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Full Length Research Paper

Molecular evidence for the occurrence of two new luteoviruses in cool season food legumes in Northeast Africa

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Some legume samples with yellowing and stunting symptoms from Ethiopia and Sudan that serologically reacted with a broad-spectrum luteovirus monoclonal antibody did not react or very weakly reacted with virus-specific antibodies suggesting the occurrence of new luteovirus variants. Reverse transcriptase (RT)-PCR amplification, cloning, nucleotide sequencing and analysis of coat protein (CP) gene of a luteovirus isolate from chickpea in Sudan indicated that it shares a closest predicted amino acid sequence identity of only 66% with *Soybean dwarf virus* (SbDV). Since this is less than the accepted threshold value of 90% recommended for discriminating luteovirus species, the isolate is suggested to represent a distinct luteovirus for which the name Chickpea yellows virus (CpYV) is proposed. Similarly, a lentil isolate from Ethiopia shared a closest CP amino acid sequence identity of 86% with viruses of the Beet western yellows virus subgroup. Following the same criteria, this isolate represents another distinct luteovirus species for which the name Lentil stunt virus (LStV) is suggested. From faba bean, CP sequences of *Turnip yellows virus* were amplified from Egyptian and Moroccan samples whereas partial CP sequences of SbDV were amplified from Ethiopian, Syrian and Chinese samples. The study indicated that legume luteoviruses in northeast Africa are highly diverse.

Key words: Phylogenetic relationship, sequence alignment, *Polerovirus*, *Luteoviridae.*

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. Yellowing and stunting diseases primarily caused by luteoviruses are considered to be the most destructive viral diseases of these crops worldwide including northeast Africa (Bos et al., 1988). Luteoviruses belong to family *Luteoviridae* that has three genera namely *Luteovirus*, *Polerovirus* and *Enamovirus* (D'Arcy and Domier, 2004). Their genome is a linear ssRNA of ca. 6 kb that has five to six major open reading frames (ORFs). Like for most plant viruses, serological tests using virus-specific antibodies and sequence information of ORF3 encoding the coat protein (CP) gene are the two most important criteria in discriminating among species of family *Luteoviridae* while ORF1/2 fusion protein including polymerase gene are also used particularly for distinguishing the genera (D'Arcy and Domier, 2004). In some cases as in beet poleroviruses, ORF0 sequence information is also used in species discrimination (Hauser et al., 2000).

Among luteoviruses, *Bean leaf roll virus* (BLRV), *Beet western yellows virus* (BWYV) *Soybean dwarf virus* (SbDV), *Pea enation mosaic virus-1* (PEMV-1) and a recently described Chickpea chlorotic stunt virus (CpCSV) have been reported to commonly cause yellow-

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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
Mo-22	Faba bean	Ouled Ayyad, Morocco	2001	TuYV
EVp7-93	Faba bean	Fayoum, Egypt	1993	TuYV
EV2-94	Faba bean	Fayoum, Egypt	1994	TuYV
EV3-94	Faba bean	Fayoum, Egypt	1994	TuYV
SL1-94	Faba bean	Tel Hadya, Syria	1994	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 in this work); CpYV, Chickpea yellows virus (proposed in this work).
*Virus isolates from Ethiopia were collected by the first author while others were obtained from previous collection maintained at BBA, Braunschweig, Germany as dried leaves.

ing and stunting symptoms of cool season food legume crops in countries of northeast and north Africa and west Asia (Makkouk et al., 1988, 1994; Abraham et al., 2000; Abraham et al., 2006; Tadesse et al. 1999 and Fortass et al., 1997). It should be noted that polerovirus isolates previously considered as BWYV have been recently re-classified as four distinct virus species, namely BWYV, *Beet chlorosis virus* (BChV), *Beet mild yellowing virus* (BMYV) and *Turnip yellows virus* (TuYV) based primarily on 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 important for their classification, the generic term BWYV subgroup will be used in this paper.

Understanding the diversity of viruses existing in legume crops in northeast Africa is considered as a pre-requisite for designing effective management strategies as those based on disease resistance or cultural practices. Previous virus surveys conducted by various authors in the region have established that luteoviruses are the major causes of yellowing and stunting of legume crops in the region (Makkouk et al., 1988, 1994; Abraham et al., 2000; Abraham et al., 2006, Tadesse et al., 1999 and Fortass et al., 1997). However, in most of these works, a significant portion of samples with virus-like symptoms gave no serological reaction with available virus-specific antibodies and hence the causal agents remained unidentified. This was suspected to be due to the fact that some of them belonged to yet unrecognized viruses for which specific antibodies are not yet available. Sequence information which is the most reliable and accurate means for identifying virus isolates, is in most cases lacking for legume luteoviruses in the region.

During a recent study to determine the diversity of legume luteoviruses in northeast Africa and west Asia (Abraham, 2005, Abraham et al., 2006), several samples collected from different countries in Africa and Asia that show yellowing and stunting 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 the viruses associated with various legume samples in the region, attempts were made to serologically test the samples using virus-specific antibodies and also 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 some of the samples.

MATERIALS AND METHODS

Origin of samples and serological tests

The country of origin, the year of collection of the luteovirus-infected samples used are presented in Table 1. The leaf samples were preserved by drying over $CaCl_2$ and maintained at 4°C at BBA, Germany. Serological tests were conducted as TAS- (Franz et al., 1996) and DAS-ELISA (Clark and Adams, 1977) using antibodies of varying specificity including a broad spectrum monoclonal antibody to a luteovirus (2-5G4) (Katul, 1992) and known luteovirus-specific antibodies to BLRV, CpCSV, SbDV and BWYV subgroup. The

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 and to each other.

Virus/Isolate	Database Access. No.	Sequenced isolates		
		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)	AY956384	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	-

source and specificity of the antibodies used has been described by Abraham et al. (2006).

Reverse transcriptase (RT)-PCR, cloning and sequence analysis of coat protein gene

The studies with samples positive with CpCSV antibodies are reported earlier (Abraham et al., 2006). Selected samples that reacted positively only with 2-5G4 but did not or very weakly reacted with antibodies specific to CpCSV were used for this study. Total RNA extraction from these samples, amplification of viral RNA by RT-PCR, cloning and sequencing of the coat protein gene was essentially done as described by Abraham et al. (2006). To be able to amplify a diverse range of luteovirus sequences, two sets of degenerate primers designed from conserved sequences of known luteovirus sequences obtained from the database (Table 2) were used. The first set included a combination of a sense primer S1 (5'GCTCTAGAATTGTTAATGARTACGGTCG3') and antisense primer AS3 (5'CACGCGTCIACCTATTTIGGRTTITG3') (I = inosine) expected to flank the whole coat protein gene of poleroviruses (Abraham et al., 2006). 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, that is 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 of the clones obtained was done by a commercial company (MWG Biotech, Germany) 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), USA (U51448 and L20835) and Germany (EF466131 and EF466132).

RESULTS

Serological tests

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 (Table 2). Those not reacting with CpCSV antibodies included some faba bean samples from Egypt and Morocco that reacted specifically to polyclonal BWYV antibodies and those from Ethiopia, Syria and China that reacted with SbDV polyclonal antibodies. A chickpea sample from Sudan (Su-cp-8) reacted with 2-5G4 but showed a very weak reaction, which was less than the ELISA threshold value with SbDV, and accordingly was inconclusive. Similarly, a lentil sample from Ethiopia (Et-le-3) that reacted with 2-5G4 did also show a very weak and doubtful reaction with antibodies to BWYV group. None of the samples reacted with BLRV-specific monoclonal antibody.

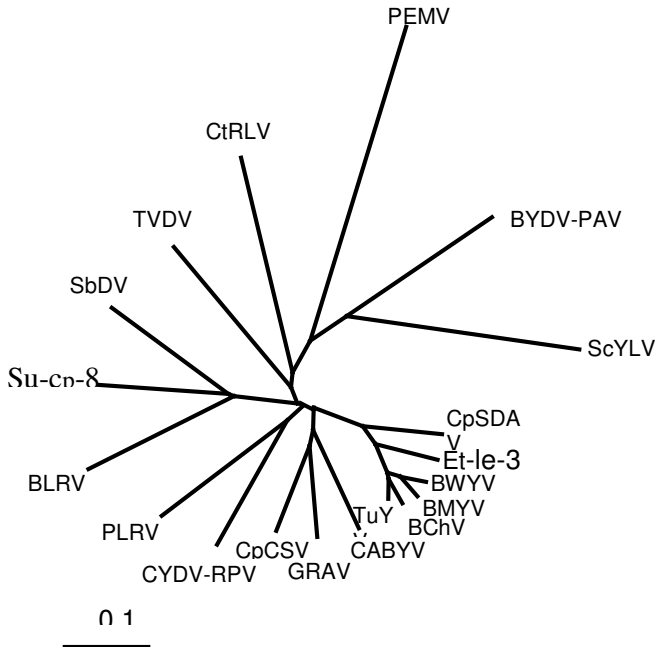

Figure 1. Unrooted dendrogram showing the phylogenetic relationship of the predicted coat protein amino acid sequences of two distinct luteovirus isolates (Su-cp-8 and Et-le-3) with those of other luteoviruses from the database. Database accession number of the luteovirus sequences and virus acronyms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

RT-PCR Amplification and sequence analysis

When those 2-5G4-positive samples that did not react with CpCSV antibodies were subjected to RT-PCR, full or partial CP-gene sequences were amplified (Table 1) based on the primer pair used. Using the primer pair S1/AS3, amplicons of similar size (ca. 600 bp) were obtained from some faba bean, chickpea and lentil samples from Ethiopia, Sudan, Egypt and Morocco. Cloning and sequence analysis however revealed 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 and each other as shown in Table 2. A sequence from a chickpea isolate 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 (Figure 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. Multiple alignment of the predicted amino acid sequence encoded by Su-cp-8 with those of SbDV and BLRV is shown in Figure 2. It can be seen that 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.

A luteovirus sequence obtained from a lentil isolate 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 (Figure 1) but appears to be less related to some species of this subgroup than they are to each other. Multiple alignment of predicted amino acid sequences encoded by Et-le-3 with viruses of BWYV subgroup and CpSDaV (Figure 3) shows that most of the amino acid differences are concentrated at N-terminal part of its CP.

CP sequences of luteovirus isolates very similar to TuYV were also amplified from three faba bean samples from Egypt and two from Morocco (Table 2). 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 also had high level of identity (92%) with BWYV and BMYV sequences (Table 2).

All the 2-5G4 positive isolates including those amplified by S1/AS3 were amplified with primer pair S2/AS3 and gave a product of ca. 340 bp. However, when those amplified only by S2/AS3 were cloned and sequenced, SbDV sequences of ca. 340 nt from the C-terminal part of the CP gene were obtained from faba bean samples from Ethiopia, Syria and China. The percentage nucleotide sequence identity between the isolates ranged from 90 - 100%. Phylogenetic comparison of the nucleotide sequence of the isolates with those of SbMV from the database showed that the grouping is roughly correlated to the geographical origin of the samples. 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 highly homogeneous among themselves and formed their own cluster (Figure 4).

DISCUSSION

The serological and sequence data presented here revealed the association of some luteovirus isolates with distinct CP sequences that are clearly different from those previously described. In particular, Su-cp-8 and Et-le-3 isolates that 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

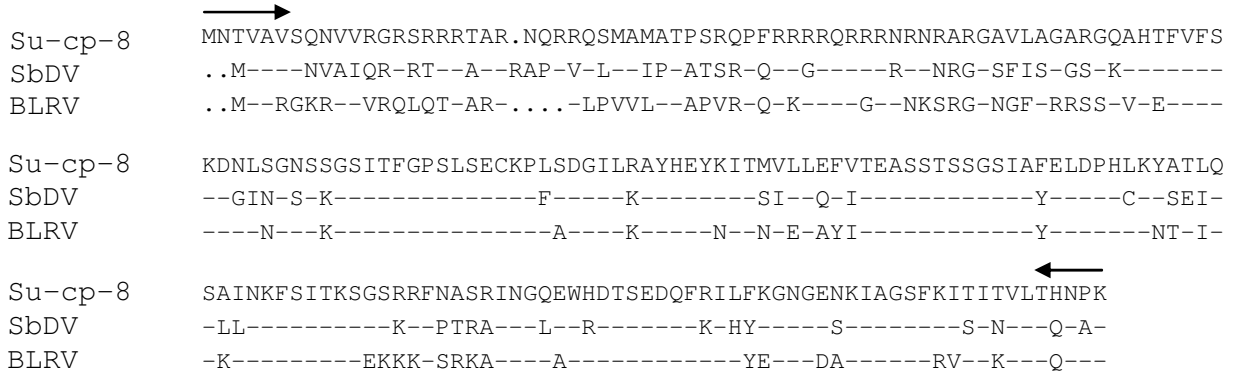

Figure 2. Alignment of the predicted coat protein amino acid sequence of isolate Su-cp-8 with those of *Soybean dwarf virus* (SbDV) and *Bean leaf roll virus* (BLRV). The arrows indicate the primer sequence sites. Database accession numbers of SbDV and BLRV sequences used in this alignment are indicated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

Figure 3. Alignment of the predicted coat protein amino acid sequence of isolate Et-le-3 with those of *Turnip yellows virus* (TuYV), *Beet western yellows virus* (BWYV), *Beet chlorosis virus* (BChV), *Beet mild yellowing virus* (BMYV) and *Chickpea stunt disease associated virus* (CpSDaV). The arrows indicate the primer sequence sites. Database accession number of the luteovirus sequences used aregiven in Table 2.

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. 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*, further information on their biological properties is required.

Phylogenetically, CpYV is most closely related to SbDV

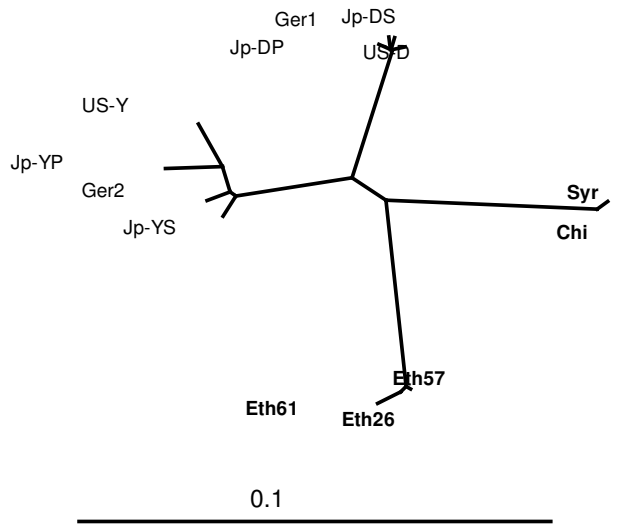

Figure 4. Unrooted dendrogram showing phylogenetic relationship among partial coat protein nucleotide sequences of SbDV isolates. Sequenced isolates are from Ethiopia (Eth26, Eth57 and Eth61), Syria (Syr) and China (Chi) while sequences from Japan (Jp-YP, -YS, -DS and –DP), USA (US-Y, US-D) and Germany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

and BLRV (Figure 1), both members of genus Luteovirus and may belong to the same genus. However, final assignment to the genus should await sequence information of the polymerase gene which is the major criteria for distinguishing the genera in the family *Luteoviridae* (D'Arcy and Domier, 2004). In this work, although CP sequences were repeatedly amplified using CpYV RNA extracts, our further attempt to amplify sequences from other genomic regions of the virus using primer pairs designed from other conserved regions of other luteoviruses was not successful, possibly because there is poor match of the primer in the region used. It should also be noted that the CP of SbDV and BLRV are not amplified by the S1/AS3 primers pair that amplified CpYV and other poleroviruses since the 5' end is highly divergent. This primer pair may therefore be useful to differentiate between these viruses.

In previous work, 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, a recent study (Abraham et al. 2006) suggested that CpCSV is the most common and that no BWYV subgroup viruses were detected in several samples serologically or by molecular means suggesting that this group of viruses is at least not widespread in the country. Given that LStV is phylogenetically closer to BWYV subgroup than other poleroviruses, it would be helpful to investigate further its distribution and importance in Ethiopia and elsewhere. Although LStV is clearly a polerovirus, obtaining further sequence information particularly at the 5' end of the genome including ORF0 will clarify its exact taxonomic position in relation to other members of BWYV subgroup.

At the beginning of this study, the only legume (faba bean) luteovirus isolate from BWYV subgroup for which ORF0 sequence data is available in the database is from France, that based on 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, but the sequence is not available in the database. Pairwise comparison of this sequence with that of our faba bean isolates 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 have to be considered as TuYV isolates.

The data also confirms the occurrence of SbDV in faba bean in Ethiopia, China and Syria using more robust molecular techniques as most previous reports are based mostly on serology which is not that reliable in luteoviruses identification. Together with our recent report of this virus in faba bean and clover in Germany (Abraham et al., 2007), results of this study suggest that SbDV has wider geographical distribution than it was once thought (Figure 2). Sequence and biological information on more isolates from different geographical areas would help to obtain a better understanding of the nature of variation of SbDV isolates from different crops and geographical origin.

The fact that the new viruses described here were not encountered in the previous legume serology-based virus surveys in various countries in the region suggests that the identification techniques used were unable to reveal the occurrence of new viruses. In serological tests, viruses that do not react with a set of specific antibodies used are normally excluded. In addition, some of the antibodies may cross react with other viruses sharing common epitope leading to erroneous identification. In

the present study, the combined use of broad spectrum and virus-specific antibodies followed by sequence analysis revealed the occurrence of new and known viruses. The results underline that virus identification based on serology should be supported by molecular techniques including sequence information of at least part of the virus genome particularly when a virus or its isolate are reported for the first time from a country, a region or a new host.

It is very likely that both CpYV and LStV also occur in other legume growing regions of the world and hence should be included in future surveys. 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, suitable virus propagation and purification protocols need to be developed. Finally, since the reported two viruses are proposed as "new" based only on information from serology and CP gene sequence, further studies on their biological properties is necessary.

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