-	68.2	66.4	92.9	94.8	75.3	44.8
Eth-231	88.4	88.4	89.0	-	67.3	65.6	67.5	71.3	42.4
FBNSV-Eth	98.8	83.7	84.7	87.2	-	65.1	68.4	69.3	44.8
FBNYV-Eg	84.3	95.9	95.9	88.4	83.7	-	93.5	74.0	42.1
FBNYV-Sy	83.7	95.3	95.3	87.5	83.1	99.4	-	74.0	45.5
MDV	83.7	84.3	83.7	82.6	83.7	83.7	83.1	-	46.5
SCSV	56.2	57.1	57.1	56.7	55.6	55.6	55.3	53.6	-

From sample Eth-183 which, based on serological data, is suspected of having a mixed infection, sequences similar to those of Eth-218 (serogroup B) were amplified (Table 5, Fig. 4**).** This result corroborated our prediction from serological results that this sample has a mixed infection with serogroup A and B isolates although only serogroup B but not A sequences were obtained. Eth-183 and Eth-218 are very similar to each other in both CP and U1 protein amino acid sequences and both appear to be similar to typical FBNYV isolates from Egypt or Syria (Table 5, Fig. 4). In line with this result, the phylogenetic analysis of two further Eth-218 proteins encoded by DNA-R and -U2 (Fig. 4) confirmed the assignment of Eth-218 to FBNYV.

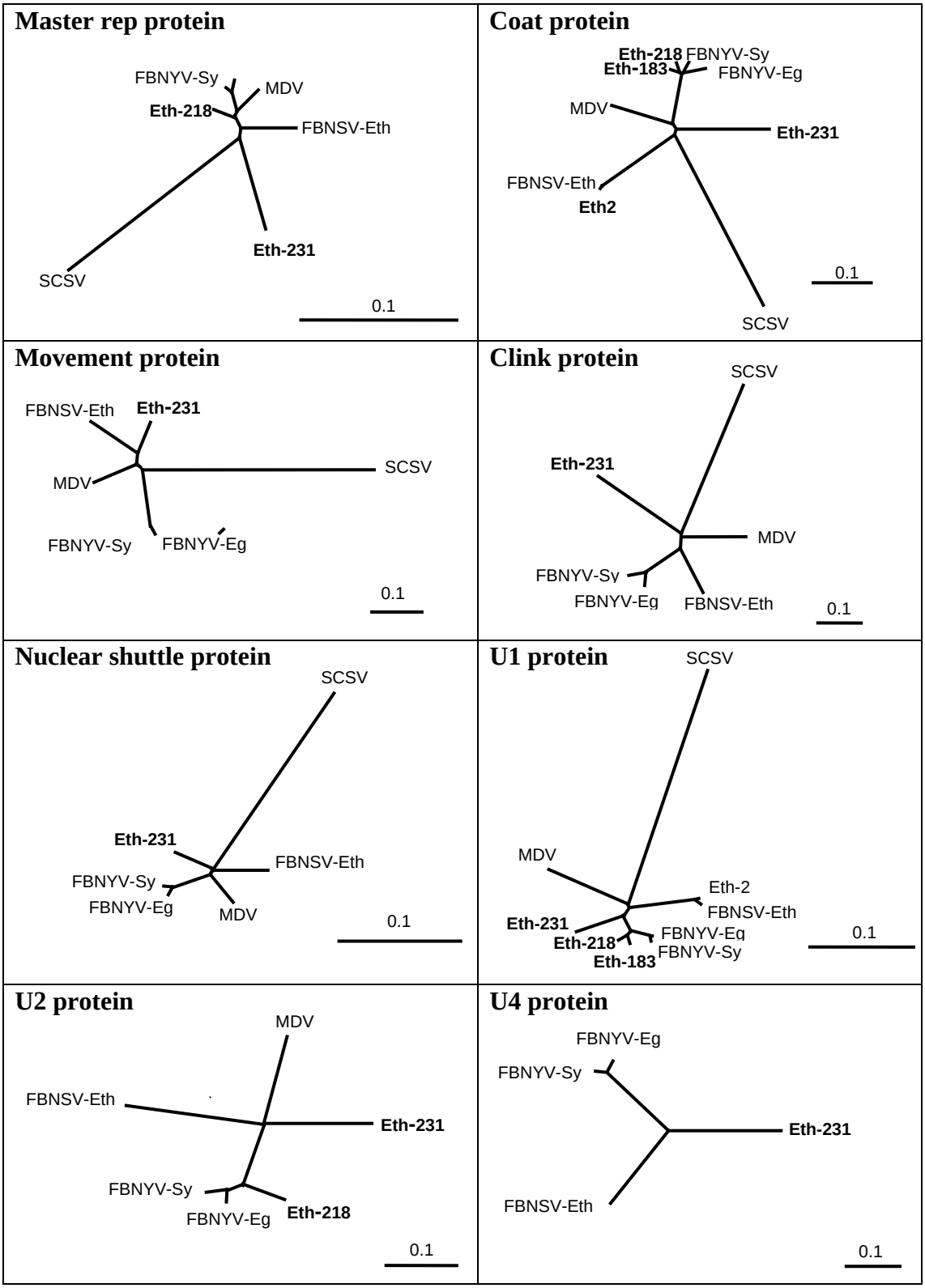

Fig. 4. Phylogenetic relationship of proteins encoded by the eight DNAs of Eth-231 with those of the other nanovirus isolates including those of the other Ethiopian serogroups (in bold)

Genome sequence of the Eth-231 isolate and its relationship to other nanoviruses

Since analysis of DNA-S and -U1 sequences of Eth-231 (Table 5) suggested that it represents a novel nanovirus isolate never reported before, its eight genomic DNAs were completely sequenced and analysed. The eight ssDNAs thought to make up the entire nanovirus genome ranged in size from 972 nt (DNA-U4) to 1002 nt (DNA-R), amounting to a total genome size of 7935 nt (Table 6). All eight DNAs appear to be structurally similar in having a common stem loop sequence and one major positive-sense ORF that potentially encodes one of the eight distinct nanovirus proteins. Comparison of the total nucleotide sequence of Eth-231 with that of other nanoviruses revealed an overall nucleotide identity of c. 70% (Table 7). This is significantly below the nucleotide sequence identity threshold of 75% currently accepted as a major criterion for nanovirus species demarcation by the International Committee of Taxonomy of Viruses (ICTV) (Vetten et al. 2004). The result thus suggested that Eth-231 represents a distinct nanovirus.

Table 6. Sizes of the eight ssDNAs, their encoded proteins, positions and sequence of some transcription units of the Eth-231 isolate and.

DNA component [a]	Size bases	TATA-box position [b]	ORF				
			Initiation position [c]	Intiation codon and its context	No. of aa	Protein size (kDa)	Termination codon
R	1002	74	120	AATATGG	286	33.1	TGA
S	1001	278	316	AAAATGG	183	20.5	TAA
M	980	297	353	CGAATGG	112	12.7	TAG
C	995	283	323	AAAATGG	173	20.1	TGA
N	1000	299	341	AAAATGG	153	17.3	TAA
U1	990	285	376	TTCATGG	154	17.9	TAA
U2	995	297	366	CTTATGG	121	14.8	TAA
U4	972	262	360	TTTATGG	106	12.2	TAA

[a] Primer pairs used to amplify the DNAs were 1-4, 7-13 as shown in Table 1.

[b] Position of TATA box is the number of nucleotides from the first nucleotide of the stem loop to the first base of the TATA box.

[c] Postion of the ORF is the number of bases from the first base of the stem loop to and including the first base of the initiation codon.

Analysis of the noncoding regions of the Eth-231 DNAs

Each of the DNAs has a highly conserved region of 32 bases capable of forming a putative stem-loop structure at its noncoding region as has been already reported for other nanoviruses (Katul et al. 1998, Sano et al. 1998, Burns et al. 1995). The nonanucleotide sequence TAGTATTAC forming the loop and shown to include a viral origin of replication (Timchenko et al. 1999) is perfectly conserved in all genomic DNAs of the nanoviruses sequenced. The stem loop sequence of all eight DNAs of Eth-231 starts with nucleotides CT followed by GC-rich sequence and ends with a matching AC at positions 31 and 32 (Fig. 2). This is different from related nanoviruses, such as FBNYV, FBNSV (Katul et al. 1998, Katul and Vetten, 1999, Timchenko et al. 1999) and MDV (Sano et al. 1998), whose DNA-C and -U1 stem loop sequences lack these bases, making Eth-231 unique in this respect. In addition, whereas the stem loop regions of six of the Eth-231 DNAs are perfectly identical with those of other nanoviruses, two of its DNAs (DNA-S and DNA-N) have a stem sequence that differs in four bases, CA at positions 5 and 6 and the matching nucleotides TG at positions 27 and 28 (Fig. 2). Moreover, the common sequence motifs flanking the stem loop that are suggested to contain specificity elements for recognition of the master rep protein (Timchenko et al. 1999) are also somewhat different in these two DNA components as compared to those of other FBNYV isolates, FBNSV and MDV (Fig. 2).

As previously described for the Moroccan (Abraham et al. in preparation, 2005b) and other nanovirus isolates (Katul et al. 1997, Sano et al. 1998, Hughes, 2004), the nucleotide sequence of the noncoding region of the seven non-rep DNAs shows some relationships. DNA-U1 and -C share an identity of 91% while DNA-M and -U2 have an identity of 93%. The noncoding region of DNA-N is the most deviating from that of all the others whereas that of DNA-S and -U4 are more divergent from each other although they share a higher identity with DNA-C and U1. Because of the large ORF of DNA-R, this component has a very small noncoding region in addition to the stem loop sequence and thus was not included in the comparison.

For each DNA, the total nucleotide size, the properties of the ORFs, i.e. position and context of initiation codon, the number of amino acids and the stop codons are presented in Table 6. The potential TATA-box sequence (TATAAA) is conserved in all DNAs with a distance between the box and the initiation codon varying from 38 nt for DNA-S to 98 nt for DNA-U4 (Table 6). The possible polyadenylation signal AATAAA or similar sequence required for efficient termination of transcription (Rothnie et al. 1994) are appropriately located downstream of the ORFs in all the DNAs.

Comparison of the Eth-231 proteins with those of other nanoviruses.

Each of the DNAs has only one ORF potentially encoding a protein ranging in size from 12 to 33 kDa (Table 6). These proteins are homologous to those of other described nanovirus isolates. The amino acid sequence identities resulting from pairwise comparisons of the eight proteins of Eth-231 with those of other nanoviruses are presented in Table 7. When considering all eight proteins, Eth-231 shares an overall protein amino acid sequence identity of ≤ 74% with other nanoviruses. If the U4 protein which has not yet been identified from nanoviruses other than FBNYV and FBNSV is excluded from the comparison, the seven proteins share an overall identity of 76-77% with both FBNYV isolates and MDV, indicating that Eth-231 is as different from FBNYV and FBNSV as it is from MDV. It is more distantly related to SCSV with mean amino acid sequence identities of 55% for the six known homologous proteins, ranging from 37.5% for DNA-U1 to 82.2% for DNA-R.

DNA-R codes for the master replication initiator (Rep) protein that contains an NTP-binding motif (GGEGKTS) that is conserved in all Rep proteins of nanoviruses (Timchenko et al. 1999). With a Rep protein amino acid sequence identity ranging from 53.1% for BBTV to 92.0% for MDV (Table 7), this protein appears to be the most conserved. On the other hand, the U4 protein is the least conserved when compared to its homologues of known FBNYV isolates with an identity ranging from 54.7-57.1%. In general, the proteins encoded by the three DNAs (DNA-U1, -U2 and -U4) whose function is unknown, are more variable than those encoded by the DNA-S, -R, -M, -N and -C.

The Clink protein encoded by DNA-C is unusually divergent when compared to the equivalent protein of FBNYV, FBNSV, and MDV, sharing amino acid sequence identities of 58-60%. This is partly due to a stretch of 17 amino acids (positions 101-117) which is strikingly different from the corresponding region in the Clink protein of other known nanovirus isolates, although this domain is a fairly conserved Clink domain for members of genus Nanovirus (Fig. 3). Most notably, the LxCxE motif, which invariably occurs in the Clink protein of all other members of the family Nanoviridae and has been demonstrated to interact with retinoblastoma-like plant proteins for cell-cycle regulation (Aronson et al. 2000, Wanitchakorn et al. 2000), is absent from the amino acid sequence deduced from the DNA-C sequence of Eth-231. This makes the Eth-231 Clink protein unique among all other nanoviruses.

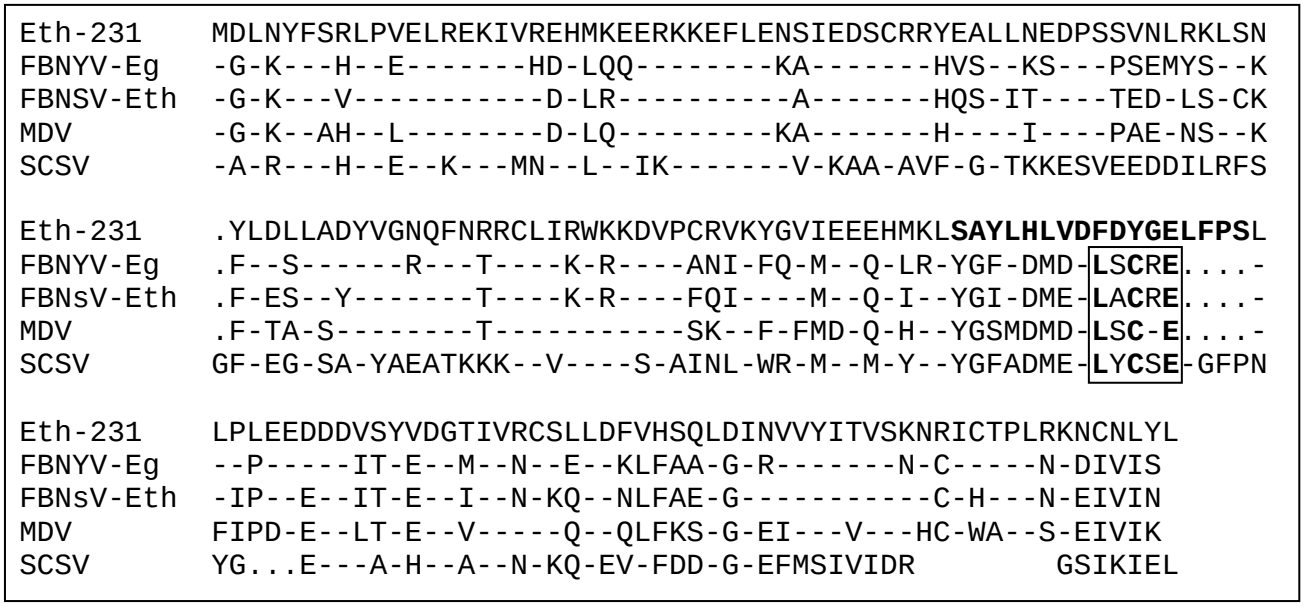

Fig. 3. Alignment of the predicted amino acid sequences of the Clink protein of Eth-231 with that of members of the genus *Nanovirus*. Note that the LxCxE motif conserved in all other nanoviruses (boxed) is not present in corresponding region of Eth-231 (bold).

Phylogenetic analysis of the proteins encoded by each of the DNAs identified from the four Ethiopian isolates including the eight proteins encoded by Eth-231 is shown in Fig. 4. As expected from the serological data (Table 3) and sequence comparisons (Table 7), isolate Eth-2 was grouped with FBNSV while Eth-183 and Eth-218 were grouped with FBNYV on the basis of the CP and U1 protein sequences. In the case of Eth-231 when all the eight proteins were compared, some proteins (e.g. Clink protein, Rep protein) show lower similarity to the FBNYV or FBNSV than to MDV.

Table 7. Total nucleotide and amino acid (coding) sequence identities between homologous DNAs of Eth-231 and other members of the family Nanoviridae.

Isolate/ Virus	DNA components																Mean	
	R		S		M		C		N		U1		U2		U4			
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
FBNSV-Eth	87.5	90.9	75.3	87.2	68.4	81.3	70.0	60.4	75.8	91.5	70.0	65.6	60.1	65.3	62.4	54.7	70.4	74.6
FBNYV-Eg	88.2	90.9	78.0	88.4	64.8	80.4	70.3	58.0	75.8	89.5	69.0	65.6	58.6	61.2	57.4	55.2	70.4	73.6
FBNYV-Sy	88.9	91.3	77.7	87.5	66.4	75.9	70.5	58.0	77.0	90.8	67.4	67.5	60.7	62.8	57.5	57.1	70.4	73.8
MDV	86.6	92.0	78.4	82.6	65.3	79.5	73.8	59.2	76.5	90.8	71.3	68.7	69.7	60.3	- *	-	74.5	76.1
SCSV	77.7	82.2	63.8	56.7	62.5	45.0	59.5	40.5	63.9	68.0	58.8	37.0	-	-	-	-	64.4	54.9
BBTV	56.9	53.1	48.4	17.4	49.7	17.6	52.7	17.0	55.5	44.1	-	-	-	-	-	-		

* Dashes denote that a comparison was not possible due to the fact that a homologous DNA components has not yet been identified from the other nanovirus.

DISCUSSION

The serological and sequence data presented here indicate that although serogroup A isolates similar to the previously characterized Ethiopian nanovirus isolate (FBNSV-Eth) are the most prevalent in Ethiopia, legume nanoviruses infecting faba bean in the country are highly diverse. The occurrence of typical FBNYV isolates represented by serogroup B in this country is reported for the first time. In addition, srogroup C have never been reported from anywhere before. The occurrence of all previously known and new FBNYV-like viruses together with the report on the occurrence of at least two distinct nanovirus species in Morocco (Abraham et al. in preparation, 2005b) indicates that previous suggestion about geographically associated variation of FBNYV isolates (Franz et al. 1996) is incorrect and appears to be result of inadequate sampling.

Our results revealed a high diversity of legume nanovirus isolates including the already known (serogroups A and B) and novel (serogroup C) nanovirus species in a very confined geographical area in the Gedeo zone of southern Ethiopia, where faba bean or legumes in general are grown only at limited scale. Furthermore, some of these serotypes appear to occur in mixed infection suggesting that they occupy similar ecological niches. It is not clear why only serogroup A (FBNSV) isolates occur in the main faba bean growing areas while a diverse group of nanoviruses occur in the restricted area Gedeo, a non-traditional faba bean growing area. One possible explanation is that the nanovirus isolates in this area evolved from a common ancestor but diversified to genetically distinct groups possibly in indigenous legumes species in which they have coevolved and cause symptomless infection. It is thus conceivable that when a susceptible host like faba bean is introduced, they cause severe damage in single or mixed infection. More intensive study of the genetic structure of nanovirus populations in southern Ethiopia and elsewhere based on a large number of samples will help in further revealing a more complete picture of nanovirus diversity in legumes.

Distinction between viruses or their strains is critical in epidemiological surveys as the isolates may differ in their pathogenicity, host range, vector specificity and geographical distribution. However, only few studies were carried out on the variability of nanoviruses worldwide. Karan et al (1995) and Bell (2002) studied the genetic variability of the master rep DNA of BBTV isolates from different countries and different parts of Vietnam, respectively. Such kind of information has been lacking for legume nanoviruses. In addition to the recent study on Moroccan nanovirus isolates (Abraham et al. in preparation, 2005b), this work provides useful information on the diversity or variability of legume nanoviruses within countries. Our results also indicate that there is a good correlation between serological typing using differentiating Mabs and difference

in CP and U1 gene sequences indicating that the two techniques were complementary. Similar conclusions were reached after detailed comparison of serological and molecular techniques for typing of RNA plant viruses such as Rice yellow mottle virus (Fargette et al. 2002) and Plum pox virus (Candresse et al. 1998). Therefore, large scale serological analysis of samples from a region followed by sequencing of one or more genes of isolates representing different serogroups as done in our work would be the best approach to understand the diversity or variability of legume nanoviruses in a country or a region.

Like the other previously sequenced isolates of FBNYV and FBNSV, eight distinct circular ssDNAs were found associated with Eth-231 infection, supporting the notion that these eight components are integral parts of the nanovirus genome (Vetten et al. 2004). However, the number of DNAs required for obtaining an infection similar in symptomatology and aphid transmission to that of a wild-type isolate has not yet been experimentally demonstrated. Further work using all or different combinations of cloned DNAs would be helpful in achieving this goal.

DNA-U4 was first reported from FBNSV-Eth (Franz et al. 1999, Katul and Vetten 1999) and was at the same time identified from FBNYV-Eg and FBNYV-Sy (Vetten et al. 2004, Katul and Vetten, unpublished data). It has not yet been identified from other nanoviruses. The occurrence of two possible initiation (ATG) codons in close proximity to each other at the start of the coding region in all DNA-U4 sequences obtained so far raised the question as to which of these two start codons is the correct one (Vetten et al. 2004). The DNA-U4 ORF of Eth-231 lacks the second ATG and has only one initiation codon at the start of its coding sequence. The absence of the second ATG suggests that DNA-U4 of Eth-231codes for a 12 kDa but not for a 10 kDa protein.

According to the current ICTV criteria for nanovirus species demarcation (Vetten et al. 2004), distinct nanovirus species should have an overall nucleotide sequence identity of $\leq$ 75 % and CP amino acid sequence of $\leq$ 85%. In addition to FBNSV as the predominant faba bean-infecting nanovirus in Ethiopia, a typical FBNYV isolate (e.g. Eth-218) is reported here from Ethiopa for the first time. This indicates the occurrence of a second nanovirus in faba bean in Ethiopia. Finally, Eth-231, a previously unrecognized nanovirus species shares overall nucleotide sequence identities of only 70-71% and CP amino acid sequence identities of 87-88% with its closest relatives among the nanoviruses. In addition, Eth-231 considerably differs in its noncoding sequence from other FBNYV isolates and MDV (Fig. 2) and is also unique among all known nanoviruses in lacking an LxCxE motif in its Clink protein. These data provide strong molecular evidence for regarding Eth-231 as a distinct nanovirus species for which the name Faba bean yellow leaf virus (FBYLV) is proposed. Therefore, based on molecular criteria, three distinct nanoviruses i.e.

FBNYV, FBNSV and FBYLV occur in Ethiopia. Further data on their variation in biological properties such as symptom variation, host range and aphid vectors are required prior to considering these viruses a definitive member of genus Nanovirus.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

A number of new viruses were found to be associated with legume samples that showed yellowing and stunting symptoms in countries of Northeast and North Africa and West Asia indicating a high diversity of viruses in this region. The fact that these viruses have not been recognized in previous legume virus surveys suggests that the virus identification techniques used were not adequate to reveal the occurrence of new viruses. Such surveys mostly relied on serological tests alone (e.g. Abraham et al. 2000, Fortass and Bos, 1991, Makkouk et al. 1994, 1995, 2003a, 2003b, Tadesse et al. 1999) with no supplementary biological and molecular data. As a result, a significant proportion of samples with clear virus-like symptom did not react with virus-specific antibodies and hence the associated viruses remained unidentified. The use of serological methods alone in virus identification has a drawback in that only viruses for which specific antibodies are already available can be detected and the possible new viruses are mostly overlooked. In addition, antibodies that are produced to specific virus often cross react with other related but distinct virus species that share certain common epitopes in their coat protein leading erroneous virus identification. This phenomenon is particularly common for luteoviruses as has been shown for CpCSV and BWYV antibodies in the present study (Abraham et al. submitted, 2005) and also previously reported for many luteovirus combinations (e.g**.** Martin and D'Arcy, 1990, Fortass et al. 1997). In the present study, the combined use of serological tests employing broad-spectrum and virus-specific antibodies to luteoviruses and nanoviruses followed by sequence analysis revealed the occurrence of hitherto unknown viruses infecting legumes in the region covered in this study. The results underline that virus identification based on serological tests should be supported by sequence information of at least part of a virus genome particularly when a virus or virus isolate is reported for the first time from a country, a region or a new host.

Of the three new luteoviruses discovered and tentatively named in this study, Chickpea chlorotic stunt virus (CpCSV), appears to be the most frequently encountered in the samples studied. The virus appears to have two geographically differentiated strains differing in molecular, serological and biological properties. Although the number of samples used in this study is not enough to make firm conclusion, the data suggests that there is a geographically associated variation among CpCSV isolates that could be categorized into two groups. If the existence of such variability could be confirmed with further studies on larger number of sam-

ples from different countries, it may have important practical value for crop improvement programs since sources of resistance obtained in one country may not be used for another as they could be strain-specific.

Sequence information of the complete genome of CpCSV and phylogenetic analysis indicated that CpCSV belongs to the genus *Polerovirus* of the family *Luteoviridae*. Furthermore, recombination analysis provided evidence that CpCSV genome may be result of an unusual kind of recombination between *Cucurbit aphid-borne yellows virus* (CABYV)-like and *Soybean dwarf virus* (SbDV)-like ancestral viruses in part of its readthrough protein. In agreement with the recombination model suggested by Miller et al. (1995, 1997), it appears that the recombination hotspot is located approximately at the start site of subgenomic RNA2 with reference to CABYV (Ashoub et al. 1998). It should however be noted that the lack of sequence information for the major part of the genome of *Groundnut rosette assistor virus* (GRAV) (Scott et al. 1996), the closest relative of CpCSV (Abraham et al submitted, 2005) did not allow us to compare the entire genomes the two viruses. Sequencing of the genome of GRAV will provide further information on the phylogenetic relationship of these two closely related but distinct legume luteoviruses.

Chickpea yellows virus (CpYV) and Lentil stunt virus (LStV) are proposed to be new viruses only on the basis of sequence information of the CP gene. Therefore, information on serological and biological properties such us the host range and aphid vectors are necessary to firmly establish these viruses as new species in the family *Luteoviridae*. For such studies, the development of reliable and sensitive diagnostic procedures for specific detection and identification of these viruses in single or mixed infection is important. It is also possible that there are other yet unrecognized or inadequately characterized luteoviruses infecting legumes. For example, Fortass et al. (1996) described a luteovirus isolate from Morocco that has serological properties similar to BWYV but behaved like BLRV in hybridization test. Similar virus isolates were not encountered in this study. Sequence analysis of more isolates from different geographical areas is therefore likely to reveal further the diversity of luteoviruses infecting legumes.

Serological and molecular analysis of faba bean nanovirus isolates from Ethiopia and Morocco revealed a high viral diversity to such an extent that the isolates characterized can be considered as new nanovirus species. It was earlier suggested that the two distinct nanovirus isolates previously referred to as FBNYV evolved as distinct groups in geographical isolation in Ethiopia and in other countries (Franz et al. 1996). Sequencing of the genomic DNAs of these two groups of isolates later indicated that although isolates from Egypt and Syria are typical

FBNYV isolates, the isolate from Ethiopia is in fact a distinct nanovirus for which the name Faba bean necrotic stunt virus has been proposed (FBNSV) (Katul and Vetten, 1999, Katul and Vetten, unpublished). The finding that both FBNYV and FBNSV isolates occur in Morocco and Ethiopia indicates that these viruses may occur everywhere else. This is in sharp contrast contrary to a notion (Franz et al. 1996) that the two nanoviruses are geographically isolated. In addition, the discovery new nanovirus tentatively named Faba bean yellow leaf virus (FBYLV) from Ethiopia indicates that legume nanoviruses are much more diverse than once thought. It is most likely that further serological and molecular analysis of samples originating from different countries as done in this work would reveal new nanoviruses and/or their strains and also provide information on the geographical distribution of already known nanoviruses.

Sequence analysis of the putative genome of two nanovirus isolates from Morocco and one from Ethiopia consistently indicated that eight distinct ssDNAs are associated with each the isolates. This results support the earlier suggestion (Vetten et al. 2004) that these eight ssDNAs are integral parts of the nanovirus genome. The lack of LXCXE , a retinoblastoma binding motif, in the Clink protein of the newly discovered FBYLV makes this virus unique among all nanoviruses known so far. This finding suggests that as it has been demonstrated in the case of mastreviruses (Xie et al. 1995) and begomoviruses (Arguello-Astorga et al. 2004), nanoviruses may also use different mechanism to interact with retinoblastoma-like protein. This finding is likely to stimulate further studies on the significance of the presence or absence of this motif in the Clink protein of nanoviruses and its requirement for replication and virus-host interaction.

Irrespective of their taxonomic grouping, luteoviruses and nanoviruses infecting cool season food legumes share similar ecological niches under natural condition. They naturally infect the same group of hosts (legumes), are phloem limited in their host and cause similar symptoms, share aphid vectors by which they are persistently transmitted, and are not seed-transmitted. In particular, CpCSV and FBNYV, the two viruses frequently detected from the countries covered in this study, are both persistently transmitted by *Aphis craccovora* which is possibly their major natural vector (Abraham et al. submitted, 2005, Franz et al. 1998), and their major hosts are cool season food legumes and in which they often occur in mixed infection (Abraham et al. submitted, 2005, Franz et al. 1997). Hence, the disease epidemiology in the field is likely to be similar. Therefore, information generated for the management of better studied luteoviruses and FBNYV can be useful for the development of possible control measures to CpCSV..

In conclusion, this work has provided a substantial amount of information on a diverse range of luteo- and nanoviruses associated with yellowing and stunting diseases of cool season food

legumes, in particular sequence data on three new luteoviruses and one new nanovirus. The sequence information generated here is not only of fundamental importance for future molecular studies on these two groups of plant viruses but also, together with the information on virus diversity and variability, is invaluable for breeding for resistance to specific viruses or strains in a certain area. Finally, the antibodies and the group-, virus- or isolate-specific primers described here will be useful for resistance breeding and studies of virus epidemiology and ecology. Therefore, these diagnostic tools can be helpful in efforts to understand and develop suitable management strategies for yellowing and stunting diseases caused by luteo- and nanoviruses in cool season food legumes.

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SUMMARY

The production of cool season food legumes is limited by a number of biotic and abiotic factors including diseases caused by viruses. Yellowing, stunting and leaf roll diseases generally thought to be caused predominantly by luteo- and nanoviruses are among the most destructive viral diseases of these crops worldwide. However, information on the exact identity and the biological and genetic diversity of the causal viruses has been limited particularly in countries of Northeast and North Africa and West Asia since virus identification has often been based solely on serological diagnosis of field samples with no information on biological or molecular properties. When legume samples with yellowing and stunting symptoms collected in Ethiopia, Sudan, Egypt, Morocco, Syria, Germany and China were serologically analyzed, many of them reacted with broad-spectrum antibodies to luteoviruses and/or nanoviruses, indicating luteo- and nanovirus infections in the legume crops surveyed. Since further serological analysis with specific antibodies suggested the occurrence of uncommon or unknown viruses in these crops, partial or complete nucleotide sequences of the virus isolates in representative samples were determined to establish the exact identity of the causal viruses. These attempts led to the discovery of a diverse range of luteo- and nanoviruses, some of which were characterized in greater detail.

A new luteovirus tentatively named Chickpea chlorotic stunt virus (CpCSV) and phylogenetically related to, but distinct from, *Groundnut rosette assistor virus* (GRAV) [with a coat protein (CP) amino acid identity of 78%] was detected in several samples in most countries. Biological studies showed that CpCSV is transmitted by *Aphis craccivora* but not by *Aphis fabae*, *Acyrthosiphon pisum* or *Myzus persicae* and has a host range limited to a few cool season food legume species. Electron microscopy of purified preparation showed isometric particles measuring 28 nm in diameter. A rabbit polyclonal antiserum and 10 mouse monoclonal antibodies were produced, characterized and evaluated for use in routine detection and identification of the virus. Sequencing of the complete RNA genome of an Ethiopian isolate of CpCSV revealed that the viral RNA is 5900 nucleotides and is arranged in six major open reading frames. The CpCSV genome shares genomic organization and phylogenetic relationships with members of the genus *Polerovirus* of the family *Luteoviridae*. Recombination analysis suggested that most of CpCSV genome possibly evolved from *Cucurbit aphid-borne yellows virus*- and/or GRAV-like ancestors, but that it has acquired a stretch of ca. 90 amino acids in its readthrough domain from an ancestral *Soybean dwarf virus* (SbDV)-like virus. Analysis of the coat protein gene sequence of 18 CpCSV isolates from five countries indicated that the virus occurs as two

five countries indicated that the virus occurs as two geographically differentiated strains referred to as NE (Northeast Africa) and WN (West Asia and North Africa). The strains also differed in symptom severity caused in faba bean and in reactions with monoclonal antibodies raised against an Ethiopian isolate of CpCSV.

Analysis of the CP gene sequences of luteovirus isolates associated with other legume samples provided evidence for the possible occurrence of two further previously unrecognized luteoviruses. The CP sequence of a luteovirus isolate from chickpea in Sudan indicated that it is a distant relative of SbDV with which it shares a CP amino acid identity of only 66%. Since this warrants its classification as a distinct luteovirus, the name Chickpea yellows virus (CpYV) is proposed. An isolate from lentil in Ethiopia shared a CP amino acid identity of 86% with viruses of the Beet western yellows virus subgroup. Due to the fact that this is less than the currently accepted threshold value of 90% in amino acid sequence identity for discriminating different luteovirus species, this isolate also appears to represent another distinct luteovirus species for which the name Lentil stunt virus (LStV) is proposed. In addition to these new luteoviruses, almost identical CP gene sequences of *Turnip yellows virus*-like viruses were amplified from faba bean samples from Egypt and Morocco. CP gene sequences strikingly closely related to those of SbDV were amplified from faba bean samples from Ethiopia, Germany, Syria and China, suggesting that SbDV has a much wider geographic distribution than originally thought. SbDV was reported here for the first time from Germany and Europe.

The use of monoclonal antibodies (Mabs) raised against the nanovirus *Faba bean necrotic yellows viru*s (FBNYV) has provided evidence for the occurrence of FBNYV or its close relatives in legume crops in many countries of West Asia and North Africa. Moreover, discriminating Mabs have indicated a serological variability among FBNYV or nanovirus isolates. To obtain a better understanding of the genetic basis of the variability among FBNYV or nanovirus isolates within a geographic area, the eight distinct ssDNA components thought to form the nanovirus genome were completely sequenced for two Moroccan isolates (Mor5 and Mor23) which differed strikingly in epitope profiles. Mor23 resembles typical FBNYV isolates from Egypt and Syria. However, analysis of the Mr5 sequences showed that Mor5 is very similar to the previously described Ethiopian isolate (ETH) which is suggested to belong to a distinct nanovirus species named Faba bean necrotic stunt virus (FBNSV). These results provided first molecular evidence for the occurrence of two distinct nanovirus species within a country.

Serological analysis with eight differentiating Mabs of 73 nanovirus-infected samples collected from faba bean crops in Ethiopia indicated that the isolates could be categorized into at least three distinct serogroups designated A, B and C**.** The coding sequence of at least two DNA components (DNA-S and -U1 encoding CP and U1 protein, respectively) of each serotype were determined to allow for genetic comparisons. These studies demonstrated striking differences in amino acid sequences of these proteins. Serogroup A isolates are the most prevalent in Ethiopia and similar to the previously described isolate ETH referred to as nanovirus FBNSV. Serogroup B isolates are similar to typical FBNYV isolates reported to predominate in Egypt and Syria, but appeared to be absent from Ethiopia. The third group designated serogroup C represents a novel nanovirus type which possessed serological and molecular properties never reported from anywhere else before. To understand the genetic properties of this virus and to fully assess its relationship to other nanoviruses, all eight genomic ssDNA components of Eth-231, a representative serogroup C isolate, were sequenced. Sequence analysis indicated that Eth-231 shares an overall nucleotide sequence identity of only 70%. Since this is less than the currently accepted level of percentage identity (75%) of distinct nanovirus species, serogroup C isolates are proposed to be named Faba bean yellow leaf virus (FBYLV). Furthermore, the Clink protein of Eth-231 is unusually divergent and unlike all previously known nanoviruses, lacks the LXCXE motif involved in cell cycle regulation. The serological and sequence data presented provided evidence for the occurrence of three distinct nanovirus species infecting faba bean in Ethiopia; FBNSV (serogroup A isolates), FBNYV (serogroupB isolates) and FBYLV (serogroup C isolates).

In conclusion, this work has provided a substantial amount of information on a diverse range of luteo- and nanoviruses associated with yellowing and stunting diseases of cool season food legumes, in particular sequence data on three new luteoviruses and one new nanovirus. The sequence information generated here is not only of fundamental importance for future molecular studies on these two groups of plant viruses but also, together with the information on virus diversity and variability, is invaluable for breeding for resistance to specific viruses or strains in a certain area. Finally, the antibodies and the group-, virus- or isolate-specific primers described here will be useful for resistance breeding and studies of virus epidemiology and ecology. Therefore, these diagnostic tools can be utilized for the management and control of the yellowing and stunting diseases caused by luteo- and nanoviruses in cool season food legumes.

ZUSAMMENFASSUNG

Die Wirtschaftlichkeit des Anbaus von Körnerleguminosen, wie Fababohne, Linse, Kichererbse, etc. ist durch abiotische und biotische Faktoren, wozu auch Viruskrankheiten gehören, gefährdet. Vergilbungs-, Stauche- und Blattrollkrankheiten, die vermutlich vorwiegend durch Luteo- und Nanoviren verursacht werden, gehören weltweit zu den schädlichsten Krankheiten dieser Kulturen. Jedoch ist das Wissen über die genaue Identität sowie über die biologische und genetische Variabilität der verursachenden Viren besonders in westasiatischen und nord- (und nordost-) afrikanischen (WANA) Ländern sehr beschränkt. Das ist vor allem darauf zurückzuführen, dass hier eine Virusidentifizierung oft allein auf serologischen Methoden basierte, ohne dass auch biologische und/oder molekulare Eigenschaften bestimmt wurden.

Als Proben von Körnerleguminosen mit Vergilbungs- und Stauchesymptomen aus Äthiopien, Ägypten, Marokko, Sudan, Syrien, Deutschland und China serologisch analysiert wurden, reagierten viele von ihnen mit Breitspektrum-Antikörpern zum universellen Nachweis von Luteoviren bzw. Nanoviren, was als Hinweis für das Vorkommen dieser Viren in den inspizierten Körnerleguminosenkulturen angesehen wurde. Als weitere serologische Untersuchungen unter Verwendung von spezifischen Antikörpern das Vorkommen von ungewöhnlichen oder unbekannten Viren in diesen Kulturen anzeigten, wurden partielle bzw. vollständige Nukleotidsequenzen von repräsentativen Virusisolaten bestimmt, um auf diese Weise die genaue Identität der verursachenden Viren feststellen zu können. Diese Versuche führten zur Entdeckung einer Reihe sehr unterschiedlicher Luteo- und Nanoviren, von denen einige hier näher charakterisiert wurden.

Aus mehreren Leguminosenproben aus fünf WANA-Ländern gelang der Nachweis eines neuen Luteovirus mit dem vorläufigen Namen Chickpea chlorotic stunt virus (CpCSV). Es ist phylogenetisch am nächsten verwandt mit *Groundnut rosette assistor virus* (GRAV), von dem es jedoch aufgrund einer Hüllprotein(CP)-Aminosäuresequenzidentität von 78% klar unterscheidbar ist. Biologische Untersuchungen am CpCSV zeigten, dass es nur von der Blattlaus *Aphis craccivora*, nicht aber von *Aphis fabae*, *Acyrthosiphon pisum* oder *Myzus persicae* übertragen wird. Sein Wirtskreis beschränkt sich auf einige wenige Körnerleguminosen. Elektronenmikroskopisch ließen sich in gereinigten Viruspräparaten isometrische Partikeln mit einem Durchmesser von 28 nm im sichtbar machen. Ein CpCSV-spezifisches Antiserum und 10 monoklonale Antikörper (Mab) wurden hergestellt, charakterisiert und im Hinblick auf ihren Einsatz unter Routinebedingungen und zur Virusidentifizierung bewertet.

Die Sequenzierung des vollständigen RNA-Genoms eines CpCSV-Isolates aus Äthiopien zeigte, dass die virale RNA aus 5900 Nukleotiden besteht und sechs Offene Leseraster (ORF) aufweist. Die CpCSV-RNA hat eine Genomorganisation, die typisch für Arten der Gattung *Polerovirus* (Familie *Luteoviridae*) ist, mit denen es auch phylogenetisch eng verwandt ist. Eine Rekombinationsanalyse deutet darauf hin, dass sich der überwiegende Teil des CpCSV-Genoms möglicherweise aus *Cucurbit aphid-borne yellows virus- u*nd/oder GRAV-ähnlichen Vorfahren entwickelt hat. Jedoch hat es in seinem P5-Gen einen ca. 90 Aminosäuren umfassenden Bereich aus einem entsprechenden Genom-Bereich eines Vorfahren oder noch unbekanntem Verwandten des *Soybean dwarf virus* erlangt. Eine Analyse der CP-Sequenzen von 18 CpCSV-Isolaten aus fünf Ländern zeigte, dass sich die Isolate in zwei Gruppen (=Stämme) einordnen lassen, die mit der geographischen Herkunft der Isolate (Nordost-Africa bzw. Westasien und Nordafrica) in Beziehung zu stehen scheinen. Die beiden Stämme unterschieden sich auch in der Symptomstärke in Fababohnen und in der Reaktion mit Mabs, die gegen ein äthiopisches Isolat des CpCSV hergestellt worden waren.

Die Analyse der CP-Sequenzen von Luteovirusisolaten aus anderen Körnerleguminosenproben lieferte Hinweise für das mögliche Vorkommen von zwei weiteren, noch unbekannten Luteoviren. Die CP-Sequenz eines Luteovirusisolat von Kichererbse aus Sudan zeigte, dass es sich dabei um die Sequenz eines entfernten Verwandten des SbDV handelt, mit dem es eine CP-Aminosäuresequenzidentität von nur 66% hat. Da dies seine Klassifizierung als eigenständiges Luteovirus rechtfertigt, wird für dieses Virus der Name Chickpea yellows virus (CpYV) vorgeschlagen. Ein Luteovirusisolat aus Linse in Äthiopien wies eine CP-Aminosäuresequenzidentität von 86% mit Viren aus der Beet western yellows virus (BWYV)-Untergruppe auf. Weil dieser Wert (86%) unter dem Wert von 90% für Aminosäuresequenzidentitäten liegt, der als derzeitiger Schwellenwert für die Unterscheidung von Isolaten einer Virusart bzw. Luteovirusarten gilt, scheint dieses Linsenisolat eine weitere neue Virusart darzustellen, für die der Name Lentil stunt virus (LStV) vorgeschlagen wird. Neben den CP-Sequenzen dieser zwei neuen Luteoviren, wurden aus Fababohnenproben von Ägypten und Marokko auch CP-Sequenzen erhalten, die fast identisch mit denen von Viren aus der BWYV-Untergruppe waren. Ein RT-PCR-Nachweis von CP-Sequenzen mit auffälliger Ähnlichkeit zum SbDV gelang aus Fababohnenproben aus Äthiopien, Deutschland, Syrien und China. Dies ist ein deutlicher Hinweis dafür, dass SbDV eine viel weitere geographische Verbreitung hat als bisher angenommen (Japan, USA). SbDV wurde damit erstmals für Deutschland und auch Europa nachgewiesen.

Die Verwendung von Breitspektrum-Mabs, die einstmals gegen das Nanovirus *Faba bean necrotic yellows viru*s (FBNYV) hergestellt worden waren, hat Hinweise für das Vorkommen von FBNYV und/oder nahe verwandten Nanoviren in einigen Körnerleguminosenkulturen vieler WANA-Länder geliefert. Darüber hinaus hat der Einsatz von anderen, mehr spezifischen Mabs eine beträchtliche serologische Variabilität zwischen FBNYV- oder nahe verwandten Nanoviren aufgezeigt. Um ein erweitertes Verständnis für die genetische Basis dieser Nanovirusvariabilität innerhalb eines begrenzten geographischen Gebietes zu erlangen, wurden die acht verschiedenen ssDNA-Komponenten, die als integrale Bestandteile des Nanovirusgenoms angesehen werden, von zwei marokkanischen Isolaten (Mor5 and Mor23) bestimmt, die sich auffällig in ihren Epitopprofilen unterschieden. Die Sequenzen des Mor23 waren denen von typischen Isolaten des FBNYV aus Ägypten und Syrien sehr ähnlich. Die Analyse der Mor5-Sequenzen zeigte jedoch, dass sie denen eines früher aus Äthiopien beschriebenen Nanovirusisolates sehr ähneln, das sich von typischen FBNYV-Isolaten so auffällig unterschied, dass der Name Faba bean necrotic stunt virus (FBNSV) für dieses andersartige Nanovirus inzwischen vorgeschlagen worden ist. Diese Resultate liefern die ersten molekularen Hinweise für das Vorkommen von zwei verschiedenen Nanovirusarten an Fababohnen innerhalb eines Landes und sogar innerhalb eines begrenzten Gebietes.

Die serologische Analyse von 73 nanovirusinfizierten Fababohnenproben aus Äthiopien unter Verwendung von sieben spezifischen Mabs zeigte, dass die Isolate in wenigstens drei verschiedene Kategorien mit den Bezeichnungen Serogruppe A, B und C eingeteilt werden konnten. Die kodierenden Sequenzbereiche von zwei DNA-Komponenten (DNA-S und -U1) einer jeden Serogruppe wurden zum Zwecke eines genetischen Vergleichs bestimmt. Diese Studien zeigten auffällige Unterschiede zwischen den abgeleiteten Aminosäuresequenzen der Nanovirusproteine auf. Isolate der Serogruppe A waren am weitesten in Äthiopien verbreitet und am ähnlichsten dem früher beschriebenen äthiopischen Isolat des FBNSV. Isolate der Serogruppe B waren zwar selten, aber ähnlich den typischen FBNYV-Isolaten aus Ägypten und Syrien, die man in Äthiopien bisher nicht nachgewiesen hatte. Die Isolate der Serogruppe C stellen ein neuartiges Nanovirus dar, das bisher unbekannte serologische und molekulare Eigenschaften aufweist. Zum besseren Verständnis dieses Virus wurden die acht DNA-Komponenten von Eth231, einem typischen Vertreter der Serogruppe C, vollständig sequenziert. Sequenzanalyse des Eth231-Genoms zeigte, dass seine Gesamtnukleotidsequenz nur eine Identität von 70% mit der von anderen Nanoviren aufweist. Da dies weniger als der derzeitige Schwellenwert von 75% für unterschiedliche Nanovirusarten ist, sollten Isolate der Serogruppe C als neues Nanovirus mit dem Namen Faba bean yellow

leaf virus (FBYLV) betrachtet werden. Außerdem ist Eth231 auch dahingehend ungewöhnlich, weil in der Aminosäuresequenz seines Clink-Proteins das LxCxE-Motiv fehlt, das in allen anderen Clink-Proteinen der Nano- und Babuviren vorkommt. Die serologischen und genetischen Daten über äthiopische Nanoviren liefern den ersten Hinweis für das Vorkommen von drei verschiedenen Nanovirusarten an Fababohnen in Äthiopien; FBNSV (Serogruppe A), FBNYV (Serogruppe B) and FBYLV (Serogruppe C).

Zusammenfassend haben unsere Untersuchungen eine beträchtliche Menge an Informationen über eine mannigfaltige Reihe von Luteo- und Nanoviren geliefert, die mit Vergilbungs- und Stauchekrankheiten an Körnerleguminosen im Zusammenhang stehen. Insbesondere wurden die Nukleotidsequenzen für drei neue Luteoviren und ein neues Nanovirus neben den zahlreichen Teilsequenzen für schon bekannte oder hier erstmals beschriebene Viren erhalten. Die vorliegenden Sequenzinformationen sind einerseits von fundamentaler Bedeutung für zukünftige molekulare Studien an diesen beiden Gruppen von Pflanzenviren. Andererseits sind sie wegen der Daten zur Diversität und Variabilität der hier untersuchten Viren von großem Wert für die Virusresistenzzüchtung in bestimmten Gebieten und WANA-Ländern. Schließlich werden die bereitgestellten Antikörper und hier beschriebenen gruppen-, virus- oder isolatspezifischen Primer nicht nur für die Resistenzzüchtung, sondern auch für Untersuchungen zur Epidemiologie der Viren nützlich sein. Somit können die nun vorhandenen diagnostischen Möglichkeiten zur Vermeidung und Bekämpfung der durch Luteo- und Nanoviren verursachten Vergilbungs- und Stauchekrankheiten an Körnerleguminosen sinnvoll eingesetzt werden.

APPENDIX I

The complete sequence of the RNA genome of Chickpea chlorotic stunt virus (CpCSV), a new luteovirus and its encoded proteins (isolate FBV from Ambo, Ethiopia)

```
1       ACAAAAGAAAGCAAGAGGGAAAACTTCACGCTGAGTATCTCGCGAAGTTTATGAACGTGTTGATTAACAAATTCACTTCAAATTTTGAGA
                                                 ORF0   M  N  V  L  I  N  K  F  T  S  N  F  E

91      TCAATTTCTCACCCTCACTTTCTCTCGCAACCAGAAGATCGTTAATTTGCTTGTTCCTTGCAAACTCAAGCGAATTTATTAAAATATCAA
        I  N  F  S  P  S  L  S  L  A  T  R  R  S  L  I  C  L  F  L  A  N  S  S  E  F  I  K  I  S  N

181     ACGAGCGAGATGGCACTGACAAAAGTACTTGCTATCGCTCTCTTCTGCTTTTGCTTCCATTTCTTTTTGGGAGCTTCGAGTTCTTTGGCG
             ORF1 M  A  L  T  K  V  L  A  I  A  L  F  C  F  C  F  H  F  F  L  G  A  S  S  S  L  A
        ORF0 E  R  D  G  T  D  K  S  T  C  Y  R  S  L  L  L  L  L  P  F  L  F  G  S  F  E  F  F  G  D

271     ACCAACTGGTCGTCCCCTGGAATGGCGGGAATAACCGCCATCACGGGCTTTTCCAACGATTACTTACAAACACCAGGTTTTGTACCTTAC
          T  N  W  S  S  P  G  M  A  G  I  T  A  I  T  G  F  S  N  D  Y  L  Q  T  P  G  F  V  P  Y
            Q  L  V  V  P  W  N  G  G  N  N  R  H  H  G  L  F  Q  R  L  L  T  N  T  R  F  C  T  L  R

361     GTTTACAACCTGGTAGAGAAGCCCGTTACAATCCCTCAATCCAAATTACCCCTACCAGAGTTGAACTATACAGATATATTCAAAGTCCTC
          V  Y  N  L  V  E  K  P  V  T  I  P  Q  S  K  L  P  L  P  E  L  N  Y  T  D  I  F  K  V  L
            L  Q  P  G  R  E  A  R  Y  N  P  S  I  Q  I  T  P  T  R  V  E  L  Y  R  Y  I  Q  S  P  L

451     TGGTTGAAGGGTTATCAGGACACCAGGACCTGCTTAGTCTTGGCCTTCACCACTTCACAAAGCTCGTTGGCGCATATGTACGACAACTTG
          W  L  K  G  Y  Q  D  T  R  T  C  L  V  L  A  F  T  T  S  Q  S  S  L  A  H  M  Y  D  N  L
            V  E  G  L  S  G  H  Q  D  L  L  S  L  G  L  H  H  F  T  K  L  V  G  A  Y  V  R  Q  L  E

541     AGCGAAATAACTTCTGCCTGCGCTCTGAGATTATCTTGGGCGATAGTGTCGATCTGGACGTTAGTCATCTGGGCATTTTGCTCATGGATG
          S  E  I  T  S  A  C  A  L  R  L  S  W  A  I  V  S  I  W  T  L  V  I  W  A  F  C  S  W  M
            R  N  N  F  C  L  R  S  E  I  I  L  G  D  S  V  D  L  D  V  S  H  L  G  I  L  L  M  D  G

631     GTTCGGATCATAACAACACACACCATGCTTATAGTGGCAGTAGGGTTATTAATTGCTTGCACCGTTGCTACGGCCAAGCTCTTACATCTG
          V  R  I  I  T  T  H  T  M  L  I  V  A  V  G  L  L  I  A  C  T  V  A  T  A  K  L  L  H  L
            S  D  H  N  N  T  H  H  A  Y  S  G  S  R  V  I  N  C  L  H  R  C  Y  G  Q  A  L  T  S  D

721     ATATTTGGGAGCTTTTCGGTGTGGATTATTGTCCCTGTTTACAGGTCCCTAGCTTTCCTTTGGAAGCTGAGATCCCCGAAGACAGTTTCC
          I  F  G  S  F  S  V  W  I  I  V  P  V  Y  R  S  L  A  F  L  W  K  L  R  S  P  K  T  V  S
            I  W  E  L  F  G  V  D  Y  C  P  C  L  Q  V  P  S  F  P  L  E  A  E  I  P  E  D  S  F  Q

811     AATTCGATGAAGATAGTGAAGGAGAAGATGACGAAGGGTTTTGGGAGCTATGATATGATTATGTCCCCTCCAAAAAGTTGCGTGCTCGAA
          N  S  M  K  I  V  K  E  K  M  T  K  G  F  G  S  Y  D  M  I  M  S  P  P  K  S  C  V  L  E
            F  D  E  D  S  E  G  E  D  D  E  G  F  W  E  L  *

901     ATGCTTCACGATGATGAACAACATTGTGGTTATGCGAGCTGCATATTATTAGCTGATGGAACAGTGGGGTTGTTGACTTCATATCACGTC
          M  L  H  D  D  E  Q  H  C  G  Y  A  S  C  I  L  L  A  D  G  T  V  G  L  L  T  S  Y  H  V

991     ATGGAGGAAGCTTACTGGGTTAAGTCTAACAAGACTGGAAATAAAATTAAGACCAGTGACTTTCGACCACTTACTCAATCCCAAAACGCT
          M  E  E  A  Y  W  V  K  S  N  K  T  G  N  K  I  K  T  S  D  F  R  P  L  T  Q  S  Q  N  A

1081    GATTTGTCTATTTTGGTAGGTCCACCCAACTGGCAAGGGCTTTTGGGCTGTTCAGCAGCCCAATATGTTACAGTAAAACATCTAGCCGCC
          D  L  S  I  L  V  G  P  P  N  W  Q  G  L  L  G  C  S  A  A  Q  Y  V  T  V  K  H  L  A  A

1171    GGTGACGCTCGTATCTTCTACCGTAAAAACGGTGAATGGTACTCTGGAGTGGCTAAGTTGGTAGGACCTCACAAATTGAACTTCGTCAAT
          G  D  A  R  I  F  Y  R  K  N  G  E  W  Y  S  G  V  A  K  L  V  G  P  H  K  L  N  F  V  N

1261    GTCCTGTCAAATACCGAGCCTGGGTTCAGCGGCACCCCTTATTTTAGTGGAAACAAAATAGTTGGAGTACACACAGGAGGGGATGAAGAA
          V  L  S  N  T  E  P  G  F  S  G  T  P  Y  F  S  G  N  K  I  V  G  V  H  T  G  G  D  E  E

1351    GAGAATAGAAATTATATGGCCGCTATCCCTCATCTTGAAGGTTTGACTGCGAGCAAATATATATATGAAACGACTGCTCCCAAAGGTCGA
          E  N  R  N  Y  M  A  A  I  P  H  L  E  G  L  T  A  S  K  Y  I  Y  E  T  T  A  P  K  G  R

1441    ATCTTTGACGAAGATTTATACCAAGAATTGCTAGAAGAGTTTTCCACGCAAGAAGCACGATCGATAATGAAACATAAAGGCTTCGATATG
          I  F  D  E  D  L  Y  Q  E  L  L  E  E  F  S  T  Q  E  A  R  S  I  M  K  H  K  G  F  D  M

1531    GAATGTTCTGGAAAGTTTAAAGGCAGATGGGTTTGGATAGATTGTAATAATGATCTCACCCCTGCCGAGATAAATAACATTCTCTCTAGC
          E  C  S  G  K  F  K  G  R  W  V  W  I  D  C  N  N  D  L  T  P  A  E  I  N  N  I  L  S  S

1621    AAAGGGAAAACCAGGCTTGAGTCAGAAAGCCCGCGATTCTTCGAAGATGCCGAAGAATTTTATGATGCCGCTGACTCGATGGAACTTGAG
          K  G  K  T  R  L  E  S  E  S  P  R  F  F  E  D  A  E  E  F  Y  D  A  A  D  S  M  E  L  E

1711    ACTTCAAAGAAGGCTAATTTAAACGGGCTTCGAGGCGCCGACCTCGTAAAAACCGGAAAAGAAGGCTCCACCCAAAAGACCAAGAGCGAC
          T  S  K  K  A  N  L  N  G  L  R  G  A  D  L  V  K  T  G  K  E  G  S  T  Q  K  T  K  S  D
ORF2 (fusion prot) *  F  K  R  A  S  R  R  R  P  R  K  N  R  K  R  R  L  H  P  K  D  Q  E  R  R
```

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1801  GATGGAGACACTGTCCAGAAAGTGATAGAGGCTCTCGTCGCGAAAATGAATGTTCAAGAGTTGGAGAAACAAGTGATCTCCAAGGTGGCT
       D  G  D  T  V  Q  K  V  I  E  A  L  V  A  K  M  N  V  Q  E  L  E  K  Q  V  I  S  K  V  A
         W  R  H  C  P  E  S  D  R  G  S  R  R  E  N  E  C  S  R  V  G  E  T  S  D  L  Q  G  G  S

1891  CAGAAGGTGCAGAAGAATACAAGCACCCCACCAAAGAAGAACAGAAGGCGCAGAGGAAAGCGTGGAGGAGAGAGCAAGCAAATAGCTTTA
       Q  K  V  Q  K  N  T  S  T  P  P  K  K  N  R  R  R  R  G  K  R  G  G  E  S  K  Q  I  A  L
         E  G  A  E  E  Y  K  H  P  T  K  E  E  Q  K  A  Q  R  K  A  W  R  R  E  Q  A  N  S  F  N

1981  ACTACTTCTTCGCCTCCCAGTACAAGTGGGAAGTACCTGCCGCCTCAGAAGAGGCCCCAGGGTTTGAAGCCTGTGGAAAAATCCCCCAAT
       T  T  S  S  P  P  S  T  S  G  K  Y  L  P  P  Q  K  R  P  Q  G  L  K  P  V  E  K  S  P  N
         Y  F  F  A  S  Q  Y  K  W  E  V  P  A  A  S  E  E  A  P  G  F  E  A  C  G  K  I  P  Q  F

2071  TCTACCATCCAAAGCAAAAACAACAAGGTAGATGGGGAGAGAAAGTCGTCGCCCAACACCCAGAGATGGGTGAAAAAGTCAGAGGCTTCG
       S  T  I  Q  S  K  N  N  K  V  D  G  E  R  K  S  S  P  N  T  Q  R  W  V  K  K  S  E  A  S
         Y  H  P  K  Q  K  Q  Q  G  R  W  G  E  K  V  V  A  Q  H  P  E  M  G  E  K  V  R  G  F  G

2161  GCTGGCCAGAGTTCGGCAAAGAAGCTGAACTGAAATCCCTGCGGTTGCAAACCGCGAGGTGGCTCAAACGCGCTGAGTCAGCTAAAATCC
       A  G  Q  S  S  A  K  K  L  N  *
         W  P  E  F  G  K  E  A  E  L  K  S  L  R  L  Q  T  A  R  W  L  K  R  A  E  S  A  K  I  P

2251  CGTCGTCTGAGGAACGGGAGCGCGTTATCGGTAAAACAGTAGAGGCATATCAAAATGTGAAATCAAATTGCCCTGCTGCAACAAGGCTGA
         S  S  E  E  R  E  R  V  I  G  K  T  V  E  A  Y  Q  N  V  K  S  N  C  P  A  A  T  R  L  N

2341  ACGAGCTGAGTTGGGATCAATTTCAAAAATCCTTTCAACCAGCCGTTCACTCCCTCGAACTTGATGCTGGAATTGGCGTTCCTTATATAG
         E  L  S  W  D  Q  F  Q  K  S  F  Q  P  A  V  H  S  L  E  L  D  A  G  I  G  V  P  Y  I  A

2431  CATATGGTCTCCCCACCCATCGAGGATGGGTTGAGAACCACGAACGCCAGCTTCTCCCTATTTTGGCTCAGTTGACCTACGACCGACTAA
         Y  G  L  P  T  H  R  G  W  V  E  N  H  E  R  Q  L  L  P  I  L  A  Q  L  T  Y  D  R  L  K

2521  AGAAGATGTCGCAGGTTAACTTTGAAGACATGAGTGCTGAGGAGTTAGTACAGAAGGGTCTATGTGACCCCATCAGAGTATTCGTCAAGG
         K  M  S  Q  V  N  F  E  D  M  S  A  E  E  L  V  Q  K  G  L  C  D  P  I  R  V  F  V  K  G

2611  GAGAACCGCATAAACAGGCAAAACTTGATGAGGGTCGTTACCGCCTCATCATGTCGGTTAGCCTTGTAGACCAATTGGTAGCCCGGGTAT
         E  P  H  K  Q  A  K  L  D  E  G  R  Y  R  L  I  M  S  V  S  L  V  D  Q  L  V  A  R  V  L

2701  TGTTTCAAGAACAAAATAAGCTAGAGATAACTCTCTGGCGATCAATACCAAGCAAACCCGGAATGGGGCTTTCTACGGATGCCCAAGTGA
         F  Q  E  Q  N  K  L  E  I  T  L  W  R  S  I  P  S  K  P  G  M  G  L  S  T  D  A  Q  V  T

2791  CGGAGTTCATGAGTTCTCTGTCCCAACATGTGCAAGTACCAGTCGAAGACTTGGTATATGACTGGGAAAAGCATGTGTTGCCCACAGATT
         E  F  M  S  S  L  S  Q  H  V  Q  V  P  V  E  D  L  V  Y  D  W  E  K  H  V  L  P  T  D  C

2881  GCTCCGGATTTGACTGGAGCGTCTCCGACTGGCTCCTCCAAGATGAAATGGAGGTTAGAAATCGCCTCACTGAGAATAACAACGACCTTA
         S  G  F  D  W  S  V  S  D  W  L  L  Q  D  E  M  E  V  R  N  R  L  T  E  N  N  N  D  L  T

2971  CCAAGCGCCTTCGTGGCTGCTGGTTGAAGTGTCTATCTAACAGTGTCTTGGCGTTATCCGATGGCAGCCTCTTTGCCCAACGCGTACCAG
         K  R  L  R  G  C  W  L  K  C  L  S  N  S  V  L  A  L  S  D  G  S  L  F  A  Q  R  V  P  G

3061  GAGTTCAGAAGTCTGGAAGCTACAACACTAGCTCGACCAACTCTAGAATCCGCGTAATGTGTGCTTTTCACGCTGGCGCCTCGTGGTGTA
         V  Q  K  S  G  S  Y  N  T  S  S  T  N  S  R  I  R  V  M  C  A  F  H  A  G  A  S  W  C  I

3151  TAGCCATGGGAGATGATGCTTTGGAGTCTGTGGACACTGACCTATCAGTGTATAAAGATATAGGTTTAAAAGTCGAGGTTTCAGGACAAC
         A  M  G  D  D  A  L  E  S  V  D  T  D  L  S  V  Y  K  D  I  G  L  K  V  E  V  S  G  Q  L

3241  TGGAATTTTGCTCTCACATTTTTGAGAAGCCTGACCTCGCCATTCCGGTAAACGTAGGAAAAATGTTGTACAAACTCATCTACGGGTACA
         E  F  C  S  H  I  F  E  K  P  D  L  A  I  P  V  N  V  G  K  M  L  Y  K  L  I  Y  G  Y  N

3331  ATCCGGAATGTGGATCCATCCAGGTCCTCAGGAATTATATTGACGCTTGCACCTCAGTGCTAAATGAATTGCGTCACGACCCTGAGATGG
         P  E  C  G  S  I  Q  V  L  R  N  Y  I  D  A  C  T  S  V  L  N  E  L  R  H  D  P  E  M  V

3421  TCCAACTCCTCTACTCGTGGCTCCTTGATCCAGTGTTGCCACAAAATTAATCAAGAGAGAGGAGTACAGAGATAGCCGACTAACTAGTTG
         Q  L  L  Y  S  W  L  L  D  P  V  L  P  Q  N  *

3511  CGAGATCGGAGTCTCAGTCTAGTTCATTGCAAAGATAGATTACAAATTTTTAGCTGGTTTTGCCGCTGGCTTTGTGACATCAATCCCAAT

3601  ATCTGTCTGTGCAATATATATCATCTACCTTAAAATTTCAACCCACGTGAGATCAATAGTCAATGAATACGGTCGTGGTTAGAAATAATG
                                                                    ORF3  M  N  T  V  V  V  R  N  N  G
                                                                    ORF4  M

3691  GCAGAAGGAGGAGAAATAGGCGTACTGTTCAGCGGGCTAGGCGCCGCAACCCAGTGGTTGTGGTCGAAGCCCCTAGGCAGCCACAGCGCG
         R  R  R  R  N  R  R  T  V  Q  R  A  R  R  R  N  P  V  V  V  V  E  A  P  R  Q  P  Q  R  G
       A  E  G  G  E  I  G  V  L  F  S  G  L  G  A  A  T  Q  W  L  W  S  K  P  L  G  S  H  S  A

3781  GAAGACGACGAAGAAGAAACCGTCGACGCGCTTCAGGAAGAAGCACAGCTGGAAGACGAGGGTCTAGCGAGACATTTGTGTTTTCGAAAG
         R  R  R  R  R  N  R  R  R  A  S  G  R  S  T  A  G  R  R  G  S  S  E  T  F  V  F  S  K  D
       E  D  D  E  E  E  T  V  D  A  L  Q  E  E  A  Q  L  E  D  E  G  L  A  R  H  L  C  F  R  K

3871  ACAACCTCGCGGGTAGTTCCTCAGGAAGTATCACGTTCGGGCCGAGTCTATCAGACTGCCCAGCTTTCAGCTCTGGAATACTCAGGGCCT
         N  L  A  G  S  S  S  G  S  I  T  F  G  P  S  L  S  D  C  P  A  F  S  S  G  I  L  R  A  Y
       T  T  S  R  V  V  P  Q  E  V  S  R  S  G  R  V  Y  Q  T  A  Q  L  S  A  L  E  Y  S  G  P
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3961  ACCATGAATATAAAATCTCAATGGTCAAGTTGGAGTTCATCTCCGAGGCCGCTTCCACCTCCTCAGGGTCGATCGCTTACGAGCTTGATC
        H  E  Y  K  I  S  M  V  K  L  E  F  I  S  E  A  A  S  T  S  S  G  S  I  A  Y  E  L  D  P
       T  M  N  I  K  S  Q  W  S  S  W  S  S  S  P  R  P  L  P  P  P  Q  G  R  S  L  T  S  L  I

4051  CCCACTGCAAATCCACTTCGCTTGGGTCCTACATCAATAAATTTGGAATCACCTCGAATGGACAAAGGACTTTTGCAGCGAGGCTTATTA
        H  C  K  S  T  S  L  G  S  Y  I  N  K  F  G  I  T  S  N  G  Q  R  T  F  A  A  R  L  I  N
       P  T  A  N  P  L  R  L  G  P  T  S  I  N  L  E  S  P  R  M  D  K  G  L  L  Q  R  G  L  L

4141  ACGGAATCGAATGGCACTCCTCAGACGAGGACCAGTTCAGGATACTTTATAAAGGAAATGGAGGCTCCGCCATCGCGGGGTCATTCAGGA
        G  I  E  W  H  S  S  D  E  D  Q  F  R  I  L  Y  K  G  N  G  G  S  A  I  A  G  S  F  R  I
       T  E  S  N  G  T  P  Q  T  R  T  S  S  G  Y  F  I  K  E  M  E  A  P  P  S  R  G  H  S  G

4231  TCACCATCAAGTGCCAAACTCAGAATCCGAAATAGGTAGATGACAGTTCCCCCCCCGGACCAAGTCCAACACCACCTCCACCTCCCCCAG
        T  I  K  C  Q  T  Q  N  P  K  *  V  D  D  S  S  P  P  G  P  S  P  T  P  P  P  P  P  P  A
       S  P  S  S  A  K  L  R  I  R  N  R  *     ORF5 readthrough

4321  CACCAGCTCCAGAACCACAACCTTGTAAGAAATATAGGTTTTGGGGTTATGAAGGTGTGCCACAGAATAAGATAGTCACAGCGCAGAACG
        P  A  P  E  P  Q  P  C  K  K  Y  R  F  W  G  Y  E  G  V  P  Q  N  K  I  V  T  A  Q  N  D

4411  ATAGAAATATCGATGTGCGTGGCTTGAATTATGTTAAGTTTTATAAATGGGAGGATGATAATTGGACGGAAGTCAACCTTCAAGCCAATT
        R  N  I  D  V  R  G  L  N  Y  V  K  F  Y  K  W  E  D  D  N  W  T  E  V  N  L  Q  A  N  Y

4501  ATTCAGTGAACAATTCGCAGTATGCAGAACCATACATGATCATCCCCGCATCTAAAGGCAAGTTCCACGTCTACCTTGAATGTGATGGAC
        S  V  N  N  S  Q  Y  A  E  P  Y  M  I  I  P  A  S  K  G  K  F  H  V  Y  L  E  C  D  G  Q

4591  AGATGGCTGTCAAAAGTGTGGGTGGAAAAGCGGATAATTCCTGGAGAGGCTTGATTGCGTATGACACCTCCAGAAGAATGTGGAATGTTG
        M  A  V  K  S  V  G  G  K  A  D  N  S  W  R  G  L  I  A  Y  D  T  S  R  R  M  W  N  V  G

4681  GTAATTACAAAGGCTGTACCATAGAAAATTATAGGAAAACGGACAGCTTTGTGCTTGGTCACCCTGATGTTGAAGTCAATGATTGCAAAT
        N  Y  K  G  C  T  I  E  N  Y  R  K  T  D  S  F  V  L  G  H  P  D  V  E  V  N  D  C  K  F

4771  TTGATAAAGCCAGGGGAGTAGAAGCAGATTGGTACGCGTCGTTCCAGTTAACTTGTGATGACGACGAAGGATCTTGGATCCTGTACGCCC
        D  K  A  R  G  V  E  A  D  W  Y  A  S  F  Q  L  T  C  D  D  D  E  G  S  W  I  L  Y  A  P

4861  CACCTATCCCAAAGGACAGCTTGTATAACTATACTGTCTCTTATGGAGAATATACAGAAAATATGTGTGAATGGGGCGCCGTATCAATAT
        P  I  P  K  D  S  L  Y  N  Y  T  V  S  Y  G  E  Y  T  E  N  M  C  E  W  G  A  V  S  I  S

4951  CAATTGATGAAGACAACAGCTCTACAGGGAACGAAGTCAAAATTAAACCCGGAAGGGGGCATTTAGTGCACCGGGCTCTGCCAGAAGGAA
        I  D  E  D  N  S  S  T  G  N  E  V  K  I  K  P  G  R  G  H  L  V  H  R  A  L  P  E  G  T

5041  CACTGGAACAACAACCCTTGGAGGATGTCCAAGTAAAAGAATACTTCTGGAAGGAAAACCAGTCTGAAACCACAAGCGATTCGGACGGTG
        L  E  Q  Q  P  L  E  D  V  Q  V  K  E  Y  F  W  K  E  N  Q  S  E  T  T  S  D  S  D  G  E

5131  AGTCATTCATTAGCAAAATCAAGGGAAAATTACCTATGACGACTAAACTCCCGCCGAAAGGCTTTCTCTCGCGTCTTAAACCTTCGGAAA
        S  F  I  S  K  I  K  G  K  L  P  M  T  T  K  L  P  P  K  G  F  L  S  R  L  K  P  S  E  K

5221  AAGAAGAAACAGCCAGATCAAAAGAATCTGAGGTCAAGCCGGAGGATGTCGACAACCTGGTTCGAGCCGCAGGCAAAGAATTCCAGTATG
        E  E  T  A  R  S  K  E  S  E  V  K  P  E  D  V  D  N  L  V  R  A  A  G  K  E  F  Q  Y  G

5311  GCATTTATGATGATGCTAGAGAGAGGCTCCATAATAAGGAATTTAACCAGAACATGGAAGAACTGGAGTCAGACCTTGAAGAAATAAATC
        I  Y  D  D  A  R  E  R  L  H  N  K  E  F  N  Q  N  M  E  E  L  E  S  D  L  E  E  I  N  R

5401  GCTTGGAACCTCCTGACATTGATGTTTGGCGAGGCAAGGACACAGCTGAAACTGTAGCTGTGTTCGAAGATCCGTGGGAGTTTTTCCGTA
        L  E  P  P  D  I  D  V  W  R  G  K  D  T  A  E  T  V  A  V  F  E  D  P  W  E  F  F  R  K

5491  AACAGGAAGATCCCAATCCACCTAAATTAAAAGGAACATTATCAAAAATAGGTTCATCCATAGGTGGAGGGTCTCTCTCAGGAGGGAACT
        Q  E  D  P  N  P  P  K  L  K  G  T  L  S  K  I  G  S  S  I  G  G  G  S  L  S  G  G  N  L

5581  TGAGAAGAGCCGCTGAAAGTGTTAACGAAGATTCTATGAAATTTAAACTGTCGACCACTGAGCGCAACCAGTACGAAAGAATTCGGAAAT
        R  R  A  A  E  S  V  N  E  D  S  M  K  F  K  L  S  T  T  E  R  N  Q  Y  E  R  I  R  K  S

5671  CAAAAGGTGAAACAGCAGCTCGTGTGTACTTACGCAGCAGGTTCTCTTCATAGTGGTATATCAACCGCCCACTCTAAAGATACGGTTTTC
        K  G  E  T  A  A  R  V  Y  L  R  S  R  F  S  S  *

5761  ACCAAATATGGGTGAGTTCATATTTCCTAGCTTCTGGATTTGAAGCAAACTCGGGCCGCAGAGGATAAATGTCGGAACGAAAGCGAGGAG

5851  CGAGTAGGCCCCAGTGTTATACGTGGGTATTTCCTACGGCACTGCGGTGT
```

APPENDIX II

The complete sequence of the eight ssDNAs making up the genome of Faba bean yellow leaf virus (FBYLV), a new nanovirus, and their encoded protein (Isolate Eth-231, collected from Gedeo, Ethiopia)

DNA-R, encoding the master replication associated (M-rep) protein

```
 1        CTGGGGCGGGGCTTAGTATTACCCCCGCCCCAGGTTCACGGGATCATCACGTGAATTGCACATGTACTATGGCTA
76        TAAATAGCTTATGTTGTAATATTTTATCATTCATTCAATAAAATATGGCTCGACAAGTTATATGTTGGTGCTTTA
                                                  M  A  R  Q  V  I  C  W  C  F  T
151       CATTAAATAATCCTCTCTCTCCTCTCTCTCTTCATGAATCAATGAAGTACCTTGTTTATCAAACTGAACAAGGTG
             L  N  N  P  L  S  P  L  S  L  H  E  S  M  K  Y  L  V  Y  Q  T  E  Q  G  D
226       ATTCTGGAAATATTCATTTCCAGGGTTATATTGAAATGAAGAAACGTACGTCTCTTGCTGGTATGAAGAAACTGA
             S  G  N  I  H  F  Q  G  Y  I  E  M  K  K  R  T  S  L  A  G  M  K  K  L  I
301       TACCAGGTGCTCACTTTGAGAAGAAGAAAGGCACACAAGGACAGGCCAGAGCGTATGCAATGAAAGAAGATACAA
             P  G  A  H  F  E  K  K  K  G  T  Q  G  Q  A  R  A  Y  A  M  K  E  D  T  R
376       GAGTTGAAGGTCCATGGGAGTATGGTGAGTTCATTCCTACCATTGAAGATAAGCTCAGAGATGTTATGCAGGACA
             V  E  G  P  W  E  Y  G  E  F  I  P  T  I  E  D  K  L  R  D  V  M  Q  D  M
451       TGAAGAACACAGGGAAGAGACCCATAGAGTATATTGAAGAGTGTTGTGATACGTATGACAAATCTGCAAGTACTC
             K  N  T  G  K  R  P  I  E  Y  I  E  E  C  C  D  T  Y  D  K  S  A  S  T  L
526       TTAGGGAATATCGAGGAGAGTTAAAGAAAAAACAAGCTATTGCAAGTTGGGAGTTGCAGAGGAAGCCATGGATGG
             R  E  Y  R  G  E  L  K  K  K  Q  A  I  A  S  W  E  L  Q  R  K  P  W  M  D
601       ATGAGGTAGATGCTATGCTTGAGGAGAGAGATGGAAGAAGAATCATTTGGGTATATGGCCCACTTGGTGGAGAAG
             E  V  D  A  M  L  E  E  R  D  G  R  R  I  I  W  V  Y  G  P  L  G  G  E  G
676       GGAAAACCTCTTACGCTAAGCATCTCGTAAAGACGCGTGATGCTTTTTATTCGACAGGTGGAAATACAGCCGACA
             K  T  S  Y  A  K  H  L  V  K  T  R  D  A  F  Y  S  T  G  G  N  T  A  D  I
751       TAGCTTTTGCATGGGACCACCAAGAGTTAGTGCTCTTCGACTTTCCACGTAGCTTCGAGGAGTACGTTAACTATG
             A  F  A  W  D  H  Q  E  L  V  L  F  D  F  P  R  S  F  E  E  Y  V  N  Y  G
826       GAGCCATTGAACAATTAAAGAATGGTATAATCCAATCAGGAAAATACCAAAGTGTAATTAAGTATACAGATTATG
             A  I  E  Q  L  K  N  G  I  I  Q  S  G  K  Y  Q  S  V  I  K  Y  T  D  Y  V
901       TAGAGGTTATTGTATTTGCTAATTTTACTCCGCGAAGCGGCATGTTTAGTGATGATAGGATTGTCTTTGTATACG
             E  V  I  V  F  A  N  F  T  P  R  S  G  M  F  S  D  D  R  I  V  F  V  Y  A
976       CATGACGTCACTTGATCCTATGATGAG
```

DNA-S, encoding the structural (coat) protein

```
1         CTGGCACGGGGCTTAGTATTACCCCCGTGCCAGGTTCACGGGATCACTCTTGTGGCCGTTGGATTATAGTGACAC
76        GTGGACGATCAGGATCCGTGATTACATCTGACGGAAGATCGTTCACTCCTTCCACGAAGCTTCGTGGTAGGGCCC
151       TATAAGCGCGTATACTTTAACTTTACTTTAGTAAAGTAAAGATTGATGTGACGTTAGTAATATTCGTTGCTTAGT
226       GGATTACAGCTGTCTTTGCTTCGTCTCGAAGCAAAGGTGTTTTTTTTTTGTCTATAAATACTCTTCCTTTTCGTTG
301       TTTCACACAACGAAAATGGTTAGCAATTGGAATTGGTCTGGTAAGAAAGGGAGAAGAACTCCACGTCGTGGTTAT
                        M  V  S  N  W  N  W  S  G  K  K  G  R  R  T  P  R  R  G  Y
376       TCCAGGCCATATAAATCTGCTGTTCCTACGACGAGGGTTGTTGTTCATCAATCCGCAGTGCTGAAGAAAGATGAA
           S  R  P  Y  K  S  A  V  P  T  T  R  V  V  V  H  Q  S  A  V  L  K  K  D  E
451       GTTTCTGGTAGTGAAATCAAACCGGAAGGTGATGTTGCTCGTTATAAGATGAAGAAGGTGATGCTAACATGTACG
           V  S  G  S  E  I  K  P  E  G  D  V  A  R  Y  K  M  K  K  V  M  L  T  C  T
526       TTGAGGATGCCTCCAGGAGAGTTAGTGAACTACCTTATTGTTAAGTGTAATTCACCAATTGTTAATTGGTCTGCT
           L  R  M  P  P  G  E  L  V  N  Y  L  I  V  K  C  N  S  P  I  V  N  W  S  A
601       GCTTTTACCTCTCCTGCGTTGTTAGTTAAGGAGAGTTGTCAAGACATGATTACAATTATTGGTAAAGGAAAGGTT
           A  F  T  S  P  A  L  L  V  K  E  S  C  Q  D  M  I  T  I  I  G  K  G  K  V
676       GAGTCCAATGGAGTTGCTGGGACAGATTGTACTAAGTCGTTTAATAGATTTATTAAATTAGGTGTTGGTGTTAGT
           E  S  N  G  V  A  G  T  D  C  T  K  S  F  N  R  F  I  K  L  G  V  G  V  S
751       CAAACACGACATGTATATGTTGTATTGTATACTAGTGTAGCTTGTAAGGCTGTATTAGAACATAGAATGTATATT
           Q  T  R  H  V  Y  V  V  L  Y  T  S  V  A  C  K  A  V  L  E  H  R  M  Y  I
826       GAAGTGTAAATCATTTGTAATGAAGAACACATGAATAATAAATAAATGAATTCCATTTGATCTTGGTACTCTGCG
           E  V  *
901       AAGCAGTGTTGTTTGTTGTTTTTATTTCCTTATTTGCCCTTCATTAATGAAGGGGATTTGTCTAAAATACCCCTG
976       GGAATACTGTTTGATCCTGGGACAGC
```

DNA-C, encoding the Clink protein

```
1         CTGGGGCGGGGCTTAATATTACCCCCGCCCCAGGTTCACGGGATCACTACGTGGACGACCTATGACCATTGGATG
76        AACATAACACGTGGACGATCAGGGTCTGTGATTACATCTGACGGAAGATCGTTCACACCTTCCACGAAGCTTCGT
151       GGTAGGGCCTATTAGTGCGTATGCTTTAGCTTTACTTTTTTAAAGTAAAGAATTGATGTGACGTTAACTATTGTA
226       TGGTTAATTGAATACAGCTGTCTTTGCTTCGTCTCGAAGCAAAGGATATATGTGCGTCTATAAATAGTTTTCTTT
301       TYCGTTGTGTGAAACAACGAAAATGGATCTGAATTATTTTTCTCGTCTTCCTGTCGAATTGAGAGAGAAGATTGT
                                M  D  L  N  Y  F  S  R  L  P  V  E  L  R  E  K  I  V
376       ACGTGAGCATATGAAAGAGGAAAGGAAGAAGGAATTCCTTGAGAATTCAATTGAAGATAGTTGTAGAAGGTATGA
            R  E  H  M  K  E  E  R  K  K  E  F  L  E  N  S  I  E  D  S  C  R  R  Y  E
451       AGCTCTATTAAATGAAGATCCCTCATCTGTAAATTTGCGTAAGTTAAGTAATTATTTAGATTTATTAGCTGATTA
            A  L  L  N  E  D  P  S  S  V  N  L  R  K  L  S  N  Y  L  D  L  L  A  D  Y
526       CGTTGGTAATCAGTTTAATAGGAGATGTCTAATTAGATGGAAGAAGGATGTACCCTGCAGAGTTAAATATGGAGT
            V  G  N  Q  F  N  R  R  C  L  I  R  W  K  K  D  V  P  C  R  V  K  Y  G  V
601       TATTGAAGAAGAACATATGAAGCTCTCTGCATATCTGCATCTTGTGGATTTTGACTATGGGGAGTTGTTTCCTTC
            I  E  E  E  H  M  K  L  S  A  Y  L  H  L  V  D  F  D  Y  G  E  L  F  P  S
676       ATTACTTCCTTTGGAGGAAGACGATGATGTATCGTATGTAGATGGTACGATTGTTAGATGTAGTCTACTAGATTT
            L  L  P  L  E  E  D  D  D  V  S  Y  V  D  G  T  I  V  R  C  S  L  L  D  F
751       TGTACATAGTCAATTAGATATTAATGTAGTGTATATTACTGTAAGTAAGAATCGTATTTGTACTCCTCTGAGGAA
            V  H  S  Q  L  D  I  N  V  V  Y  I  T  V  S  K  N  R  I  C  T  P  L  R  K
826       GAATTGTAATTTGTATTTATGAATAAATAATACTTGAATAAATACTGATTTTGATCTGTTTACTCTGCGAAGCAG
            N  C  N  L  Y  L  *
901       CGTGTTATGTTGTTTATGTTTCCATCTATGCCCTTCATTAATGAAGGGGAATTGTCTAAAATGACCTTGCTGACG
976       TCATTTGATCCTATGATGAG
```

DNA-M, encoding the movement protein

```
1         CTGGGGCGGGGCTTAGTATTACCCCCGCCCCAGGCTCACGGGATCACTCTTGTGGCCGTTGGATTATAGTGACAC
76        GTGGACGATCAGGATCCGTGATTACATCTGACGGAAGATCGTTCACACTTACCACGAAGCTTCGTGGTAGGGCCC
151       TATTAGCGCGTATGCTTTTGCTTTACTTTACTAAAGTAAAGCAAGATGCTTTGCTTTATACTATTCGCTGTTGTT
226       GAGTACAGCTGTATTTAATTATTTAATAAATAATTAAATGTTGTCTTGCAGAGTAAGCTTGCACTTTCCTCTATA
301       AATACGCAAGCTTCATTTATCATTCGTCTTGTTTTTGTTTCATCGAGAGCGAATGGCTGATGCAGGTTATTATGA
                                                                  M  A  D  A  G  Y  Y  E
376       AGGTTATCAAGACGATGCTGATGTTGCATCAGAGAAGCGCCATCAAGCGTTGTATTTGATAGGTATAATTATTTT
            G  Y  Q  D  D  A  D  V  A  S  E  K  R  H  Q  A  L  Y  L  I  G  I  I  I  L
451       AATTATGGTATGTATTGTTATTCTATGGGTATGTATTATGCTTGCGTGTTATGTTCCTGGATTTGTGAAGAAGAC
            I  M  V  C  I  V  I  L  W  V  C  I  M  L  A  C  Y  V  P  G  F  V  K  K  T
526       GCTTGAAGCTTGGTTGAGTTCTTCACCGTTGATGAAGAGAAGAGTTGCATCAACTCTAACAAGAACACCATTTGA
            L  E  A  W  L  S  S  S  P  L  M  K  R  R  V  A  S  T  L  T  R  T  P  F  E
601       AGCAACTGGACCTGAGAGAGAAAGAAATTGGGATGCAAGAAGACAAACAACACCTGTAGTTAGTCAACCTGTTAA
            A  T  G  P  E  R  E  R  N  W  D  A  R  R  Q  T  T  P  V  V  S  Q  P  V  N
676       TACAAGTGTATTTTAGAATAGGCATAATTAATTGTAATTAACGTTATCGTTAATTGTATATTGTATTTATTTGAT
            T  S  V  F  *
751       ATAAATGAAGATTATATTTACTTTGTGTATTGTGTATTGTTGTTATTTATTTACCATTAGATGAAAATGATAAGA
826       GTAAATAATTGATTAACCATGGTAAAATGTAACTTAGTAATTATATAATTTGTGTTAATTACTCCGCGAAGCGAT
901       ATGGATCGTACTCTTAGGCCCAATAGCACTTAGGCCCAATTGTAATTACATTGAATGACGTCATTTGATCCTTTG
976       CTGAG
```

DNA-N, encoding the nuclear shuttle protein

```
1         CTGGCACGGGGCTTAGTATTACCCCCGTGCCAGGTTCAAGGGATCAAAGGAGTCAAATAATGACCGTTAGATCAA
76        ATAATAAATGAACGATCAGGATCTGTGATTACATCTGACGGAAGATCGTTCATGTATTATATTCTATACACGTGG
151       AGAGTTTTTATTGGTAGTTAGTTAGTGGATGCTTTTTAATACGCGTTATGCTTTTTTAACGCGCTTTGGTGTTCA
226       AGTGGGTCCTATGTTGCTTTTGCTTTTCAAAAGCAGATGCTATCTTCGCCATTAAGCAACGTATGTTTTTCCTAT
301       AAATACGCCTTTCTTCTTCGCATTTCAGCGAAGAAAAAAAATGGCAGATTGGTTTGCGAGTCCTCTGAAGACATG
                                                  M  A  D  W  F  A  S  P  L  K  T  C

376       TACTCATGTCTGTGATTTTGTTTCTCTTGCTGGTAATCCACAGCAAGAGATTATGTGCTGTGATAGTACGAAGGA
            T  H  V  C  D  F  V  S  L  A  G  N  P  Q  Q  E  I  M  C  C  D  S  T  K  D
451       TAAATTAAATGATTCAAGGAAGGTTCTGCTTGTTAGTTGCGGTGTGAGCTTCAATGGAAGCTTCTATGGTGGAAA
            K  L  N  D  S  R  K  V  L  L  V  S  C  G  V  S  F  N  G  S  F  Y  G  G  N
526       TAGAAATGTTCGTGGTCAGCTGCAGATATCGATGGTTGAAGATGATGGTGTCTGTAGACCAATTGGTTATGTTCC
            R  N  V  R  G  Q  L  Q  I  S  M  V  E  D  D  G  V  C  R  P  I  G  Y  V  P
601       AATTGGTGGGTATTTGTACCATAATGATTATGGTTATTATCAAGGTGAGAAGACGTTCAATCTGGATATTGAATC
            I  G  G  Y  L  Y  H  N  D  Y  G  Y  Y  Q  G  E  K  T  F  N  L  D  I  E  S
676       TCAATATCTGAAGAAAGATGAAGATTATAATAGGAAGTTTATTGTAACCATTCTCAATGAGAATGGATTAGATAG
            Q  Y  L  K  K  D  E  D  Y  N  R  K  F  I  V  T  I  L  N  E  N  G  L  D  S

751       TCAATGTGATTTGAAGGTGTTTATTGTACATTCATTAAGGATTAAGGTGTAATTAGTATACATAATGATTTAATT
            Q  C  D  L  K  V  F  I  V  H  S  L  R  I  K  V  *
826       ATTGTATTGTAATTATTGTATTTATTAAATGAATAATACATGATTAATATTGTGTGTCGTATTTATATTTTCTTT
901       TAATTACTCCCGCGAAGCGATATGGATCGGATTGTTAGGCCCAATAACACTTAGGCCCAATAGAAATACACTGAG
976       GGACACTGTTTGATCCTTGGACAGC
```

DNA-U1, encoding a protein of unknown function (U1)

```
1         CTGGGGCGGGGCTTAGTATTACCCCCGCCCCAGGTTCACGGGATCACTACGTGGACGACCTATGACCATTGGATG
76        AACATAACACGTGGACGATCAGGATCCGTGATTACATCTGACGGAAGATCGTTCACACCTTCCACGAAGCTTCGT
151       GGTAGGGCCTATTAGTGCGTATGCTTTAACTTTACTTTAAGTAAAGTAAAGAATTGATGTGACGTTAACTATTAT
226       ATGGTTAATTGAATACAGCTGTCTTTGCTTCGTCGCGAAGCAAAGGATTTATGTTTGTCTATAAAAGCTATGAAT
301       TGTGTAGATTAATTCGTCGTTGTTTAATATCGATTAAACCATATTCTCTCTCTGTTTCATCAACCTAAAGCTTTC
376       ATGGCTATTACGCATGTTAGTGATTCTCTATTGGTTGATGAAGCTTCTGAAGAAATAATAACGATCGAAAGAAAA
            M  A  I  T  H  V  S  D  S  L  L  V  D  E  A  S  E  E  I  I  T  I  E  R  K
451       CTGAAGAGTGTTGAGTGTCACGATGATATTAATCAAGTGATAAACGTCAAAGTTGAAGATGTTGATCTTGATATG
            L  K  S  V  E  C  H  D  D  I  N  Q  V  I  N  V  K  V  E  D  V  D  L  D  M
526       CGAGACAGGGTTGTATTGAAGCTGCAGTTCAGATTATGTTACACATACAAGAAGAAGCTGGACATAACGCTTCTG
            R  D  R  V  V  L  K  L  Q  F  R  L  C  Y  T  Y  K  K  K  L  D  I  T  L  L
601       GGTTGTCGTTTGAAGGTTCATACAGAGTTGAAGAACACAAGTGCTGCTGTAGTGAAGAGTATTTTGCAGAAGAGG
            G  C  R  L  K  V  H  T  E  L  K  N  T  S  A  A  V  V  K  S  I  L  Q  K  R
676       ATGAACATGATATGTAATGGTAATTATGTAATAGGTATTAGATTATTTTTTATTAATATTAATCAGTTGATTAAT
            M  N  M  I  C  N  G  N  Y  V  I  G  I  R  L  F  F  I  N  I  N  Q  L  I  N
751       ACTTGTAAATGGATTATACGTATAGAAGATGTATATCCAATATGTACGCTGTATCATATGAATAATAATACAGAT
            T  C  K  W  I  I  R  I  E  D  V  Y  P  I  C  T  L  Y  H  M  N  N  N  T  D
826       GTAATTTGTATTTAAGAAATAAATAATACTTGAATAAATACTGATTTTGATTTGTTTACTCTGCGAAGCAGCGTG
            V  I  C  I  *  E  I  N  N  T  *
901       TTATGTTGTTTATGTTTCCATTTATGCCCTTCATTAATGAAGGGGAATTGTCTAAAATGACCTTGTGATGTCATT
976       TGATCCTATGATGAG
```

DNA-U2, encoding a protein of unknown function (U2)

```
1         CTGGGGCGGGGCTTAGTATTACCCCCGCCCCAGGCTCACGGGATCACTCTTGTGGCCGTTGGATTATAGTGACAC
76        GTGGACGATCAGGATCCGTGATTACATCTGACGGAAGATCGTTCACTCCTTCCACGAAGCTTCGTGGTAGGGCCC
151       TATTAGCGCGTATGCTTTTGCTTTACTTTACTAAAGTAAAGCAAGATGCTTTGCTTTATACTATTCGCTGTTGTT
226       GAGTACAGCTGTATTTAATTATTTAATTAATAATTAAATGTTGTCTTGCAGAGGAAGCTTGTACTTTCCACTATA
301       AATACGCTGTTGCTGAATGAAATGATTGCTTTTATTTCATTCTTCTCTCTTCTTTTATTGTTCTTATGGTTTCGC
                                                                          M  V  S  H
376       ATAGGCGCTTGAAGCTCTCGTTGAGAGAGATTACTCAGTTGAAAGAAGAACAAGATGAGTTCTGGGTTTCTTATG
             R  R  L  K  L  S  L  R  E  I  T  Q  L  K  E  E  Q  D  E  F  W  V  S  Y  E
451       AAACATATCTTCGTGCTCACGAAGATGTTCTTGGGGAAATATGCAGATATCATGGAAGAAGAGTTAAAGCTTATC
             T  Y  L  R  A  H  E  D  V  L  G  E  I  C  R  Y  H  G  R  R  V  K  A  Y  P
526       CAAAGCTTCCCAGTTATGCTCCAACACGTTGGGTCCTTAGGCTTAGGACTGTATATGATGTTAGAGTAGATGAGT
             K  L  P  S  Y  A  P  T  R  W  V  L  R  L  R  T  V  Y  D  V  R  V  D  E  C
601       GTAAGCGCTGTAAGGAAGAAGAAGTAATTAGGCAATATAGTAACCCAGTTAGAGAAGAAGGGTTAAATGATTTGT
             K  R  C  K  E  E  E  V  I  R  Q  Y  S  N  P  V  R  E  E  G  L  N  D  L  Y
676       ATGATTATGGTAATTATAGATATCAAGTGTATTATACAAATTCTAATTGTAATTAACGTTATCGTTAATTGTATA
             D  Y  G  N  Y  R  Y  Q  V  Y  Y  T  N  S  N  C  N
751       GTGTAATTATTTGATATAAATGAAAATTATATTTATATTGTGTATTGTGTATTGTTGTTATTTATTTACCATTAG
826       ATTAAAATGGTAAGAGTAAATAATTGATTAACCATGGTAAAATGTAACTTAGTAACTATATAATTTGCGTTAATT
901       ACTCCGCGAAGCGATATGGATCGTACTCTTAGGCCCAATAGCACTTAGGCCCAAATGTAATTACATTGAATGACG
976       TCATTTGATCCTTTGCTGAG
```

DNA-U4, encoding a protein of unknown function (U4)

```
1         CTGGGGCGGGGCTTAGTATTACCCCCGCCCCAGGCTCACGGGATCACTCTTGTGGCCGTTGGATTATAGTGACAC
76        GTGGACGATCAGGATCCGTGATTACATCTGACGGAAGATCGTTCACACCTTCCACGAAGCTTCGTGGGAGGGCCC
151       GCTAGTTACTTTAACTTTACTTTAAGTAAAGTAAAGAATTGATGTGACGTTATTGTTTAATTGATTACAGCTGTC
226       TTTGCTTCGTCTCGAAGCAAAGGACTTTTGTACGTCTATAAAAGCTGTGGATTGTGCAGATGAAAAATTCATTTT
301       TTTTATTTCGCTAAATCTCTTGTTGTTGTTGTTTCGTTTAAACGAAAGAGGAATATTTTATGGAACCCAGGGTTC
                                                                M  E  P  R  V  L
376       TATTATCTCTTCTTCTCGTTGTGATTGTATTCAATCCTTCCTTCGTGTTGAATTTGATTATTGGTTATATTATGG
             L  S  L  L  L  V  V  I  V  F  N  P  S  F  V  L  N  L  I  I  G  Y  I  M  G
451       GTATTATGAGTAGGAGTAATATATCGAAGCTGAAGGCGATGTGTTTAGGTAAGAAAGAAGAACACAACGAAGAAG
             I  M  S  R  S  N  I  S  K  L  K  A  M  C  L  G  K  K  E  E  H  N  E  E  E
          AAGAATCTCTTATAACCCAGAAGAAGAATCCCTTTGAAGATGCAGATCCAGATGTATTGCAACACTTGAAGACGT
             E  S  L  I  T  Q  K  K  N  P  F  E  D  A  D  P  D  V  L  Q  H  L  K  T  L
601       TGGGTTTGGATACGAAGGTGGACGATGAAGATTTGGAATATTTGCAACGTGTCTGGAAATCAATTAGATGTAATA
             G  L  D  T  K  V  D  D  E  D  L  E  Y  L  Q  R  V  W  K  S  I  R  C  N  K
676       AGTAAATGTAAATTTACGGATTTTATGTAAATAGATGTTGATATGTATTTAAATGTACAAATGTATATATATAGT
             *
751       ATTATTATTGTATTAGTTCGTAGATTGTACTCGTATGTCGTATTAGAACATAGATGTATTGTATGTAACCATATG
826       CATGAAGAACATATGAACAATAAATAAATATCATTTGATCTACTTACTCCGCGAAGCGGTGTGGTTTCGTGTTTT
901       GTTCCTTATATGCCCTCATTATGAAGGGGAATGTCTAAATGACCTTGTGACGTCATTTGATCCTTTGCTGAG
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