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Full Length Research Paper

Serological and RT-PCR detection of *cowpea mild mottle* carlavirus infecting soybean

M. Tavasoli[1], N. Shahraeen[2] and SH. Ghorbani[1]

[1]Division of Microbiology, College of Science, Alzahra University, Tehran, Iran.
[2]Plant Virus Research Department, Iranian Research Institute for Plant Protection (IRIPP), Tehran, P.O. Box- 19395-1454, Iran

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During 2006-2007 growing seasons, survey was carried to identify a disease of possible viral etiology causing mosaic of soybean in the soybean field. Leaf samples showing symptoms of mild mosaic were collected from soybean fields in Dezful region (Khozestan province, Southern Iran). Electron microscopy, DAS-ELISA serological tests and RT-PCR assays with specific pairs of primers were applied to identify and determine the viral etiology of the agent. Flexuous particles of ca. 15 x 650 nm were present in the leaf dip preparations examined by electron microscopy. On the basis of serology, RT-PCR assays and reaction of indicator host plants, the causal virus of these mild mosaic symptoms on soybean in the Southern Iran was identified as *Cowpea mild mottle virus*- CPMMV, a whitefly-transmitted carlavirus.

Key words: Cowpea mild mottle virus, serology, RT-PCR, detection, Southern Iran

INTRODUCTION

As soybean (*Glycine max* (L.) Merril) cultivation increases in Iran (106000 ha in 2005) (Anonymous 2005) occurrence of virus and virus-like diseases can limit production (Golnaraghi et al., 2002a,b, 2004; Tavasoli et al., 2007). Soybean (*Glycine max*) is susceptible to infection by several viruses, which substantially reduce yield and quality and at the moment it is known that it is a natural host for 35 potentially important viruses (Edwardson et al., 1991).

*Cowpea mild mottle virus (*CPMMV) was first reported on cowpea (*Vigna unguiculata*) in Ghana (Brunt and Kenten, 1973). Subsequently it was reported from several tropical regions of Africa (Brunt and Philips, 1981; Thouvenel et al.,1982; Anno-Nyako ,1984; 1986; Mink and Keswani, 1987) , Asia (Antignus and Cohen ,1987; Nolt and Rajeshwari, 1987; Shahraeen , 1989; Reddy, 1991;), Brazil and Argentine (Laguna et al., 2006; Almeida et al., 2005) and from Ivory Coast in a diverse range of plant species that include leguminous and Solanaceous food crops (Hartman et al., 1999). CPMMV is reported to be transmitted by the whitefly, *Bemisia tabaci* (Homoptera: *Aleyrodidae*), in a non-persistent manner (Jeyanandarajah and Brunt, 1993; Memelink et al., 1990). CPMMV has filamentous particles of approximately 650 ×15 nm in size with a coat protein of 32-36 KDa (Demski et al., 1989). CPMMV is a member of genus *Carlavirus* which has recently been classified under the plant virus family Flexiviridae (Memelink et al.1990; Giovanni et al., 2007). CPMMV causes mosaic, chlorosis, necrosis and distortion in a range of indicator host plants (Iwaki et al., 1986; Demski and Kuhn, 1989). Soybean, (*Glycine max* L Merill, groundnut (*Arachis hypogaea* L.), cowpea (*Vigna unguiculata* (L.) Walp.), tomato (*Lycopersicon esculen0-tum* Mill. , broad bean (*Vicia faba* L.) and *Nicotiana clevelandii* Gray. have been reported as diagnostic hosts of CPMMV (Demski and Kuhn., 1989; Reddy,1991). The CPMMV genome consists of a single-stranded RNA of Mr 2.5×10^6 with six open reading frames (ORF) encoding for the following putative proteins: methyltransferase (Mt), papain-like protease (P-Pro), helicase (Hel), RNA-dependent RNA polymerase (Pol), coat protein (CP), nucleic acid binding protein (NB), plus three triple gene block (TGB) (Nolt et al., 1997). CPMMV-S and CPMMV-M, are

*Corresponding author. E-mail: shahraeen@yahoo.com.

Table 1. Primers used in RT-PCR and predicted amplicon size for detection of CPMMV

Tm(°C)	Primer position	primer sequence
56.8	CPMMV Upstream1778-1799 bp	5' - CAC TTG GAA TTT TAT GTT GAC - 3'
58.8	CPMMV Downstream1982-2002bp	5' - TCA TTT CGA TTG GAC CTA TC - 3'

two reported strain of the virus. Mild to severe systemic symptom appearance is reported by CPMMV in different hosts (Laguna et al., 2006; Tavassoli et al., 2007). There have bean a few previous studies on soybean viral diseases and their distribution in Iran, where a few important viruses from nepo-, poty-, bromo-, cucumo- and tospoviruses group have been reported (Shahraeen et al., 2005; Golnaraghi et al., 2000, 2002a,b, 2004, 2007; Ghorbani et al., 2007).

Field symptoms associated with virus infection includeing mosaic, mottling, vein clearing and leaf crinkle were observed in soybean fields in Dezful region. CPMMV has been reported to be transmitted by the whitefly, *Bemisia tabaci*)(Laguna et al.,2006). Recently a disease causing stem necrosis of soybean in Brazil and Argentina has also been attributed to CPMMV or a related carlavirus (Almeida et al., 2005; Laguna et al., 2006). In Iran CPMMV has been reported to infect cowpea in Guilan (Northern) province of Iran (Ghorbani et al., 2007) where mix cropping pattern (pulses and solanaceous food crop is a common practice and a large population of the whitefly vector *Bemisia tabacci* Genn. may be responseble for the transmission and spread of pathogen to other crop .

Investigation of incidence and distribution of soybean viruses are very important in developing appropriate control measures. During this study limited number of infected sample were collected and tested using DAS-ELISA serology, RT-PCR and electron microscopic studies for the presence of CPMMV in the sample.

Material and methods

During 2006-2007 growing seasons, infected leaf samples of soybean (*G. max* cv. Clark) plants with mild mosaic and leaf defoliation symptoms were collected from fields in Dezful territory (Khozestan province). For virus detection and host range studies, the sap extracted from infected soybean leaf samples using 0.1 M phosphate buffer (pH 7.2) containing 2% PVP and 1% Na-DIECA (Golnaraghi et al., 2004) were mechanically inoculated to several host plants: soybean, cowpea, groundnut, bean, (*Phaseolus vulgaris* L.) , broad bean tomato, *Chenopodium amaranticolor* Coste & Reyn, *C. quinoa* Willd., *Datura stramonium* L., *Gomphrena globosa* L., *Vigna radiata* (L) R. Wilcz , *Vigna aconitifolia* (Jacq.) Marechal and *Nicotiana rústica* L., tobacco (*N. tabacum* L.) and *Cassia tora* L.. Seeds from infected filed plants were sown and grown under greenhouse conditions.

DAS-ELISA (Clark and Adams, 1977; Lister, 1987) serological test was performed in order to identify CPMMV in the samples. CPMMV antibody kit (plus positive control) was gifted by Dr. Winter (DSMZ, Braunschweig, Germany) and used as prescribed. RT-PCR test was performed (Tavasoli et al., 2007), where total RNA was extracted by TRI-reagent solution as described (Sigma Company). 30-50 mg. of infected tissue was grounded to a fine powder using a wooden applicator stick, in a 1.5 ml Eppendorf tube cooled in liquid nitrogen. Grounded leaf tissue was extracted with 500 µl of TRI-reagent buffer, after 10 min, 250 µl of phenol-chloroform was added, and mixed, followed by centrifuged at 30000 rpm for 15 min. The supernatant was transferred to a new micro tube and 300 µl of isopropanol was added. Samples were kept at -20 °C for at least 20 min, then centrifuged as above; supernatant removed and the pellet was washed by ethanol 75%, and finally dissolved in 20 µl distilled water. For RT-PCR reaction the specific pair of CPMMV primers were designed (Table 1) applying Gene Runner and Blast programs in 1778-2002bp sequences region of two CPMMV strains in GenBank (Accession number AF024628 and AF024629, pair of primers were synthesized (Isogene company , Netherland, Figure 1). Reverse transcription reaction was followed using RevertAid TM first strand cDNA synthesis Kit (BioNeer Co. Korea, Kit-1621), following the specific protocol by applying reverse primer. PCR amplification was performed using lyophilized PCR micro tubes (Accupower PCR PreMix (BioNeer Co., Korea) under the following conditions: (denaturation: 94°C , 30 s ; annealing: 56.5°C , 30 s ; extension: 72°C , 30 s) for 35 cycles, finally 5.5 min of extension at 72°C. Amplified DNA fragments were separated by eletrophoresis in 1% (w/v) agarose gels in 0.5 x TBE and visualized at 302nm after staining in ethidium bromide (0.5 ug/ml).

For virus particle morphology observation (Edwardson et al., 1991), applying leaf dip preparation method (Milne ,1984) young soybean symptomatic trifoliate leaf sample infected by CPMMV were selected and prepared using 400 mesh carbon coated grids, stained with 2% uranyl acetate and examined under a transmission electron microscope (TEM-Philips-301G Model).

RESULTS

The virus isolated from soybean in the Southern part of Iran was identified as CPMMV based on serological reaction, particle morphology, experimental host range (Edwardson et al., 1991) and RT-PCR test with specific pair of primers. Soybean CPMMV infected few indicator plants and produced necrotic local lesions followed by systemic mosaic, mild mottling and veinal necrosis on soybean cv. Clark, chlorotic local and systemic vein clearing and mosaic on cowpea (local cultivar), bean cv. Tender Green reacted with mosaic and leaf distortion while cv. Top Crop with mild mottling, Yellow vein clearing downward leaf rolling in groundnut (cv.NC-2), chlorotic local lesion on *Chenopodium amaranticolor* and *C. quinoa.* The virus isolate did not produce any visible symptoms in *Vigna radiata, V. aconitifolia,* and tomato but they were shown to be infected and gave positive results in the in ELISA recovery test. On the other hand, CPMMV could not infect broad bean, *Datura stramonium* and tobacco (Table 2). The symptoms induced following mechanical inoculation of herbaceous host plants resembled those described for CPMMV-S (Laguna et al., 2006),

Figure 1. Upstream and downstream specific primers by matching CPMMV-S and CPMMV-M sequences from GenBank using GeneRunner software. (*) regions are indicating of similar sections.

Table 2. Reaction of selected host plants to inoculation with CPMMV the soybean isolate from Southern Iran.

Host plants	Symptoms		ELISA Test results
	Local reaction	Systemic reaction	
C. amaranticolor	CLL	-	+
C. quinoa	CLL	-	+
A. hypogaea (cv. Local)	Ylvc,Cr,Ld		+
Glycine max	NLL	M, Mot,Vn	+
P. vulgaris (cv. Top Crop)	-	MMot	-
P. vulgaris (cv. Tender Green)	CLL	M,Ld	+
V. unguiculata	CLL	M,Ld	+
V. radiatae	(-)	(-)	+
V. aconitifolia	(-)	(-)	+
L. esculentum	-	MMot	+
V. faba	-	-	_
D. stramonium	-	-	_
N. tabacum (cv. Rustica)	-	-	_
G. globosa	-	-	-

CLL= Chlorotic local lesion, NLL=Necrotic local lesion, YIVc=Yellow vein clearing, Cr=Crinkle, M=Mosaic, Mot=Mottling, Vn=Veinal necrosis, Ld=Leaf distortion, MMot=Mild mottle, (-) =Negative result, + = Positive reaction, (-)=doubtful

V. unguiculata and *Vigna radiata* were without any conspicuous symptoms, but ELISA result was positive.

no obvious symptoms were seen upon inoculation of the virus isolates on *Gomphrena globosa*, *Cassia tora* .*Nicotiana rustica*, bean (local cultivar Chitee), *D. stramonium*, and broad bean. None of 800 planted seeds from infected field soybean plants showed symptoms upon emergence. Inoculation test results were further rechecked by DAS-ELISA. The expected band of 206 bp (Figure 2) was obtained in gel electrophoresis using a 1 kb ready to use

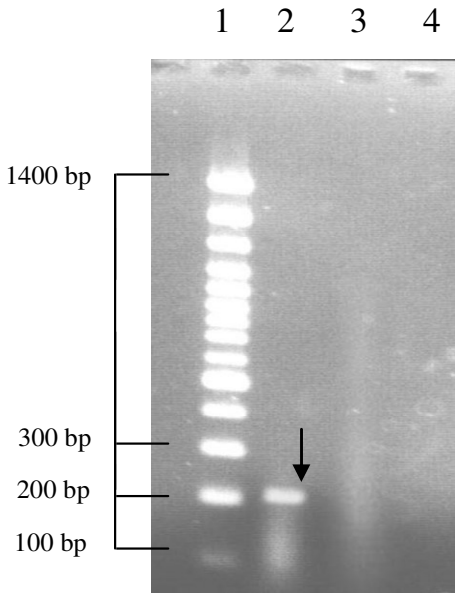

Figure 2. 1% agarose gel. Electrophoresis of CPMMV.
1-100 bp DNA ladder (Fermentas).
2- CPMMV band in 206 bp region.
3- Negative control consisting of distilled water and loading buffer
4 - Negative control using healthy soybean leaves.

marker (100 bp.) DNA ladder (Fermentas Co, Germany) and the primers designed by us. In this study for PCR reaction, the lyophilized microtube of AccuPower PCR premix (Bioneer Corporation Korea) was found more convenient replacing the needs for loading buffer compared to a common protocol. Particles of approximately 650 nm in length were observed (micrograph not shown). In leaf dip preparations of young symptomatic leaves examined in the transmission electron microscope Particle length measurement for CPMMV and of other related carlaviruses has been described elsewhere (Demski and Kuhn, 1989; Edwardson et al., 1991).

DISCUSSION

Our experimental data clearly point out that the soybean plants with mild mosaic symptoms found in the Dezful territory was infected by an isolate of CPMMV. Possibly it was being transmitted by the whiteflies present in the culture. It is not known yet how the virus came into the region. Since no seed transmission could be demonstrated under our conditions, it is likely that the virus was present in the region in some natural host or it is was introduced from the Northern region where CPMV was already identified infecting cowpea fields (Ghorbani et al., 2007; Tavasoli et al., 2007).. However, seed transmission of CPMMV is still a controversial subject. As discussed by El-Hammady et al., (2004), some authors (Brunt and Kenten, 1973; Thouvenel et al., 1982 and Iwaki et al., 1986) reported seed transmission of CPMMV is soybean, while others (Lizuka et al. 1984; Anon. 1987; Horn et al., 1991 and Rossel and Thottappilly, 1993) failed to produce such transmission. There are reports in other parts of the world that *Solanum incanum* L. may act as a perennial reservoir of CPMMV during the summer when region is virtually free from the soybean (Iwaki et al., 1986; El-Hammady et al.,2004). A survey on the plants growing near soybean field must be made to identify possible natural reservoirs of the CPMMV. Symptoms caused by this Iranian isolate of CPMMV on soybean is quite mild and similar to previous reports of infection of this crop by CPMMV and seem to be less severe that those described in Brazil which involve stem necrosis (Almeida et al., 2005). There is not yet an assessment of the yield losses on soybean caused by CPMMV associated to the plant stage when infection occurs nor the demonstration that it is being transmitted by the white flies present in the fields, which still have to be identified. The presence of CPMMV in the soybean in Southern Iran must be considered as an additional threat to this culture as well as to other legume crops since white flies populations are present everywhere. A thorough screening for resistance to CPMMV must be made on the available soybean germplasm collection of Iran and a cooperation with researchers form other parts of the world dealing with this virus on soybean must be
considered to face this menace.

On the basis of serological studies the isolate designnated as similar to CPMMV-S it is not yet clear if the cowpea isolate (Northern region, Ghorbani et al., 2007) and CPMMV isolate described in this paper are strain of one virus or distinct but serologically related viruses. In order to discriminate and determine sequence similarities between the CPMMV isolates from Iran with other reported isolates and their tentative relation to carlavirus or as a distinct virus require further molecular studies.

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