Biological and molecular characterization of *Zucchini yellow mosaic virus* from naturally infected bottle gourd

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SUMMARY: During survey, commercial fields of bottle gourd (*Lagenaria siceraria*) plants exhibited severe mosaic, interveinal chlorosis, and leaf deformation that resulted in fern-leaf appearance and severe fruit distortion in approximately 70% of the plants. The virus isolate was identified as *Zucchini yellow mosaic virus* (ZYMV) by biological and serological techniques, electron microscopy and RT-PCR. Nucleotide sequencing of the cloned PCR product showed that it was 497 bp long. Sequence analysis indicated that it contained C- terminal part of coat protein region and 3' untranslated region (UTR). The C-terminal coat protein region showed 100 % sequence identity with most of the isolates of ZYMV while 3'UTR region shared sequence identity in the range of 86.8% to 99.5% with different isolates of ZYMV indicating that the virus infecting bottle gourd is an isolate of ZYMV.

Key words: Bottle gourd, ZYMV, insect transmission, coat protein, UTR

INTRODUCTION

Lagenaria siceraria is a popular vegetable belonging to family Cucurbitaceae and is grown almost all the year round throughout India. It is a good source of vitamin B and ascorbic acid and has many medicinal uses (Robinson and Deckers-Walters, 1997). Several viruses belonging to Poty-, Cucumo-, Como- and Tobamo-virus genera have been reported to infect bottle gourd naturally (Wang and Chen, 1985; Hseu *et al.*, 1987; Al-Misa *et al.*, 1994; Choi Gug Seoun *et al.*, 2001).

Recently, occurrence of a distinct potyvirus (*Zucchini yellow mosaic virus- ZYMV)* in India causing mosaic in bottle gourd was observed (Raj Verma *et al.*, 2004 a). ZYMV was observed in the mid 1979 as occurring locally in Northern Italy (Lisa *et al.*, 1981) and within a decade it spread to all the major cucurbit growing areas in the world. The reasons for its rapid spread are still unknown (Desbiez and Lecoq, 1997). The devastating epidemic caused by ZYMV in Egypt and the severe outbreak on farms in Western New York States, Florida and California in 1983 illustrates the economic importance of the virus (Nameth *et al.*, 1983). In the present paper we report the characterization of ZYMV on bottle gourd based on bioassay, serology, electron microscopy, RT-PCR and sequence analysis.

MATERIALS AND METHODS

Survey

During survey of commercial fields of cucurbits in Pune in *kharif* 2003, leaf and fruit samples of bottle gourd were collected from plants showing a variety of symptoms ranging from mild to severe. Collected samples were tested by bio- and immunoassay, electron microscopy and RT-PCR.

Virus isolation and host range

Virus isolate was transmitted mechanically for maintenance of cultures, host range and bioassay. Sap inoculation was done by macerating leaf tissues with 0.01 M potassium phosphate buffer (pH 7.3), adding a pinch of celite powder to the extract and rubbing the extract on leaves of diagnostic species. To ensure the virus purity, the isolate was passed through three successive single-lesion transfers on *Chenopodium amaranticolor.* The isolate was maintained on *Cucurbita maxima,* which served as a source of inoculum. For host range, 18 plant species belonging to Chenopodiaceae, Cucurbitaceae and Solanaceae families were tested (Table 1). The test plants were raised in earthen pots in an insect proof glasshouse and mechanically inoculated with infective sap. The inoculated seedlings both symptomatic and asymptomatic (6 each) were observed for symptoms over a period of 4 weeks after inoculation and then tested for the presence of virus by ELISA using the ZYMV antiserum.

Insect transmission

Aphid transmission was conducted with five aphid species *viz. Myzus persicae, Aphis gossypii, A. craccivora, A. malvae* and *A. niri* maintained in insectory. The aphids were given pre-acquisition fasting for 2 hr and were allowed for acquisition access probes of 10 min on infected symptomatic pumpkin leaves. Healthy

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Table 1. Host reaction on mechanical inoculation and indexing in ELISA

Species	Reaction	ELISA
Chenopodium amaranticolor	CLL	+
Benincasa hispida	M, B	+
Citrullus lanatus	M	+
Cucumis melo	M, LD	++
Cucumis sativus	M, B	+
Cucurbita moschata	CS, M, B, VB	+++
C. pepo	VC, M, Mt, LD	+++
Lagenaria siceraria (Field Sample)	CS, VC, M, B, S, LD	+++
Luffa cylindrica	M	++
Trichosanthes anguina	M	+
Nicotiana glutinosa	NS	–
N. tabaccum L. cv. (White Burley)	NS	–
N. rustica	NS	–
Nicotiana benthamiana	NS	–
Datura stramonium	NS	–
Lycopersicon esculentum	NS	–
Vigna unguiculata	NS	–
Phaseolus vulgaris	NS	–

B= Blistering, CLL= Chlorotic local lesions, CS= Chlorotic spots, LD= Leaf distortion, M= Mosaic, Mt= Mottling, NS= No symptoms, S= Shoe-string, VC= Vein clearing, VB= Vein banding, + = Mild reaction, ++ = Moderarate reaction, +++ = Strong reaction, – = No reaction in ELISA

pumpkin leaves were used as control. Ten viruliferous aphids were released for each aphid species on each of six healthy bottle gourd plants for inoculation feeding for 20 min. Inoculated plants were sprayed with 0.1 % Endosulfan and maintained over a period of 4 weeks in an insect proof glasshouse. Thereafter all plants were tested by infectivity assay on *Chenopodium amaranticolor* and by ELISA using ZYMV antiserum. The insecticide was sprayed weekly for 4 weeks during observation of symptom development.

Electron microscopy

The field and glasshouse samples of infected bottle gourd were tested by electron microscopy following the method described by Ahlawat (1996) and Ahlawat and Verma (1997). Leaf dip preparation for electron microscopy were prepared by grinding the infected young leaves of bottle gourd showing prominent symptoms, previously fixed in 3% gluteraldehyde for 1h. A drop of sap extracted from these leaves was placed on farmvar coated grids for 1 min then rinsed it three times with sterile distilled water and stained with 2% Uranyl acetate (UA) (pH 4.5) for 10 sec and examined with JEOL-100- CX II transmission electron microscope (TEM).

Serology

For immuno-assay, direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) was conducted as described by Ahlawat (1996). Commercial polyclonal antibodies of PRSV, CMV, PVY and WMV-II were purchased from Agdia, Elkhart, Indiana, USA and of ZYMV from Bio-Rad. Samples were ground 1:10 (w/v) in extraction buffer and diluted with coating buffer. Polystyrene plates (Numc, 25. Denmark) were coated with antigen (200 ul/ml) and incubated overnight at 4^0C. Negative and positive control were used at (1: 200) (200 ul/ml) and the plates were incubated at 37^0C for 2 hr. Goat antirabbit IgG alkaline phosphate conjugate (Sigma 28.Co., St. Louis, MO) was used at 1:10000 dilution and incubated for 2hr at 37^0C. Reaction was observed 30 min after addition of para-nitrophenyl phosphate substrate at a concentration of 0.6 mg/ml in substrate buffer (pH 9.8) and was read at 405 nm by using EL X 808_{IU} Ultra microplate reader (Bio-Tek Instrument).

RNA isolation

Total RNA was isolated from 100 mg leaves of ZYMV infected bottle gourd using RNeasy Plant Mini Kit (Qiagen, USA) as per manufacturer's protocol.

Reverse-transcription polymerase chain reaction (RT-PCR)

Reverse transcription and PCR were done separately. For first strand c DNA synthesis 5 µl of elution from RNeasy column was denatured for 10 min at 65^0C and quenched on ice. RT was carried out using Omniscript RT kit (Qiagen) in 20 µL reaction mixture using oligo-dT primer (1µM) and Omniscript RT (4 units) at 42^0C for 1 h. The primers used for the amplification of C-terminal region of coat protein region (CP) and 3' UTR by PCR were derived from the Genbank accession X62662, an isolate of ZYMV from Singapore. PCR was carried out using primer pair 5' AAA TGC AGA GGC ACC ATA C (F) and AGG CTT GCA AAC GGA GTC (R). PCR amplification was performed in 50 µl reaction mixtures using 1 µM of primer, 200 µM each of dNTPs, 0.05 unit / µl of Taq DNA polymerase, 1 x reaction buffer, 1.5 mM of $MgCl_2$ and 5 µl of cDNA template. Samples were amplified for 30 cycles using a thermocycler (Biometra, Germany). Each cycle consisted of denatuaration at 94°C (30s), primer annealing at 58°C (30 s), and extension at 72°C (45s),

and a final extension of 10 min at 72°C. The PCR products were electrophoresed in 1% agarose gels in Tris-acetate EDTA followed by staining with ehidium bromide (Sambrook and Russell, 2001) and viewed in an UV transilluminator.

Cloning and sequencing

The amplified DNA was excised and eluted from the gel using QIAquick gel extraction kit (Qiagen Gmbh, Germany). The purified PCR product was ligated into pDrive cloning vector (Qiagen). Competent *Escherichia coli* (strain DH 5α) were transformed by standard molecular biology procedures (Sambrook and Russell, 2001). Recombinant clones were identified by colony PCR and also by restriction endonuclease digestion. The selected clones were sequenced at the automated DNA sequencing facility, Department of Biochemistry, Delhi University, South Campus, Delhi, India. Sequences of different isolates of ZYMV and PVY were obtained from GenBank (Table 2). Sequences were aligned using CLUSTAL W and comparison of aligned sequences was done with Bio Edit Sequence Alignment Editor (Hall, 1999).

Table 2. GenBank accession numbers of *Zucchini yellow mosaic virus* from different countries and *Potato virus Y* used for sequence analysis

Country	Host plant	Accesssion No.
Zucchini yellow mosaic virus		
India	*Lagenaria siceraria*	DQ298176 (present study)
Hungary	*Cucurbita pepo*	AJ251527
Pakistan	*Lagenaria siceraria*	AB127936
Poland	*Cucumis sativus*	AY347476
Japan	–	AB004641
Germany	–	AJ420019
China	–	AF513551
Korea	*Cucurbita moschata*	AY278998
Slovenia	–	AJ420018
Taiwan	*Cucurbita maxima*	AF127931
Italy	*Citrullus lanatus*	AY170323
USA-Connecticut	–	D00692
Israel	*Cucurbita pepo*	AY188994
USA-Florida	–	D13914
Singapore	–	X62662
Potato virus Y		
USA	–	NC-001616

RESULTS AND DISCUSSION

Symptomatology

Bottle gourd plants inoculated with the present virus isolate showed chlorotic spots, prominent veinal chlorosis followed by mosaic, vein banding and leaf distortion such as blistering and shoe-string similar to those observed in the field. No symptoms developed in control plants. Under field condition, the characteristic symptoms of the disease were severe mosaic, blisters, enations and filiformism on leaves. It also caused mosaic and distortion on fruits making them unmarketable. The symptoms resembles with those reported by Lisa *et al.* (1981).

Host range

Bio-assay revealed that the virus was easily transmitted to zucchini (*Cucurbita pepo*) a diagnostic assay host for ZYMV (a potyvirus). The virus could infect 10 out of 18 different hosts tested. The host range was mainly confined to the members of family Cucurbitaceae. *Chenopodium amaranticolor* exhibited chlorotic local lesions. No symptoms were observed on *Datura stramonium, Lycopersicon esculentum, Nicotiana benthamiana, Nicotiana glutinosa, N. tabacum* cv. White Burley, *Vigna unguiculata, Phaseolus vulgaris* (Table 1). All the inoculated plants were indexed by DAC-ELISA. All symptomatic plants gave positive reaction with ELISA and pumpkin supported maximum virus concentration (at 405 nm). The host range of the virus isolate is similar to those reported earlier in other cucurbits (Prieto *et al.*, 2001). The reactions of zucchini, muskmelon, pumpkin and *Chenopodium amaranticolor* to present isolate are similar to those induced by ZYMV (Lisa and Lecoq, 1984; Provvidenti *et al.*, 1984; Purcifull *et al.*, 1984). This also indicates that there is a very little variation in host range. ZYMV has been reported to produce symptomless infection on muskmelon, watermelon and cucumber (Wang *et al.*, 1992), but the present isolate produced systemic symptoms on all the three cucurbits. The virus under study produced very severe mosaic, filiformy and distortion of leaves like that of Connecticut isolate (Provvidenti *et al.*, 1984).

Insect transmission

The virus was transmitted in a non-persistent manner by *A. gossypii* and *M. persicae.* Symptoms induced in plants infected by aphid transmission were similar to those induced by mechanical inoculation of the virus on healthy bottle gourd plants. Maximum transmission of the virus (40%) was obtained by *A. gossypii.* However, transmissions of ZYMV isolate by other aphid species *viz. A. citricola* (Purcifull et al., 1984) and *A. middletrii, A. craccivora, Achyrthosiphon pisum, Lipaphis erysimi* and Uroleucon spp. (Brunt *et al.*, 1996) has also been reported.

Electron microscopy

Electron microscopy of the leaf dip preparation showed flexuous filamentous virus particles measuring 720-

760 nm in length similar to ZYMV isolate reported on zucchini (Singh *et al.*, 2003).

Serological reaction

The virus isolate reacted with antiserum of ZYMV and not with other antisera (PRSV, WMV-II, PVY and CMV). All the samples from field and glasshouse inoculated plants tested positive. Similar results have been reported by Davis (1986) and Desbiez and Lecoq (1997).

PCR amplification, cloning and sequencing

A product of expected size (~ 500 bp) was strongly amplified from the bottle gourd samples infected with ZYMV (Fig. 1). The nucleotide sequencing of the cloned PCR product showed that it was 497 bp long (GenBank Accession no. DQ298176). Sequence analysis indicated that it contained C- terminal part of coat protein (cp) gene and 211 bp long 3' untranslated region (UTR). The C-terminal cp region showed 100 % sequence identity with most of the isolates of ZYMV indicating that the C- terminal region of coat protein is also highly conserved in ZYMV (Table 3). However, sequence identity with C- terminal region of *Potato virus Y* was 68%, indicating that the present isolate is a distinct member of *Potyvirus*. 3'UTR region had sequence identity in the range of 88.6 % to 99.5% with different isolates of ZYMV and 26.1 % with *Potato virus Y* (Table 4). The sequence identity of C- terminal region of CP and 3'UTR with other isolates of ZYMV confirmed that the virus infecting bottle gourd is ZYMV.

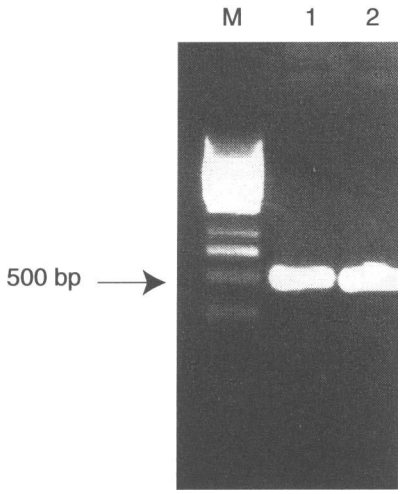

Fig. 1: RT-PCR amplification of C-terminal CP region and 3'UTR of ZYMV from leaves of bottle gourd. Lane M: 1kb DNA ladder, Lanes 1 & 2 amplified PCR product from diseased leaves of bottle gourd

Natural infection of ZYMV on some cucurbits in India has recently been reported (Raj Verma *et al.*, 2004 a, b). The introduction of ZYMV to India might have occurred through importation of infected seeds since ZYMV is seed transmissible in zucchini at a very low rate (Schrijnwerkers *et al.*, 1991). Based on particle morphology, stylet-borne aphid transmissibility, serological relationship, electron microscopy and sequence analysis of C-terminal coat protein and 3'UTR, the present virus isolate is a ZYMV.

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Table 3. Percent identity matrix of pair wise combination of amino acid sequence of C- terminal region of coat protein of different isolates of *Zucchini yellow mosaic virus* and Potato virus Y from different countries (details of GenBank accession numbers are given in table 2)

Sequence	DQ298176	AJ251527	AB127936	AY347476	AB004641	AJ420019	AF513551	AY278998	AJ420018	AY170323	AF127931	X62662	D13914	D00692	AY188994	NC_001616
DQ298176	100	100	100	100	100	100	100	100	100	100	100	98.9	100	100	100	68.0
AJ251527	—	100	100	100	100	100	100	100	100	100	100	98.9	100	100	100	68.0
AB127936	—	—	100	100	100	100	100	100	100	100	100	98.9	100	100	100	68.0
AY347476	—	—	—	100	100	100	100	100	100	100	100	98.9	100	100	100	68.0
AB004641	—	—	—	—	100	100	100	100	100	100	100	98.9	100	100	100	68.0
AJ420019	—	—	—	—	—	100	100	100	100	100	100	98.9	100	100	100	68.0
AF513551	—	—	—	—	—	—	100	100	100	100	100	98.9	100	100	100	68.0
AY278998	—	—	—	—	—	—	—	100	100	100	100	98.9	100	100	100	68.0
AJ420018	—	—	—	—	—	—	—	—	100	100	100	98.9	100	100	100	68.0
AY170323	—	—	—	—	—	—	—	—	—	100	100	98.9	100	100	100	68.0
AF127931	—	—	—	—	—	—	—	—	—	—	100	98.9	100	100	100	68.0
X62662	—	—	—	—	—	—	—	—	—	—	—	100	98.9	98.9	98.9	68.0
D13914	—	—	—	—	—	—	—	—	—	—	—	—	100	100	100	68.0
D00692	—	—	—	—	—	—	—	—	—	—	—	—	—	100	100	68.0
AY188994	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	68.0
NC_001616	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100

Table 4. Percent nucleotide sequence identity matrix of pairwise combination of 3'UTR region of different isolates of *Zucchini yellow mosaic virus* and *Potato virus Y* from different countries (details of GenBank accession numbers are given in table 2)

Sequence	DQ298176	AJ251527	AB127936	AY347476	AB004641	AJ420019	AF513551	AY278998	AJ420018	AY170323	AF127931	X62662	D13914	D00692	AY188994	NC-001616
DQ298176	100	99.0	99.0	98.5	93.3	88.6	96.6	98.1	89.5	94.3	96.2	97.6	96.2	96.2	99.5	26.1
AJ251527	—	100	99.0	99.5	94.3	89.5	97.6	98.1	90.5	94.3	97.1	97.6	97.1	97.1	99.5	25.8
AB127936	—	—	100	98.5	93.3	88.6	97.6	98.1	89.5	94.3	96.2	97.6	96.2	96.2	99.5	25.8
AY347476	—	—	—	100	93.8	89.0	97.1	98.5	90.0	93.8	96.6	97.1	96.6	96.6	99.0	25.5
AB004641	—	—	—	—	100	94.0	91.9	92.4	95.0	95.0	91.4	91.9	91.4	91.4	93.8	24.3
AJ420019	—	—	—	—	—	100	87.2	87.6	97.4	90.1	86.7	87.2	86.7	86.7	89.0	22.2
AF513551	—	—	—	—	—	—	100	95.7	88.1	94.7	95.2	95.2	97.6	98.5	97.1	27.0
AY278998	—	—	—	—	—	—	—	100	88.6	93.3	95.2	96.6	95.2	95.2	98.5	25.5
AJ420018	—	—	—	—	—	—	—	—	100	91.1	87.6	88.1	87.6	87.6	90.0	22.5
AY170323	—	—	—	—	—	—	—	—	—	100	91.9	92.8	94.3	94.3	94.7	26.1
AF127931	—	—	—	—	—	—	—	—	—	—	100	96.6	95.7	95.2	96.6	25.3
X62662	—	—	—	—	—	—	—	—	—	—	—	100	96.6	95.7	98.1	24.9
D13914	—	—	—	—	—	—	—	—	—	—	—	—	100	98.1	96.6	26.4
D00692	—	—	—	—	—	—	—	—	—	—	—	—	—	100	96.6	26.7
AY188994	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100	25.8
NC_001616	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100

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