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Research on Infectivity of *Cucumber Green Mottle Mosaic Virus* on Important Leguminous Crops

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Abstract: *Cucumber green mottle mosaic virus* (CGMMV) is one of the most important viruses affecting Cucurbitaceae crops. It has been found all over the world. Once spread, it may cause significant loss on economic crops. During a project from 2019 to 2021, occurrence of *Cucumber green mottle mosaic virus* (CGMMV) on main leguminosae crops (*Pisum sativum*, *Vicia faba*, *Vigna unguiculata*, *Glycine max*, *Phaseolus vulgaris*) was surveyed via random sampling in Ningbo, Zhejiang, China. Leaf chlorosis, yellowing (or whitening), shrinkage, spots and deformation were observed and sampled accordingly. Altogether 47 legume leaf samples were collected from 10 locations during different seasons. Total RNA was analyzed through real-time RT-PCR. Among 141 RNA extractions (3 repetitive extractions for each sample) tested, 15 were positive (corresponding to 9 samples, Ct<35), and 126 negative. Nine positive samples were further verified by conventional RT-PCR and sequencing. BLAST analysis showed that 7 sequences were over 98% identical to MP and CP regions of CGMMV isolate from Guangdong, China (Accession: MK933286), while the other 2 were over 97% identical to MP and CP regions of CGMMV isolate from Zhejiang, China (Accession: KM873783). Among 47 samples, 9 (19.15%) were positive, and out of 141 nucleic acid extractions, 15 were positive, accounting for 10.64%. Inoculation test on legume seedlings (*V. faba* var. Beidouqixing and *P. sativum* var. Jingpindawuxu) was carried out using infectious cDNA clone of CGMMV (pCB-CGMMV). Stunted growth, leaf deformation, chlorosis, and whitening were observed on both *V. faba* and *P. sativum* at 25 dpi (days post inoculation). Symptomatic leaves were diagnosed by real-time RT-PCR, which turned out to be CGMMV positive. It is speculated that leguminous plants are alternative hosts of CGMMV under natural conditions, and can serve as "virus reservoir" when cucurbitaceae plants are rare. Background research on economic significant pests is supposed to be conducted regularly. On one hand, it contributes to control of agricultural diseases and reduction of related losses. On the other, it would provide basic data for follow-up researches and supplement information for important pests.

Keywords: *Cucumber green mottle mosaic virus*, Leguminous Crops, Host Range, Disease Research

1. Introduction

Cucumber green mottle mosaic virus (CGMMV) is one of the most important viruses affecting Cucurbitaceae crops [1]. It is widely distributed around the world, including Europe, South America, Asia, etc. It has also been reported in some parts of China [2]. With the increasingly frequent trade of agricultural products, dangerous and harmful organisms such as CGMMV quietly enter China. If spread, they may cause significant losses on economic crops [3].

According to reports, host range of CGMMV is narrow. CGMMV naturally infects cucurbitaceous plants, like *Cucumis sativus*, *Citrullus lanatus*, *Cucumis melo*, *Cucurbita moschata*, *Momordica charantia*, etc [4]. It has been renewed that CGMMV could also infect *Prunus armeniaca* [5], *Ecballium elaterium* [6], *Amarantus blitoides*, *A. retroflexus* [7], and Antarctic lichens [8]. Furthermore, CGMMV exists in weeds (*Moluccella laevis*, *Withania somnifera*, *A. graecizans*, *A. muricatus*, *Ecballium elaterium*, *Chrozophora tinctoria*).

These weeds are inferred to be the "virus reservoir" of CGMMV in nature, and have a boosting effect on virus transmission [9]. Thus, host range of CGMMV includes plants in Cucurbitaceae, Solanaceae, Euphorbiaceae, and Lamiaceae.

There are many important economic crops in Fabaceae, such as soybean, kidney bean, cowpea and so on. Viral infection may cause significant loss. Therefore, it is necessary to focus on the influence of CGMMV on legumes, in the hope of taking this virus under control.

2. Material & Methods

2.1. Sample Collection and Virus Detection

2.1.1. Sample Collection

Random sampling (10 locations) was conducted for important leguminous crops in Ningbo.

2.1.2. Reagents and Instruments for Virus Detection

Primers and probes were synthesized by Liuhe BGI Technology Co., LTD (Beijing). One Step PrimeScript RT-PCR Kit (Perfect Real Time, TaKaRa), PrimeScript One Step RT-PCR Kit Ver. 2 (Dye Plus, TaKaRa), PathoScreen for CGMMV (ELISA for Cucumber green mottle mosaic virus, Agdia), 100 bp Plus DNA Ladder (Transgen Biotech), RNeasy Plant Mini Kit (QIAGEN), LightCycler 480II (Roche), ABI 2720, INGENIUS 3, NanoDrop 2000C.

2.1.3. Methods for Virus Detection

Real-time RT-PCR [10], conventional RT-PCR [11] and ELISA were employed in this study. ELISA tests were carried out according to its commercial operation manual.

2.2. Inoculation Test

2.2.1. Materials

Broad bean: *V. faba* var. Beidouqixing, Ningxia Pingluo he shengda Seed Industry Co., LTD, China.

Pea: *P. sativum* var. Jingpindawuxu, Sichuan Kesi Seed Industry Co., LTD, China.

CGMMV infectious cDNA clone: pCB-CGMMV, donated by Institute of Plant Virology, Ningbo University [12].

Nursery substrate: general organic substrate, Huaian Zhonghe Agricultural Science and Technology Development Co. LTD, China.

2.2.2. Methods

Select intact and healthy seeds for surface disinfection and breeding.

Seed disinfection

Selected seeds were subject to sterilized water for 12 h, 70% ethanol solution for 30 s and 10% sodium hypochlorite solution (diluted for 10 times) for 10 min successively, and then washed by sterilized water for 5-6 times to prepare for germination. After surface disinfection, seeds were placed in a petri dish with wet filter papers at the bottom and incubated at 25±1°C. After germination, seeds were sowed in the substrate sterilized by steam (121°C, 20 min). Cultivation was carried out under normal illumination, at the temperature of 15-25°C in the daytime and 10-15°C at night. Inoculate seedlings when the 3rd or 4th main leaf was fully unfolded.

Infection with pCB-CGMMV

The cryopreserved pCB-CGMMV solution was spread evenly onto LB solid medium containing 100 mg/L kanomycin and 50 mg/L rifampicin, cultured at 28°C for 24-48 h, until the colony grew. Single colony was transferred into 50 mL LB liquid medium (containing 100 mg/L kanamycin and 50 mg/L rifampicin) which was vibrated at 200 rpm overnight at 28°C to obtain the suspension of pCB-CGMMV. Then, the suspension was centrifuged at 4 000 rpm, 4°C for 10 min. Precipitation was suspended again via MS liquid medium (containing 100 μmol/L acetosyringone). To make the final inoculum, concentration of pCB-CGMMV suspension was adjusted to 1.0 (OD_{600}) by spectrophotometer. When the 3rd or 4th true leaf grew out, use a sterile syringe (1 mL), absorb 1 mL inoculum, and slowly press it into the 3rd or 4th leaf at the point 0.1 cm from the petiole until the whole leaf was invaded. Those plants underwent the same illumination and temperature conditions as before.

3. Result and Analysis

3.1. Sampling and Detection of CGMMV

In this survey, random sampling was conducted for economical leguminous crops in Ningbo, Zhejiang, China. Sampling was carried out in three seasons, namely spring (March to May), summer (June to August) and autumn (September to November). Correspondingly 15, 18 and 14 legume samples were collected, in a total of 47, and each sample contains at least 3 duplications (Supplementary material 1). Leaf chlorosis, yellowing (or whitening), shrinkage, spots and deformation were observed and sampled accordingly. (Figure 1).

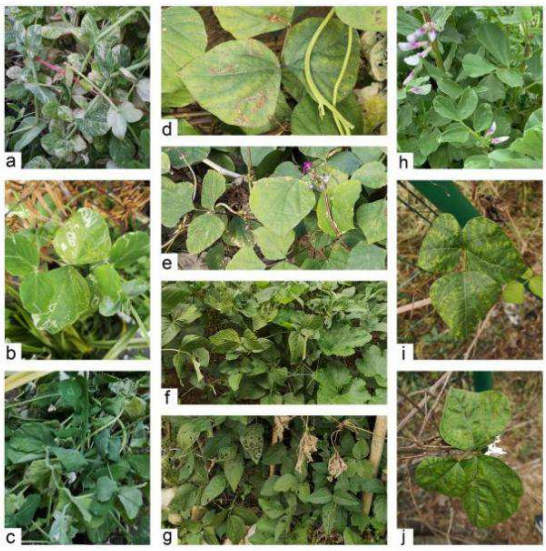

Figure 1. *Pictures of sampling. a-c Pisum sativum; d-e Vigna unguiculata; f-g Glycine max; h Vicia. faba; i-j Phaseolus vulgaris.*

Both real-time and conventional RT-PCR were used to detect CGMMV in those collected legume samples. Altogether 141 total RNA extractions (3 extractions for each sample) were tested via real-time RT-PCR firstly, among which 15 gained positive results (Ct value below 35), and 126 negative results. Those 15 positive extractions corresponded to 9 legume samples which were further verified by conventional RT-PCR and sequencing. The 654 bp amplicon was sequenced. Nine sequences (one for each positive sample) were deposited in GenBank with the accession No. of OK338437-OK338445. BLAST analysis showed that 7 sequences were over 98% identical to MP and CP regions of CGMMV isolate from Guangdong, China (Accession: MK933286), while the other 2 were over 97% identical to MP and CP regions of CGMMV isolate from Zhejiang, China (Accession: KM873783). Among 47 samples, 9 (19.15%) were positive, and out of 141 nucleic acid extractions, 15 were positive, accounting for 10.64%. In addition, serological verification of 15 positive samples by ELISA was negative.

Table 1. *Viral infection verification via real-time RT-PCR at 60 dpi.*

Plant	No.	Treatment	Ct
V. faba	1	pCB-CGMMV	20.13
	2	pCB-CGMMV	20.50
	3	pCB-CGMMV	21.10
	4	pCB-CGMMV	22.09
	5	pCB-CGMMV	23.01
	6	none	>35
	7	none	>35
	8	none	>35
	9	none	>35
	10	none	>35
P. sativum	1	pCB-CGMMV	25.21
	2	pCB-CGMMV	26.07
	3	pCB-CGMMV	24.59
	4	pCB-CGMMV	27.31
	5	pCB-CGMMV	28.45
	6	none	>35
	7	none	>35
	8	none	>35
	9	none	>35
	10	none	>35

3.2. Inoculation Test with pCB-CGMMV

Vigor tested pCB-CGMMV was inoculated onto legume seedlings via injection, followed by symptom observation and viral infection verification.

V. faba began to show symptoms at 20 dpi (days post inoculation), including stunted growth, leaf shrinkage and margin scorch, chlorosis (Figure 2). At 60 dpi, wilting was accelerated, and drought resistance was weakened compared to those control ones. Earlier blossom was observed in some inoculated seedlings. Sick leaves were sampled and verified by real-time RT-PCR, which turned out to be CGMMV positive (Table 1).

P. sativum began to show symptoms at 25 dpi, including stunted growth, leaf deformation, chlorosis, and whitening (Figure 2). At 60 dpi, those old leaves withered and yellowed faster, and drought resistance became worse than that of the control. Sick leaves were sampled and verified by real-time RT-PCR, of which results were CGMMV positive (Table 1).

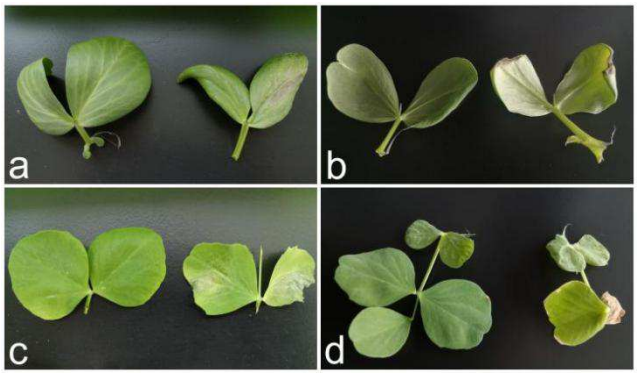

Figure 2. *Symptoms of inoculated V. faba (a-b) at 25 dpi and P. sativum (c-d) at 30 dpi, control on the left.*

4. Discussion

In this paper, we demonstrated that CGMMV infected *V. faba* and *P. sativum* by viral field investigation and inoculation tests. CGMMV infection existed in several legume crops under natural conditions and resulted in certain symptoms. CGMMV can be spread by contact as well as by seeds. Common vectors such as aphids can not transmit CGMMV. Grafting and other agricultural operations are main factors leading to prevalence in the field. Once plants get infected, soil may be contaminated if no disinfection is ever applied. The contaminated soil would become the source of viral re-occurrence [13-14]. CGMMV detected from field samples in this study had a high sequence identity with those strains from cucurbit plants (MK933286 and KM873783). In addition, some legume samples were collected in places where cucurbitaceae crops were grown nearby. Positive rate of such samples in contact with cucurbitaceae crops (42.86%) was higher than that of isolated ones (15%). Further research is supposed to focus on the transmission pattern in the field.

The detection rate of CGMMV in legumes was lower than that in cucurbitaceae [15]. Among 47 samples collected from 10 locations, 9 (19.15%) were positive, and out of 141 nucleic acid extractions, 15 were positive, accounting for 10.64%. This virus has been reported with an incidence rate of 80-100%, which delayed the growth, and caused infertility of cucumbers [16]. There was little difference on detection rate between each sampling period, and the number of positive samples in spring, summer and autumn were 3, 4 and 2, respectively. Main reason could be the rather small amount of samples. Samples collected in this survey were leaves with suspected symptoms, and low detection rate suggests leguminous crops are not preferred host of CGMMV under natural conditions. In addition, viral load was small in positive samples of which Ct values were high (>29), and ELISA results were negative. Most sampling sites chosen here are non-concentrated and

scattered ones which resemble the wild nature, while large-scale concentrated areas are rarely involved. Related research focused on high risk areas is supposed to be specially planed.

5. Conclusion

Legumes can be infected by CGMMV, which increases difficulty for virus prevention and control in the field. Legume plants may be the alternative natural host for CGMMV with low infection rate and viral load. When the amount of cucurbitaceae plants decrease, CGMMV could survive in legumes with low biomass, waiting for the next period of cucurbitaceae growth. Background research on economic significant pests is supposed to be conducted regularly. On one hand, it contributes to control of agricultural diseases and reduction of related losses. On the other, it would provide basic data for follow-up scientific research and supplement information for important pests.

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