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Detection and Characterization of *Cucumber mosaic virus* Infecting Ginger (*Zingiber officinale* Roscoe) in Malaysia

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Abstract

Ginger (*Zingiber officinale* Rosc.) is herbaceous crop belonging to the family *Zingiberaceae* and cultivated for its medicinal properties and as marketable spice. A study was carried out to investigate *Cucumber mosaic virus* (CMV) infection in Malaysian ginger plants, showing conspicuous symptoms of mosaic, stripping and yellowing. A total of 45 symptomatic and 15 non symptomatic ginger samples were collected from three States in Malaysia. Reverse-transcription polymerase chain reaction (RT-PCR) analysis using CMV specific primers showed 14 out of 60 samples were positive for CMV. Cloning and sequence analyses of the 500 bp amplicons revealed that the CMV isolates obtained were closely related to CMV isolates from China (tomato) and Thailand (cucumber) with 95% to 96% sequence similarity respectively. Phylogenetic analysis placed the Malaysian CMV isolates into Subgroup IB. This is the first report of CMV infecting ginger in Malaysia.

Keywords: Ginger; *Cucumber mosaic virus;* Detection; Virus; *Zingiberaceae;* RT-PCR.

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1. Introduction

Ginger (*Zingiber officinale* Rosc.) is native to Asia but is now cultivated worldwide. It is of culinary and medicinal importance and it is among the most valued and widely cultivated spice crops worldwide and serve as a source of revenue to Malaysia [1]. A survey of ginger farms in three different states in Malaysia showed the presence of virus-like symptoms such as mosaic, striping and yellowing. There were reports of two virus infecting ginger, *Ginger mosaic virus* (GMV) [2] and *Ginger chlorotic fleck virus* (GCFV) [3]. GMV was serologically related to *Cucumber mosaic virus* (CMV) and it remained unclear whether it was really CMV, as serology alone could not be reliably used to classify it as CMV, adding to the fact that there has not been any detail characterization of the perceived CMV on ginger since the [2] investigation. The CMV is a type species of the genus *Cucumovirus,* with single-stranded tripartite RNA genome. It has the widest host range of all known plant viruses. CMV infections can result in up to 30 % yield loss and it is among the top 10 most important plant viruses in the world [4,5,6]. Although CMV has been reported in many plant species in Malaysia but none was on CMV infection in ginger, despite the apparent viral symptoms observed on the crop across the country. This paper describes the identification and characterization of CMV from ginger expressing mosaic, striping and yellowing symptoms.

2. Materials and methods

2.1 Sampling and Nucleic acid extraction

Twenty samples comprising symptomatic (15) and non-symptomatic (5) ginger leaf samples were collected from three Malaysian States of Pahang, Selangor and Perak. Th symptoms encountered were mosaic, striping and yellowing (Figure 2), with the symptoms covering the entire infected leaf in most cases. The total nucleic acid was extracted using CTAB extraction buffer modified by [7].

2.2 Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR)

Reverse-transcription was carried out using AMV-RT reverse transcriptase system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was conducted by using CMV-specific primers [8]. The reaction was subjected to the following thermal cycling regimes; initial denaturation 94°C for 5 min, then 35 cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 60 s and 10 min of final extension at 72°C before holding the reaction at 10°C in a Thermocycler (T Personal Biometra, Germany) before resolving the PCR product on 2 % agarose gel (1st Base, Singapore) prepared and run with 1× TBE buffer (1st Base, Singapore) and visualized using Gel documentation system (Gel Doc XR, BioRad, USA) and the image captured.

2.3 Cloning, sequencing and sequencing analysis

The amplicons (500bp) were cloned using pCR^{TM} 2.1-TOPO vector (Invitrogen, CA, USA) and then sequenced (Apical Scientific, Sdn. Bhd. Malaysia). The clone sequences were compared with the virus sequences available in the GenBank database using BLASTn and representative sequence was deposited to the National Centre for

Biotechnological Information (NCBI) repository. Phylogenetic tree was constructed using MEGA 7 [9] by Neighbour-joining model with 1000 bootstrap replication using some selected subgroup IA, IB and II CMV members with *Tomato aspermy virus* (TAV) as an outgroup *Cucumovirus* member (Table 1).

2.4 Pathogenicity test

Pathogenicity of the CMV isolate was confirmed by mechanically inoculating ten tissue-cultured ginger seedlings at two-leaf stage using sap from a RT-PCR positive sample homogenized in phosphate buffer pH 7.4 and monitored for symptom development for five months and further confirmatory detection by RT-PCR assay. To rule out possible mixed infection with GCFV, RT-PCR was carried out to detect the virus using the procedure by [10].

3. Results and discussion

This study confirmed the presence of CMV through RT-PCR assay and sequencing thus corroborating a previous study that showed a virus isolated from ginger plants was positive to CMV antiserum [2]. CMV was detected from 14 ginger leaf samples by RT-PCR assay, which produced an amplicon of 500 bp of the CMV CP gene (Figure 1). The 14 samples tagged as FG, BT and TM, were sequenced and found to be homologous to one other, denoting the presence of a single CMV strain on Malaysian ginger. All 14 samples were 96 % and 95 % similar in nucleotide sequence to a Thailand (cucumber: AJ810264) and China (tomato: KX525736) CMV isolates respectively, both belonging to CMV subgroup IB, a strain confined to Asian region [11,12]. In addition, the phylogenetic analysis classified the CMV isolates obtained in this study into the subgroup I clade and specifically into subgroup IB members (Figure 3). Hence, corroborating the nucleotide sequence placement of our isolates to be closely related to CMV subgroup IB members. The nucleotide similarity with the Thailand and the Chinese strains might possibly be due the geographical location of the virus strain and the proximity between the countries. The rapid increase in the global exchange of vegetative plant propagules between regions and among the countries coupled with the fast evolution and adaptation of plant viruses in new hosts and environments could have brought the possibility of inter plant family transmission of CMV [13]. Other factors that might aided the occurrence of the viruses include, change in climate, that leads to shift in hosts and vectors population and distribution, and finally rapid evolution and adaptation of plant viruses [14]. Furthermore, host range of CMV has been expanding, year in, year out, as it remains the virus with the widest host range among all the known plant viruses [12,15,16]. Recently, CMV was isolated from a *Zingiberaceae* member, siam tulip (*Curcuma asmatifolia*) in the US, thus, pointing to the possible occurrence of the virus on other *Zingiberaceae* family members [15]. Prior to our study, CMV has been reported in Malaysia by previous researchers on other plant hosts, such as tomato, pepper and periwinkle [17]. Pathogenicity of the CMV isolate was confirmed with the appearance of mosaic, stripping and chlorosis symptoms in the inoculated ginger seedlings after five months. The presence of CMV was confirmed by RT-PCR assay, hence, proving the Koch's postulate (Figure 1). Thus, CMV was the causal agent of mosaic symptoms observed on ginger plants in Malaysia and its detection will pave a way for designing proper disease management strategies, such as seed certification, quarantine and breeding for resistance as and when due. In addition, whole genome sequencing would have revealed a more detailed and comprehensive character of the CMV isolated in ginger.

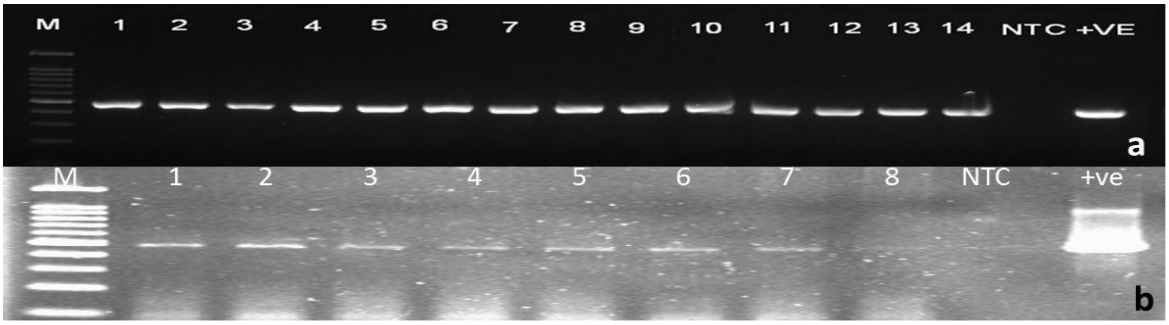

Figure 1: a) RT-PCR assay for the detection of CMV in ginger from three Malaysian States using 2 % agarose gel electrophoresis. 1 –6; Selangor State 7 – 10; Pahang 11 – 14; b) Pathogenicity test of CMV against ginger plants showing 500 bp CMV CP of expected amplicon M; 100 bp DNA marker NTC; No-template control+Ve: Positive control.

Figure 2: Symptoms of sampled ginger plants. a) Mosaic symptom developed on sample TM 1 when it was 6-month-old at Tanjung Malim b) Stripping symptom on BT 3 at 4-month-old at Bukit Tinggi c) Chlorosis and vein banding on FG 3 at 2-month-old at the faculty of Agriculture, UPM Selangor d) Healthy ginger plant at 4-month-old. Arrows indicate the symptom position.

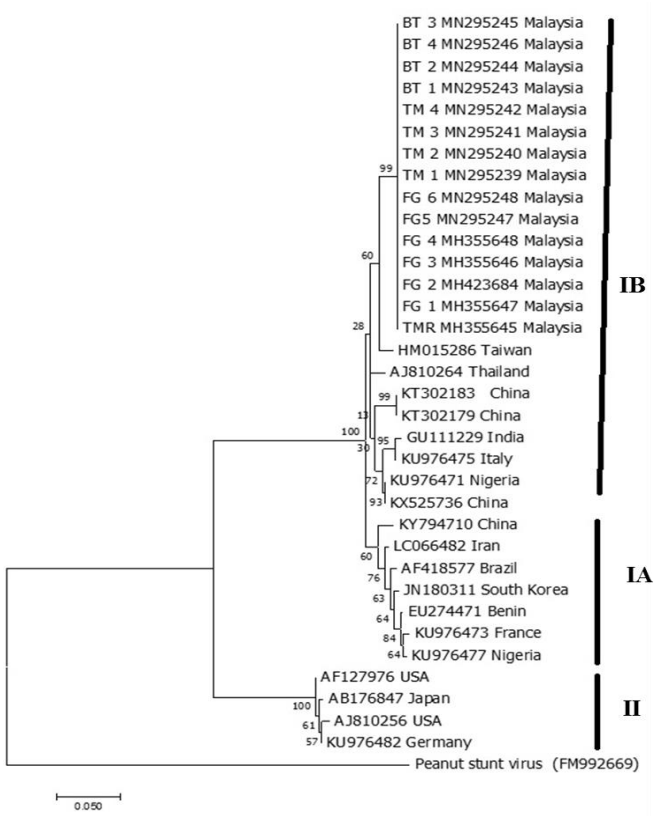

Figure 3: Phylogenetic tree showing the relationship between Malaysian CMV isolates and others inferred by Neighbour-Joining with 1000 bootstrap replicates with *Peanut stunt virus* (PSV) as out group *Cucumovirus* member.

Table 1: Sequences of *Cucumber mosaic virus* used for phylogenetic analysis from GenBank and those starting with KU in accession and PV in the strain name, were adopted from [12]

Strain	Accession number	Subgroup	Location
Met	KY794710	IA	China
IRN-TVRa26	LC066482	IA	Iran
-	AF418577	IA	Brazil
ZM	JN180311	IA	South Korea
CMV yam	EU274471	IA	Benin
PV-0475	KU976473	IA	France
PV-0445	KU976477	IA	Nigeria
BB8	HM015286	IB	Taiwan
TR15	AJ810264	IB	Thailand
DN8-3	KT302183	IB	China
DN7-1	KT302179	IB	China
New Delhi	GU111229	IB	India
PV-0473	KU976475	IB	Italy
PV-0533	KU976471	IB	Nigeria
PV-0506	KX525736	IB	China
LS	AF127976	II	USA
TN	AB176847	II	Japan
PV-0418	AJ810256	II	USA
PV-0314	KU976482	II	Germany
PSV T1	FM992669	Out group	Hungary

4.Conclusions

The study revealed the occurrence of *Cucumber mosaic virus* on ginger in Malaysia and elucidated what [2] described as *Ginger mosaic virus* as really CMV based on the RT-PCR, cloning and sequencing employed. It additionally showed the association of the observed viral disease symptoms with CMV. This finding will help farmers to take precautionary measures against CMV and infection and serve as head way for further research in breeding for resistance.

5. Recommendations

Sequencing the whole genome of the CMV isolated in ginger plants from this study will immensely provide more insight in the characteristic of the virus at molecular level, considering the fact that this is the first comprehensive molecular characterization of CMV infecting ginger in Malaysia

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