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Short Note

Characterization of a Syrian *Chickpea chlorotic stunt virus* strain and production of polyclonal antibodies for its detection

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Summary. Reverse transcription-polymerase chain reaction analysis with two primer sets of luteoviruses was used to characterize an isolate of *Chickpea chlorotic stunt virus* (CpCSV, genus *Polerovirus*, family *Luteoviridae*) (SC402-08) collected from Lattakia, Syria, during the 2007–2008 chickpea growing season. Sequence analysis revealed that the coat protein gene of the isolate shared nucleotide sequence identities ranging from 97 to 98% with the CpCSV isolates from Egypt, Morocco and Syria. The capsid protein was separated as a protein of approximately 20 kDa in sodium dodecyl sulphate polyacrylamide gel electrophoresis, and was visually detected by its reaction with CpCSV monoclonal antibody in Western blot. SC402-08 isolate of CpCSV was purified from faba bean-infected plants, and yielded 112–182 μg of purified virions kg^{-1} of infected tissue. The purified preparation was injected into a white rabbit, and an antiserum was obtained and used to detect CpCSV in infected tissues by tissue-blot immunoassay. The antiserum obtained was able to detect CpCSV by the immunoassay up to a dilution of 1:1,024,000.

Key words: CpCSV, purification, rabbit antibody.

Introduction

Chickpea (*Cicer arietinum* L.) can be affected by a number of diseases and pests, including viruses. *Chickpea chlorotic stunt virus* (CpCSV, genus *Polerovirus*, family *Luteoviridae*) is one of those and has been reported in Egypt, Ethiopia, Eritrea, Iran, Morocco, Sudan and Syria (Abraham *et al*., 2006, 2009; Kumari *et al*., 2008; Asaad *et al*., 2009b; Bananej *et al*., 2010). This virus naturally infects many legume host (e.g., chickpea, lentil, field pea, faba bean) as well as some leguminous weeds and four wild non-legume plant species (Abraham *et al*., 2006, 2009; Asaad *et al*., 2009b). CpCSV is a phloem-limited virus present in very low concentration and transmitted by aphids (*Aphis craccivora* Koch. and *Acyrthosiphon pisum* Harris) in a persistent manner (Abraham *et al*., 2006; Asaad *et al*., 2009b).

CpCSV symptoms cannot be distinguished from those caused by other yellowing viruses like *Bean leafroll virus* (BLRV, genus *Luteovirus*, family *Luteoviridae*) or *Beet western yellows virus* (BWYV, genus *Polerovirus*, family *Luteoviridae*). Robust diagnostics are therefore essential to determine CpCSV infection. So far, the only available antiserum for the detection of CpCSV was produced by Abraham *et al*. (2009).

In this investigation we characterized a Syrian isolate of CpCSV, produced a polyclonal antiserum and assessed its quality using tissue-blot immunoassay (TBIA).

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Materials and methods

Virus isolates and antisera used

CpCSV isolate SC402-08 was collected from a chickpea field in Lattakia province, Syria, during the 2007–2008 growing season. A broad set of antisera and isolates were used in this study; including polyclonal antibody (PAb) and infected faba bean plant for BLRV (SV64-95; ICARDA), PAb and infected faba bean plant for *Soybean dwarf virus* (SbDV, genus *Luteovirus*, family *Luteoviridae*) (SL1-94; Makkouk *et al*., 1997), two PAbs produced BWYV isolates, one against a Lebanese (Terbol), the other against and a Syrian isolate (Tel-Hadya) (Kawas, 1992), PAb against BWYV (No. 864, provided by BBA, Germany) and infected BWYV-faba bean plant (SC23-07; Asaad *et al*., 2009a). In addition, the following monoclonal antibodies (MAbs) were used: a broad-spectrum luteovirus monoclonal (5G4; Katul, 1992), BWYV (A5977, Agdia, USA), BLRV (4B10; Katul, 1992), SbDV (ATCC PVAS-650; from American Type Culture Collection ATCC, Rockville, Maryland, USA), a mixture of three MAbs (1-1G5, 1-3H4 and 1-4B12) produced against an Ethiopian isolate of CpCSV (CpCSV-Eth), and a mixture of three MAbs (5-2B8, 5-3D5, 5-5B8) produced against a CpCSV isolate from Syria (CpCSV-Sy) (Abraham *et al*., 2006, 2009).

Characterization of the CpCSV isolate

SC402-08 isolate was tested for viruses by tissue-blot immunoassay (TBIA) (Makkouk and Kumari, 1996) using a battery of PAb and MAb antibodies, and by RT-PCR using universal luteovirus (Lu_1 + Eco and Lu_4 + Eco) (Kumari *et al*., 2006) and CpCSV specific (Kumari *et al*., 2008) primer sets. The coat protein (CP) gene sequence obtained was studied by comparing it with sequences of other CpCSV isolates from different locations of the world and submitted to GeneBank.

The CP molecular weight of SC402-08 isolate was determined by electrophoresis. Purified virion preparations, CpCSV-infected and healthy plant extracts were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1973), followed by Western blot as described by Kumari *et al.* (2001) using CpCSV-Sy MAb and PAb produced against SC402-08 isolate in this study.

Virus purification

SC402-08 isolate of CpCSV was propagated in faba bean plants (var. Syrian local, ILB 1814) using *A. craccivora* as a vector. Twenty d after inoculation, the infected faba bean tissues were harvested, chopped and kept frozen at –20°C until use. Virus purification was carried out as described by D'Arcy *et al.* (1989), but without a macerating enzymes step.

The presence of CpCSV was tested by Dot-blot (Makkouk *et al.,* 1993) using CpCSV-Sy MAbs, and fractions free of host plant debris were pooled. The Ultraviolet absorbance ratio A_{260}/A_{280} of purified preparations was determined by using a UV-spectrophotometer, and virus concentration was estimated using an assumed extinction coefficient of 8.6 (Abraham *et al*., 2009).

Antiserum production and evaluation tests

The antiserum against the purified SC402-08 virions was produced by immunizing a New Zealand white rabbit with a primary subcutaneous injection of 40 μg virus and three intramuscular injections of 25 μg virus, administered subsequently at 2 weekly intervals. Purified virus preparations were emulsified with an equal volume of Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for subsequent injections. A booster injection was given 2 weeks after the fourth injection. Twelve blood samples were collected at weekly intervals, starting 1 week after the last injection.

The raw antiserum was evaluated for sensitivity and specificity by TBIA. Titrations were determined with twofold dilution series of antiserum ranged from 1:1000 to 1:1,024,000. Also the specificity of resulting antiserum was assessed by TBIA using a 1:1000 dilution of antiserum produced and a 1:1000 dilution of each MAb and PAb used in this experiment. The dilution of 1:3000 for both the goat anti-rabbit IgG alkaline phosphatase conjugate (A3687, Sigma-Aldrich, St. Louise, MO, USA) and the goat anti-mouse IgG alkaline phosphatase conjugate (A5153, Sigma) were used; reactions were evaluated visually using a stereo microscope at 40× magnification, 15 min after the addition of the substrate.

To reduce nonspecific background reactions, the raw antiserum was cross-adsorbed with faba bean plant extracts at 1/20 (W/V) in TBIA and Western blot tests.

Results and discussion

Characterization of CpCSV isolate

The serological TBIA test showed that isolate SC402-08 reacted positively only with CpCSV-Sy MAb (5-2B8, 5-3D5, 5-5B8) and the broad spectrum legume luteovirus MAb (5G4), but not with any of the luteovirus-specific MAbs used (Table 1).

RT-PCR analysis using specific primers confirmed the TBIA results and generated the anticipated amplicons of 530 bp and 413 bp corresponding to universal luteovirus and CpCSV-specific primers, respectively (Figure 1).

Comparison of the CP sequence of the Syrian CpCSV isolate (SC 402-08; GeneBank Accession n. FN665789) with other CpCSV-isolates revealed that the Syrian isolate clustered (97–98% homology) with group-II isolates from Egypt (EU541269), Morocco (EU541267) and Syria (EU541270) (Abraham *et al*, 2009), but showed 92% homology with group-I isolates from Ethiopia (AY956384) and Sudan (EU541263) (Abraham *et al.* 2009), and was clearly distinct from other luteovirus species (less than 85% homology). This suggests that the Syrian CpCSV isolate (SC402-08) examined in this study belongs to group-II.

SDS-PAGE analysis of purified virions gave a strong and a clear band of approximately 20 and 50 KDa, corresponding, respectively, to the major CP and the presumable read-through protein. Western blot analysis showed a strong reaction for the 20 KDa major coat protein, whereas the reaction of the 50 KDa protein was totally absent. The two proteins detected by Immunoblotting using CpCSV-Sy MAb (5-2B8, 5-3D5, 5-5B8) are similar to the major CP and the read through protein, which has been reported to occur in virions of CpCSV (Abraham *et al*., 2009).

Table 1. Homologous and heterologous tissue-blot immunoassay (TBIA) reactions of *Chickpea chlorotic stunt virus* (CpCSV) antiserum produced against Syrian isolate (SC402-04) and some other luteoviruses reactions of monoclonal and polyclonal antibodies used in this study.

Antibodies used	Virus species (isolate name)[a]			
	CpCSV (SC402-08)	BLRV (SV64-95)	SbDV (SL1-94)	BWYV (SC23-07)
Polyclonal antibodies				
CpCSV (SC402-08)	+++[b]	-	-	-
BLRV (SV64-95)	++	++	-	++
SbDV (SL1-94)	++	-	++	++
BWYV (Terbol)	++	-	-	++
BWYV (Tel-Hadya)	++	-	-	++
BWYV (# 864, Germany)	+++	+++	++	+++
Monoclonal antibodies				
5G4	+++	+++	+++	++++
BLRV (4B10)	-	+++	-	-
SbDV (ATCC-PVAS-650)	-	-	+++	-
CpCSV-Eth (1-1G5, 1-3H4, 1-4B12)	-	-	-	-
CpCSV-Sy (5-2B8, 5-3D5, 5-5B8)	+++	-	-	-

[a] CpCSV, *Chickpea chlorotic stunt virus*; BLRV, *Bean leafroll virus*; SbDV, *Soybean dwarf virus*; BWYV, *Beet western yellows* virus.
[b] +++, strong reaction; ++, intermediate reaction; - , no reaction.

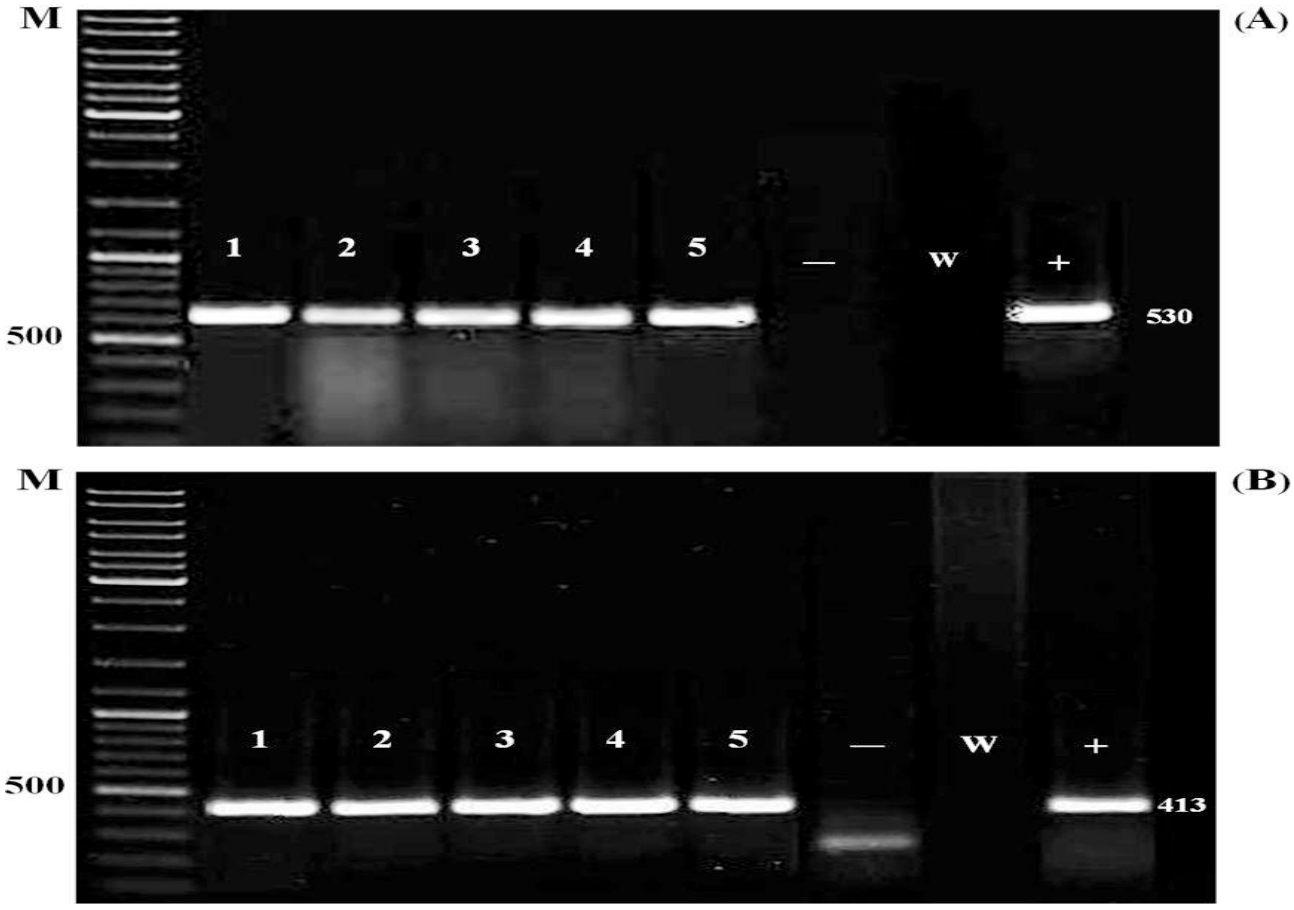

Figure 1. Detection of *Chickpea chlorotic stunt virus* (CpCSV)by RT-PCR using (A) Broad spectrum luteovirus (Lu_1 + Eco and Lu_4 + Eco) primers and (B) CpCSV-specific (CpCSV-F and CpCSV-R) primers. Lane (-), negative control; lane (W), water; lane (+), positive control; M, GeneRuler™ 100 bp DNA Ladder (Fermentas International, Burlington, ON, Canada).

Virus purification

The yield of purified virions obtained was calculated to be 112–182 μg kg^{-1} of CpCSV-infected faba bean tissues, which is close to the yield reported earlier for CpCSV purified from faba bean tissue (Abraham *et al*., 2006). Purified virus suspensions were free of host plant contaminants and had an A_{260}/A_{280} ratio of 1.54. The adequate quantity and the high purity of purified virions obtained in this study suggest that the partially modified protocol used was appropriate and can be recommended for the purification of Luteoviruses.

Quality of antiserum produced

The CpCSV antiserum produced against the Syrian isolate (6th bleeding) of CpCSV had a titer of 1:1,024,000 as determined by TBIA using goat anti-rabbit IgG alkaline phosphatase conjugate at a dilution of 1:3000, giving strong positive reactions and no background (Figure 2; Table 1).

When BWYV, BLRV, SbDV, and CpCSV isolates were tested by TBIA using antiserum produced against the Syrian isolate (SC402-08) of CpCSV, no cross-reactions were observed between CpCSV antiserum and BWYV, BLRV and SbDV, whereas cross-reactions were obtained between CpCSV and each of BWYV, BLRV and SbDV PAbs (Table 1).

TBIA results using PAbs obtained from twelve separate bleedings from the same rabbit were able to detect CpCSV efficiently with clear specific reaction and no background. The prepared antiserum from the 6th bleeding was also effective in the detection of

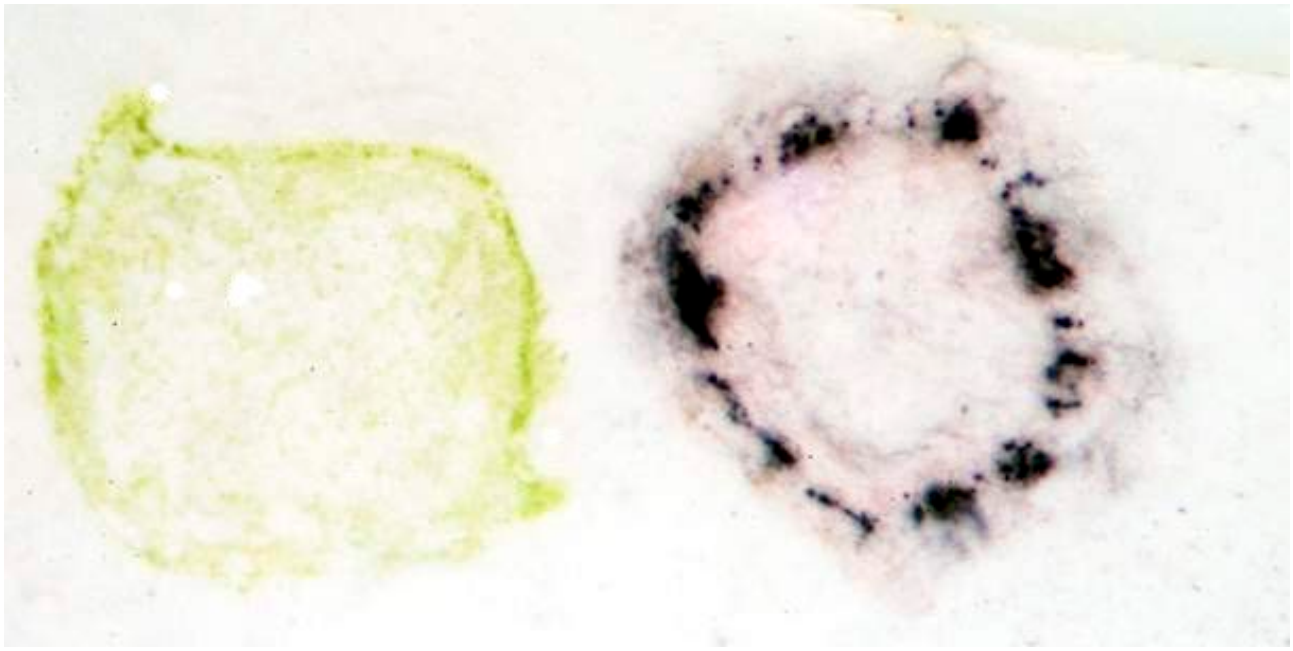

Figure 2. Detection of *Chickpea chlorotic stunt virus* (CpCSV) in prints of cross-sections of infected chickpea plant (right) and healthy plant (left), using tissue-blot immunoassay (TBIA). This test used raw antiserum dilution of 1:2000 and goat anti-rabbit IgG alkaline phosphatase conjugate dilution of 1:3000.

CpCSV in different plant species (data not shown), and had a titer of 1:1,024,000 in TBIA against CpCSV virion. The titer was calculated on the basis that at this antiserum dilution, the reaction in TBIA was still visible with no visible reaction with the respective healthy plant.

Based on the results obtained, the quality of the antiserum produced is thus acceptable for diagnostic purposes. As CpCSV is a new emerging viral disease on chickpea in Syria, fast and reliable diagnostics are needed for this virus. The good quality and the high specificity of the polyclonal antiserum produced to the Syrian isolate (SC402-08) of CpCSV, provides a practical diagnostic tool useful for many purposes, such as chickpea breeding for virus resistance through monitoring virus multiplication and routine detection of CpCSV in field surveys.

Acknowledgments

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