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Full Length Article

Biological and Molecular Characterization of *Cucumber green mottle mosaic virus* Affecting Bottle Gourd and Watermelon Plants in Saudi Arabia

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Abstract

Samples were collected from seven of each bottle gourd and watermelon plants grown in Riyadh and Hail regions of Saudi Arabia in May, 2012 showing symptom suspected to be virus infection and analyzed by a double antibody sandwich against bottle gourd and watermelon viruses. Two of watermelon and three of bottle gourd samples were found to be positive with *Cucumber green mottle mosaic virus* (CGMMV). However, variable results were obtained with other viruses infecting cucurbits. One sample of CGMMV positive samples from each region were homogenized in a mortar separately and used to make mechanical inoculation on 25 plant species which produced characteristic symptoms of tobamo virus infection. Five ELISA-positive samples from naturally infection were further tested and detected by RT-PCR assay using virus specific primers that amplified a 400 bp fragment in the coat protein gene. The nucleotide sequence of the PCR products from the Saudi isolates of CGMMV isolated from bottle gourd and watermelon were determined and demonstrated its similarity to the other isolates of CGMMV recorded in NCBI. The CP gene of CGMMV-Saudi Arabia isolates shared between 87.2% and 99.7% identity in nucleotide level. The synthesized cDNA probe for CGMMV detection was hybridized with nucleic acid extracts from infected samples collected from the above mentioned locations**.** This is the first report regarding the genetic makeup of CGMMV at the molecular level in Saudi Arabia. © 2015 Friends Science Publishers

Keywords: CGMMV; ELISA; Indicator plants; RT-PCR; Hybridization; Sequence

Introduction

The main cultivated cucurbit species (cucumber (*Cucumis sativus* L.), watermelon (*Citrullus lanatus* L.), squash, (*Cucurbita* sp.), bottle gourd (*Lagenaria siceraria*), and melon (*C. melo* L.) are important vegetable crops worldwide, especially in developing countries but are also subjected to more than 200 plant diseases.

Among these disease agents, viruses are most difficult to control and their aggressiveness depends on the host pathogen relationship and the impact of vector in the spread. Cucurbits have been reported to be infected with as many as 60 plant viruses worldwide (Nameth *et al*., 1986; Provvidenti, 1996; Zitter *et al*., 1996; Lecoq and Desbiez, 2012), at least 28 different viruses in were reported in Mediterranean region (Lecoq and Desbiez, 2012) and 15 of which were reported in Saudi Arabia (Al-Shahwan, 2003). Precise diagnosis has the prime importance in developing a management strategy of this virus. All these viruses have similar host range and thus cannot be used as a clue for the identification of the virus (Antignus *et al.*, 2001; Yoon *et al.*, 2002). CGMMV was originally described in the UK 1935 (Ainsworth, 1935). Thereafter reports were made in USA and Canada (Tian *et al.*, 2014; Ling *et al.*, 2014); Saudi Arabia (Al-Shahwan and Abdalla, 1992; Al-Saleh and Al-Shahwan, 1997); Iran (Moradi and Jafarpour, 2011), China and Greece (Zhang *et al.,* 2009; Varveri *et al.,* 2002), Korea and Myammar (Yoon *et al.,* 2008; Kim *et al.,* 2010), and in the Ukraine (Budzanivska *et al.,* 2007). Several strains of CGMMV have been reported from Europe, Pakistan, India and Japan (Komuru *et al.*, 1968; Lee, 1996; Antignus *et al.,* 1990; Francki *et al.*, 1986; Ugaki *et al.*, 1991; Shim *et al.*, 2005; Ko *et al.*, 2006). CGMMV is mechanically and seed transmitted with a narrow host range comprising of cucurbits only and is different in host range from *Tobacco mosaic virus* (TMV) whose main hosts are members of the solanaceae (Antignus *et al.*, 1990, 2001). CGMMV is a rod-shaped, microscopic (300 nm long x 18 nm wide) particle. The virus is easily sap and seed transmissible, and it survives for long periods in infected crop debris. This study aims to identification and characterization of CGMMV virus

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that was found infecting bottle gourd and watermelon grown in Riyadh and Hail regions, Saudi Arabia.

Materials and Methods

Source of samples

Seven suspected plants samples of each bottle gourd (*L. siceraria*), and watermelon (*C. lanatus*) showing a mottling and mosaic symptoms were collected from Riyadh and Hail regions, Saudi Arabia in May, 2012. Samples were tested by DAS- ELISA (Agdia Inc., France and AC diagnostic, USA) as described by Clark and Adams, 1977, to detect the important cucurbits viruses infecting (*Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* (WMV), *Alfalfa mosaic virus* (AMV) *Zucchini yellow mosaic virus* (ZYMV) and CGMMV. One sample of CGMMV positive samples from each region isolated from bottle gourd and watermelon were homogenized in a mortar separately, after adding potassium phosphate buffer (0.01 M containing 0.1% sodium sulphite (Na_2SO_3), pH 7.2 and applied on leaves of selected host range previously dusted with 600-mesh carburandum using the index finger according to Hill, 1984. Twenty five plant species were used in this test including: muskm*elon* (*Cucumis melo cv*. Russian), cucumber (*Cucumis sativus* cv. Beit Alpha), pumpkin (*Cucurbita pepo* subsp. *pepo* cv. Connecticut Field*, C. lanatus cv.* Sugar Baby*, L. siceraria,* Okeechobee gourd (*Cucurbita okeechobeensis*), *Chenopodium amaranticolor* Cost and Reyn, *petunia hybrid*, Squash (*C. pepo* cv. Vegetable Marrow), *Datura stramonium*, *Nicotiana tabacum cvs.* Samsun and Xanti-nc, *N. glutinosa*, *N. benthamiana*, *N. rustica* L.*, Solanum nigrum* L.*, Vicia faba* L., *Raphanus sativs* L.*, Brassica oleracea* L.*, Beta vulgaris* L*, Phaseolus vulgaris* cv. Black Turtel 2, *Pisum sativum cv. Alska*, *Capsicum annuum* L., *Solanum lycopersicum* and *Luffa acutangula.* Biological purification was carried out according to Kahn and Monroe, (1963) using *C. amaranticolor* as a local lesion host, whereas bottle gourd and watermelon were used as propagative host for the following experiments. Symptoms were recorded after three weeks at regular intervals and confirmation of viral infection was made through serological and molecular techniques. All plants showing no symptoms were assayed for virus infections by back inoculation of *C. pepo, C. melo, C. sativus, C. lanatus* and *C. amaranticolor* and by DAS-ELISA.

Reverse Transcription-polymerase Chain Reaction (RT-PCR) Detection

Five samples out of twelve CGMMV ELISA positive samples showing sever mottling and varying degree of mosaic were chosen as representative of the two main crops in Riyadh and Hail regions for molecular characterization. Total RNA was extracted from infected and uninfected bottle gourd and watermelon leaf samples using ISOLATE Plant RNA Mini Kit (BioLine Ltd, London, United Kingdom). The oligonucleotide primer (105 and 106) and RT-PCR condition were performed as described by (Varveri *et al.*, 2002) using one step RT-PCR kit (Qiagen) in a Thermal cycler, Mastercycler® None gradient (Eppendorf, Germany). RT-PCR products were analyzed by electrophoresis in 1% agarose gel, visualized by ethidium bromide staining and 50 bp DNA ladder (Cat No. G4521, Promega) was used to determine the size of RT-PCR amplified products (Sambrook and Russell, 2001).

Nucleotide Sequence and Analysis of CP Gene of the Saudi Isolates of CGMMV

Amplified DNA fragments of expected size (400 bp) of partial coat protein gene of each isolate (RT-PCR positive samples) were purified using QIAquick Gel Extraction Kit (Qiagean). An RT-PCR product amplified by the CP gene-specific primer of CGMMV-CP was cloned in pGEM-T easy vector (Promega Co). The nucleotide sequences were determined using an Applied Biosystems AB3730xI DNA Analyzer (Life Technologies Corporation, Carlsbad, California, USA) in the King Faisal Specialist Hospital Research Centre, Riyadh, KSA. The determined sequences of CGMMV isolates were subjected to a Blast-W search for comparison 25 previously reported CGMMV sequences, one of each of CFMMV, ZGMMV and KGMMV isolates at the GenBank databases as shown in Table 1. A phylogenetic tree and sequence homology were constructed using Laser gene DNASTAR, V5-05.

Dot Blot Hybridization Assay

The obtained RT-PCR product (400 bp) of partial CP gene of CGMMV-isolated from bottle gourd in Riyadh region were purified using QIAquick Gel Extraction Kit (Qiagean) and directly labeled with digoxigenin (DIG) labeling system according to (Feinberg and Vogelstein, 1983; Holtike and Kessler, 1990). Sap extractions were prepared from all collected samples from bottle gourd and watermelon by grinding according to (Laulhere and Rozier, 1976; Podleckis *et al.*, 1993). The DNA was fixed on the membranes by Ultraviolet cross-linked for 30 sec. Prehybridization, hybridization and immunological detection were carried out using the DIG DNA labeling and Detection kit (Roche Diagnostics) according to the manufacturer`s recommendation.

Results

Virus Isolate and Host Range

From Riyadh and Hail regions, twelve samples were detected to be positive by DAS-ELISA and five showing sever mottling and varying degree of mosaic were

confirmed to be CGMMV positive by RT-PCR and nucleotide sequence. These samples including two samples collected from bottle gourd and one sample collected from watermelon in Riyadh region were designated as CGMMV-SA-B1; CGMMV-SA-B2, and CGMMV-SA-W1 and two samples collected from each bottle gourd and watermelon in Hail region were designated as CGMMV-SA-B3, CGMMV-SA-W2, respectively. All five Saudi CGMMV samples produced systemic virus infection with similar symptoms on virus-inoculated *C. melo* cv. Russian, *C. sativus* cv. Beit Alpha, *C. pepo* subsp. *pepo* cv. Connecticut Field, *C. lanatus* cv. Sugar Baby, *L. siceraria* and *C. okeechobeensis,* while chlorotic local lesions developed on inoculated leaves of *C. amaranticolor*, and necrotic local lesions were recorded on *P. hybrid* (Fig. 1). But no evidence of infection was detected in *C. pepo* cv. Vegetable Marrow, *D. stramonium*, *N. tabacum* cvs. Samsun and Xanti-nc, *N. glutinosa*, *N. benthamiana*, *N. rustica* L., *S. nigrum* L., *V. faba* L., *R. sativs L., B. oleracea* L., *B. vulgaris* L, *P. vulgaris* cv. Black Turtel 2, *P. sativum* cv. Alska, *C. annuum* L. Symptomless systemic was observed in *S. lycopersicum* and L. *acutangula.* However, variable results were obtained when the samples were tested for the presence of other viruses including SqMV, CMV, WMV, AMV and ZYMV infecting cucurbits.

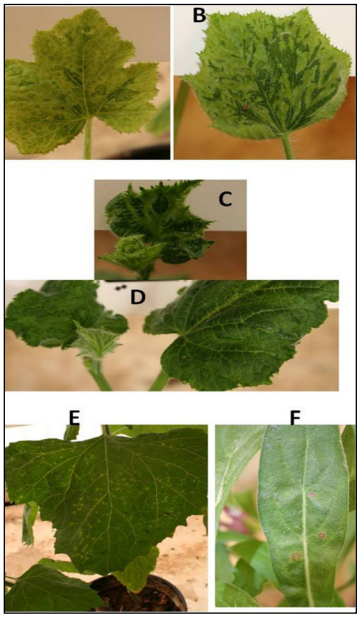

Fig. 1: Symptoms of CGMMV on some indicator plants developed in response to inoculation by the Saudi isolates of CGMMV in greenhouse condition. (A) and (B): Sever mottle on bottle gourd (*L. siceraria)*, (C) and (D): Severe mosaic and blistering on cucumber (*C. sativus*), *(*E): chlorotic local lesion on *Ch. amaranticolor,* (F): Necrotic local lesion on *P. hybrid*

RT-PCR

The RT-PCR amplified product (400 bp) of CGMMV-CP from all five isolates infecting with CGMMV isolated from bottle gourd (SA-B1 and SA-B2), watermelon (SA-W1) plants, in Riyadh regions (lane 1, 2 and 3), CGMMV isolated from bottle gourd (SA-B3) and watermelon (SA-W2) plants lanes (4 and 5) in Hail regions were obtained by using specific primer for CGMMV-CP gene, but no amplification from uninfected bottle gourd plants (lane 6) (Fig. 2).

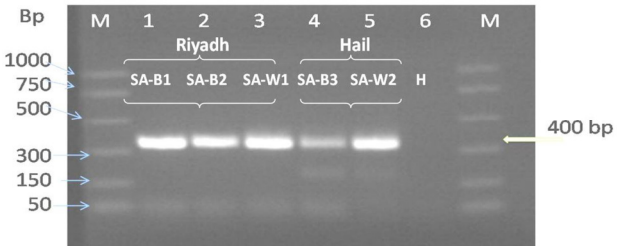

Fig. 2: Agarose gel electrophoresis (1 %) for RT-PCR amplified products (400 bp fragment) of the coat protein gene of CGMMV-SA isolates using specific primers. CGMMV isolated from bottle gourd (SA-B1 and SA-B2) and watermelon(SA-W1) plants, lanes (1, 2 and 3) from Riyadh region, CGMMV isolated from bottle gourd (SA-B3) and watermelon (SA-W2) plants lanes (4 and 5) from Hail regions. No RT-PCR amplified product was observed with uninfected watermelon leaf (Lane 6). Arrow indicates PCR products of 400 bp. Lan M: 50 bp PCR DNA marker (promega)

Generation of cDNA Probe and Dot Blot Hybridization for Detection of CGMMV

A cDNA probe for CGMMV was synthesized using the 106 and 105 primer pair. Fig. 3 illustrate dot blot hybridization of total RNA extraction from naturally symptomatic bottle gourd and watermelon plant samples collected from Riyadh and Hail regions. (A), but no hybridization between the cDNA probe and nucleic acid extraction from uninfected bottle gourd plants (Lane B: 1). (Fig. 3).

Sequence Analysis of the 400 bp Fragment of CGMMV-CP Gene

The DNA nucleotide sequences for the partial CP gene of the five Saudi CGMMV isolates from bottle gourd and watermelon plants from Riyadh and Hail regions have been determined. These five CGMMV sequences were compared with the already reported sequences in the Gene Bank by developing a phylogenetic tree of CGMMV, which showed that Saudi isolates belong to four clusters, while make 2 clusters when compared to other cucurbit viruses like CFMMV, ZGMMV and KGMMV (Fig. 4 and 5).

The phylogenetic tree was determined for the Saudi as well as the other CGMMV isolates using the DNASTAR program. Comparing the nucleotide sequences for the five

Table 1: Comparison of the nucleotide sequence identity of the coat protein gene of the Saudi isolates of CGMMV with those of other strains of the virus deposited in GenBank

ACCESSION No.	CGMMV Saudi isolates					Isolate/Strain	Original host	Country
	CGMMV-SA-B1	CGMMV-SA-B2	CGMMV-SA-B3	CGMMV-SA-W1	CGMMV-SA-W2			
CGMMV-SA-B1	100%	98.2%	98.2%	95.1%	95.1%	SA-B1	*L. siceraria*	Saudi Arabia
CGMMV-SA-B2	98.2%	100%	100%	95.4%	95.4%	SA-B2	*L. siceraria*	Saudi Arabia
CGMMV-SA-B3	98.2%	100%	100%	95.4%	95.4%	SA-B3	*L. siceraria*	Saudi Arabia
CGMMV-SA-W1	95.1%	95.4%	95.4%	100%	100%	SA-W1	*C. lanatus*	Saudi Arabia
CGMMV-SA-W2	95.1%	95.4%	95.4%	100%	100%	SA-W2	*C. lanatus*	Saudi Arabia
NC-001801	93.6%	95.4%	95.4%	98.8%	98.8%	"SH"	-	Japan
AB127937	93.9%	93.9%	93.9%	97.9%	97.9%	Pak	*L. siceraria*	Pakistan
AB369274	95.1%	94.2%	94.2%	98.2%	98.2%	Watermelon	*C. lanatus*	South Korea
AB447984	95.1%	95.7%	95.7%	99.1%	99.1%	KM4	*L. siceraria*	South Korea
AB447985	95.1%	95.7%	95.7%	99.1%	99.1%	KM7	*L. siceraria*	South Korea
AB510355	95.1%	95.4%	95.4%	99.4%	99.4%	M17C	*L. siceraria*	Myanmar
AF225984	95.1%	95.7%	95.7%	99.1%	99.1%	CGMMV-W(K)	*C. lanatus*	Korea
AF417243	94.8%	95.7%	95.7%	98.8%	98.8%	KOM	*C. melo*	Korea
AJ243831	94.5%	95.4%	95.4%	98.8%	98.8%	CGMMV-NS	-	Korea
AJ429090	93.3%	94.8%	94.8%	98.8%	98.8%	-	-	France
AJ459422	95.1%	92.7%	92.7%	93.0%	93.0%	GR5	*C. lanatus*	Greece
AJ459423	95.4%	95.4%	95.4%	99.4%	99.4%	GR7	*C. lanatus*	Greece
AJ748352	94.2%	95.7%	95.7%	99.7%	99.7%	AL1	*L. siceraria*	India
DQ767636	93.3%	94.5%	94.5%	99.1%	99.1%	-	*L. siceraria*	India
EF521882	93.9%	92.7%	92.7%	93.6%	93.6%	MC-1	*C. sativus*	Russia
EU366912	94.8%	94.2%	94.2%	97.9%	97.9%	Bangalore	-	India
FJ654658	94.5%	95.4%	95.4%	98.8%	98.8%	C	*C. sativus*	Taiwan
GQ500898	94.5%	95.1%	95.1%	98.5%	98.5%	SDAU-TANG	*C. pepo*	China
HM008919	94.5%	95.1%	95.1%	98.5%	98.5%	CCMV-TANG	*C. pepo subsp. pepo*	China
HM363013	94.5%	95.1%	95.1%	98.5%	98.5%	Yunnan	*C. lanatus*	China
HQ329106	93.6%	93.9%	93.9%	98.5%	98.5%	Kalat	*C. sativus*	Iran
HQ692886	95.1%	95.7%	95.7%	99.1%	99.1%	CGMMV11	*L. siceraria*	Taiwan
JF432067	87.2%	87.5%	87.5%	90.9%	90.1%	Wh1	*Zea maize*	China
JF838188	94.8%	95.4%	95.4%	98.8%	98.8%	Byungchun	*C. sativus*	South Korea
JN605350	94.8%	95.4%	95.4%	98.8%	98.8%	GD-GZ	C. sativus	China
NC-002633	49.4%	49.4%	49.4%	48.8%	48.8%	CFMMV	-	Israel
NC-003878	52.5%	52.5%	53.6%	52.6%	52.6%	ZGMMV-K	-	South Korea
AB015145	50.9%	50.9%	52.1%	51.0%	51.0%	KGMMV-Yodo	-	Japan

Saudi isolates indicated similarity between them that ranged between 95.1% and 100.0%. Similarity was also found between the five Saudi Arabian isolates and the remainder of the CGMMV isolates that were obtained from GenBank that ranged between 87.2.0% and 99.7%. Similarity was found between Saudi isolates SA-W1 and SA-W2 isolates and the rest of the other CGMMV isolates in a range of 90.1% to 99.7% identical for them. The results showed that the nucleotide sequence of CGMMV-SA-W1 and W2 isolates shared a high sequence identity (99.7%) with CGMMV–AL1 isolate isolated from *L. siceraria* from India (AJ748352), (99.4%) with M17C and GR7 from Myanmar (AB510355) and Greece (AJ459423), 99.1% with CGMMV-W (K) from Korea (AF225984), KM4 and KM7 from South Korea (AB447984 and AB447985) and CGMMV11 from Taiwan (HQ692886) while, low sequence identities (93% to 98.5%) were observed between our isolates and other isolates of CGMMV isolated from different countries (Table 1). The results showed that the nucleotide sequence of CGMMVSA-B1, SA-B2 and SA-B3 isolates shared a hight sequence identity (95.7%) with CGMMV–KM4, KM7 isolated from *L. siceraria* from South Koreaa and CGMMV-WK and KOM isolates isolated from *C. lanatus* and *C. melo* respectively from Korea. Low sequence identities (92.7% to 98.5%) were served with other isolates of CGMMV from isolated from different countries. Sequence homology with ZGMMV, KGMMV and CFMMV was lowest at about 48.8 to 53.6%. The nucleotide sequences of the partial CP genes of CGMMV–SA (CGMMV-SA-B1, CGMMV-SA-B2, CGMMV-SA-B3, CGMMV-SA-W1 and CGMMV-SA-W2) isolate (GenBank Accession No., KC295228, KC295229, KC295230, KC295231 and KC295232) respectively (Table 1).

Discussion

CGMMV has been reported from few Mediterranean countries: France, Greece, Israel, Spain, and Syria (Avgelis and Vovlas, 1986; Antignus *et al.*, 1990; Blancard *et al.*, 1994; Celix *et al.*, 1996; Varveri *et al.*, 2002; Kassem *et al.*, 2005). In Saudi Arabia, a severe infection outbreak was observed on watermelon and bottle gourd crops (Al-Shahwan and Abdalla, 1992; Al-Saleh and Al-Shahwan, 1997). The tobamovirus, CGMMV causes severe disease of cucurbits worldwide. The typical symptoms in watermelon

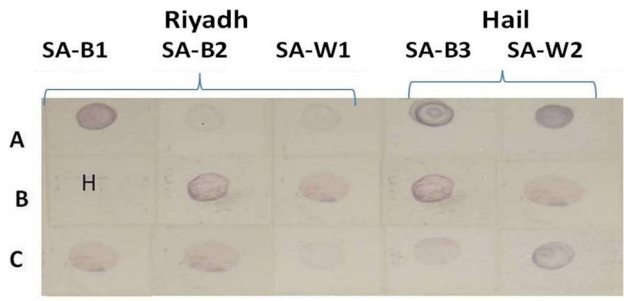

Fig. 3: Dot blot hybridization for detection of CGMMV-SA isolated from bottle gourd (SA-B1 and SA-B2), and watermelon plants (SA-W1) from Riyadh region, bottle gourd (SA-B3) and watermelon (SA-W2) plants from Hail region. No hybridization reaction was observed with uninfected watermelon leaves (Lane B: H)

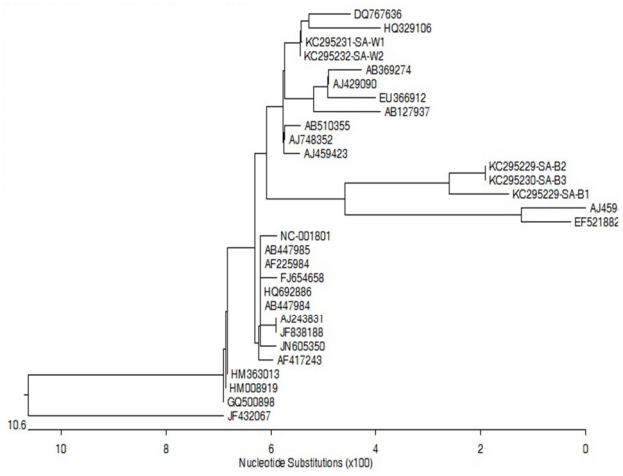

Fig. 4: Phylogenetic tree constructed from the alignment of nucleotide sequences of coat protein gene between CGMMV Saudi isolates and 25 CGMMV isolates, obtained from the GenBank database using Laser gene DNASTAR, V5-05

including leaf mottling and mosaic have been reported in greenhouse-grown plants (Varveri *et al.*, 2002; Shim *et al.*, 2005), but these symptoms are masked in plants growing in open fields (Reingold *et al.*, 2013). Serological testing by ELISA of a large number of fruits exhibiting systemic mosaic and mild to severe yellow mottling symptoms, confirmed the presence of CGMMV. Differences were observed among CGMMV isolates in their response to differential hosts (Boubourakas *et al.*, 2004). Serological and molecular variability has also been reported between European and Asian isolates.

Immunodiffusion test in agar plates was also used for detection of CGMMV isolated from infected bottle gourd plants using specific antiserum (Alshahwan and Abdalla, 1992). Although all samples showed systemic mosaic and mild to severe yellow mottling symptoms, only 41.6% of

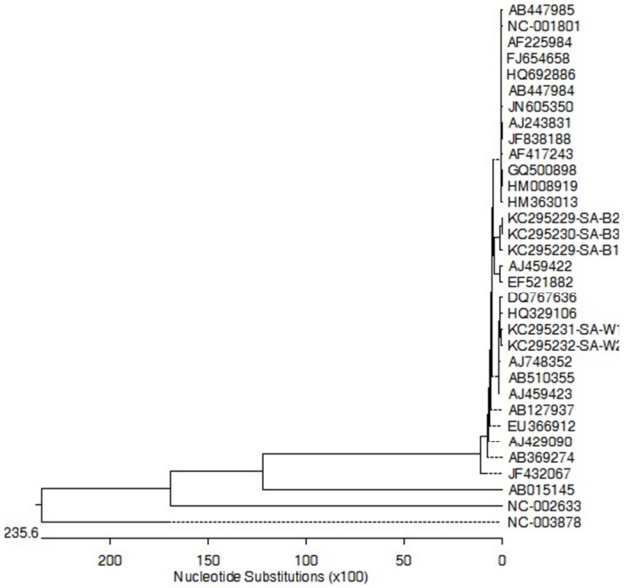

Fig. 5: Phylogenetic tree constructed from the alignment of nucleotide sequences of coat protein gene between CGMMV Saudi isolates and 25 CGMMV isolates, one of CFMMV, two of ZGMMV and three of KGMMV isolates obtained from the GenBank database using Lasergene DNASTAR, V5-05

samples were infected with CGMMV. This percentage might be due to the fact that mottle mosaic is a complex disease that can be caused by different viruses such as CMV, WMV-2, SqMV, ZYMV, as well as by CGMMV (Shabanian *et al.*, 2007; Moradi and Jafarpour, 2011) and the symptoms observed are as a result of multiple infection.

Several molecular techniques have been tested, for the identification of CGMMV. Among those detection methods, RT-PCR (Ugaki *et al.*, 1991; Shim *et al.*, 2005; Yoon *et al.*, 2008; Liu *et al.*, 2009), real time RT-PCR (Chen *et al.*, 2008), transmission electron microcopy (Alshahwan and Abdalla, 1992), immune capture-RTPCR (Celix *et al.*, 1996), ELISA (Antignus *et al.,* 1990; Celix *et al.*, 1996; Mitsuhiro *et al.*, 2006) and monoclonal antibodies (Antignus *et al.*, 2001). To establish procedure of identification and detection, mechanical inoculation, DAS-ELISA, RT-PCR, molecular hybridization and nucleotide sequence were applied in this study. The present study demonstrates the successful use of RT-PCR and sequencing to directly detect CGMMV in infected watermelon and bottle gourd plants for the first time in Saudi Arabia.

A non-radioactive dot-blot hybridization technique have been used to detect several plant viruses and have been shown to be more sensitive and more specific than serology (Hseu *et al.*, 1987; Hahm *et al.*, 1993; James *et al.*, 1999; Gioconda *et al.*, 2000; Lee *et al.*, 2001; Liu *et al.*, 2007).

In conclusion, five CGMMV isolates affecting bottle gourd and watermelon plants in Saudi Arabia were

determined based on biological reactions, RT-PCR, partial nucleotide sequences of CP gene and demonstrated its similarity. Data shown in this study represent the first report on the characterization of CGMMV at the molecular level in Saudi Arabia and will be helpful in developing effective control strategies for this virus.

Acknowledgments

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