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蚜蟲傳播彩色海芋蒟蒻嵌紋病毒之傳播生態學及海芋病毒快速檢測之研究

Transmission ecology of *Konjac mosaic virus* in calla lily by aphids and virus rapid detection

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中文摘要

彩色海芋 (*Zantedeschia* spp.) 屬於天南星科 (Araceae) 的觀賞植物，原產於非洲南部，90年代始從紐西蘭和荷蘭引進台灣，生產切花及盆花。彩色海芋因其繽紛多彩的佛焰苞，廣受到消費者的歡迎。然而，細菌性軟腐病與病毒病害一直是彩色海芋栽培上的主要限制因子。蒟蒻嵌紋病毒 (*Konjak mosaic virus*, KoMV) 屬於 *Potyvirus* 屬，是目前彩色海芋產區中導致病毒流行病的重要病原之一。遭受 KoMV 感染的海芋植株會出現嵌紋、綠島、褪綠、葉形扭曲、花梗縮短、花色斑駁等病徵，降低海芋的商品價值。本研究的第一個目的是探討蚜蟲傳播彩色海芋 KoMV 的傳播生態學；第二個研究目的是發展容易操作、快速、經濟、靈敏度高的病毒檢測法，用來偵測富含黏液植物的病毒感染。於蚜蟲傳播 KoMV 的研究中，共檢測六種蚜蟲傳播 KoMV 的能力。結果顯示，桃蚜 (*Myzus persicae*) 和棉蚜 (*Aphis gossypii*) 可以在實驗室的條件下傳播 KoMV。桃蚜和棉蚜在感染 KoMV 的葉片上取食 10 分鐘，然後轉移到健康海芋 24 小時，就能成功地傳播病毒，符合非持續性的病毒傳播模式。許多生物和非生物因素可能會影響病媒昆蟲傳播植物病毒。桃蚜和棉蚜生活史中不同的發育階段與形態，對蚜蟲傳播 KoMV 的傳播效率沒有顯著差異。環境溫度對這兩種蚜蟲傳播 KoMV 的效率也沒有影響。另外，本論文所研發的快速病毒檢測法，不僅可成功檢測感染彩色海芋的四種 potyviruses，也適用於檢測感染 KoMV 和芋頭嵌紋病毒 (*Dasheen mosaic virus*) 的彩色海芋與其他富含黏液的植物。值得一提的是新發展的病毒檢測法之偵測靈敏度高於酵素連結免疫吸附分析法(ELISA) 和以 TRIzol 試劑萃取 RNA 再進行反轉錄聚合酶連鎖反應(RT-PCR) 的方法。本研究不但提供蚜蟲傳播 KoMV 的傳播生態學相關資訊，並發展了快速且靈敏度高的病毒檢測方法，將有助於往後

研擬彩色海芋 KoMV 的防治策略。

關鍵詞：酵素連結免疫分析、具黏液植物、馬鈴薯 Y 病毒科、核糖核酸萃取、反轉錄聚合酵素鏈鎖反應、馬蹄蓮屬

ABSTRACT

Calla lilies (*Zantedeschia* spp.) are ornamental plants in the family Araceae and native to southern Africa. They were introduced into Taiwan for producing cut flowers and pot plants from New Zealand in 1990s. Their colorful bulbous flowers have won the affection of consumers. Nevertheless, bacterial soft rot and viral diseases are the major limiting factors for calla lily cultivation. *Konjak mosaic virus* (KoMV), belonging to genus *Potyvirus*, is one of the main virus causing epidemics in the calla lily-producing areas. The symptoms of KoMV-infected calla lilies are mosaic, green islands, vein chlorosis and distortion on leaves; short peduncle and discolored spots on flowers. This virus decreases the marketable value of calla lily. The first objective of this research was to study the transmission ecology of KoMV in calla lily by aphid vectors. The second objective was to develop a simple, rapid, inexpensive and sensitive sample preparation method for the detection of plant virus infection in mucilaginous plants by reverse transcription-polymerase chain reaction (RT-PCR). Six aphid species were examined for their abilities to transmit KoMV, and *Myzus persicae* and *Aphis gossypii* were

able to transmit the virus in laboratory conditions. *M. persicae* and *A. gossypii* transmit KoMV with an acquisition access period of 10 min and an inoculation access period of 24 hours, which corresponds with the features of non-persistent transmission manner. There are many biotic and abiotic factors affecting the transmission of plant viruses by insect vectors. There was no significant difference in KoMV transmission efficiency by *M. persicae* and *A. gossypii* among developmental stages and morphs. The temperature during virus acquisition and inoculation has no effect on the transmission of KoMV by these two aphid species. For developing a rapid virus detection method of calla lily-infecting viruses, crude RNA extraction with the YG buffer following with RT-PCR assay not only successfully detected the infection of four potyviruses in calla lily but also was suitable for detecting KoMV and *Dasheen mosaic virus* in calla lily and other mucilaginous plants. Furthermore, this new developed protocol was demonstrated to be more sensitive than enzyme-linked immunosorbent assay (ELISA) and TRIzol RNA extraction following with RT-PCR assay. The knowledge of transmission ecology and the development of rapid and sensitive virus detection assay

would be valuable for developing disease control strategies for KoMV in calla lily.

Keywords：ELISA, mucilaginous plant, *Potyvirus*, RNA extraction, RT-PCR, transmission efficiency, *Zantedeschia*

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Chapter 1

Transmission ecology of *Konjak mosaic virus* in calla lily by aphids

INTRODUCTION

Calla lilies (*Zantedeschia* spp.) are ornamental plants in the family Araceae and native to southern Africa. They were imported from New Zealand and the Netherlands to Taiwan in 1990s for producing cut flowers and pot plants. The colorful bulbous flowers of calla lilies have won the affection of consumers. Nevertheless, bacterial soft rot and viral diseases are the major limiting factors for calla lily cultivation (Chen *et al.*, 2003). In Taiwan, nine viruses infecting calla lily have been reported, and the major causes of viral epidemics in calla lily fields are *Konjac mosaic virus* (KoMV) and *Zantedeschia mild mosaic virus* (ZaMMV)(Chen *et al.*, 2006; Huang *et al.*, 2007). KoMV also occurs in Korea, Japan, Germany, Holland, India, China and Taiwan (Kwon *et al.*, 2002; Nishiguchi *et al.*, 2006; Huang *et al.*, 2007). KoMV belongs to the family *Potyviridae* and infects aroids including *Zantedeschia*, *Caladium*, *Dieffenbachia* and *Philodendron* species, *Typhomium flagelliforme* and *Amorphophallus konjac* (Shimoyama *et al.*, 1992; Kwon *et al.*, 2002; Manikonda *et al.*, 2011). The symptoms of KoMV-infected calla lily are mosaic, green islands and vein chlorosis on leaves, leaf distortion, short peduncle and discolored spots on flowers decreasing the value of commodity (Chen *et al.*,

2003; Huang and Chang, 2005).

Potyviruses are not only transmitted by aphid vectors in a nonpersistent manner, but also by seed, vegetative propagation, and mechanical inoculation (King *et al.*, 2011). *Aphis gossypii* is the only one identified vector of KoMV reported in Japan (Shimoyama *et al.*, 1992). Althongh one of KoMV vectors is identified, the factors affecting its aphid transmission are still unknown. KoMV, a potyvirus, infects lots of aroid plants so its transmission characteristics are assumed to be the same with other potyviruses. However, further investigation is needed to draw a conclusion. In addition, aroid plants are rich with starch, glycogen and calcium oxalate crystal (Maga, 1992). Calcium oxalate crystal acts as insect-defense function for chewing insects, but its affections on sap-sucking insects (e.g. aphids) is unknown (Korth *et al.*, 2006). To our knowledge, there is no research studying the transmission ecology of aphid –borne viruses in aroid plants. A number of important ornamental plants and food crops belong to the Araceae including calla lily, flamingo flower, taro, konjac, etc. Due to the difference of plant physiology, the transmission of aroid-infecting viruses may be different from well-studied potyviruses (e.g. *Potato virus Y*).

The objectives of this study were to identify vectors of KoMV and study the transmission ecology of KoMV by aphid vectors. I studied the transmission ecology of KoMV by two major aphid vectors, *Myzus persicae* and *A. gossypii*, in details. Species, developmental stage and morph of insect vectors were tested in this study for their transmission efficiency. In addition, the effect of temperature on virus transmission was also examined. Transmission ecology is a crucial component of disease epidemiology caused by potyviruses. The knowledge of this study would be valuable for developing disease control strategies for KoMV in calla lily fields.

MATERIALS AND METHODS

Potential aphid vectors survey

To identify potential aphid vectors of KoMV, a survey of aphids in calla lily fields was carried out in Taichung, Taiwan in September, October and November, 2008. Calla lily plots (30m × 50m) were divided into four quarters, and one yellow water trap (0.2% soap) was placed in the center of each quarter to catch migrating aphids. Trapped aphids were collected and preserved in 95% ethanol 24 hours after set-up of water traps. Crops (e.g. turnip) and weeds (e.g. giant elephant's ear) next to the calla lily plots were also searched for aphids. The specimens were brought back to the laboratory and identified to species.

Insects, viruses and plants

Aphid colonies were established from single females collected on crops and weeds in the fields. Six aphid species were used in this study: the banana aphid (*Pentalonia nigronervosa*), the cowpea aphid (*Aphis craccivora*), the green peach aphid (*M. persicae*), the turnip aphid (*Lipaphis erysimi*), the cotton aphid (*A. gossypii*) and the wheat aphid (*Rhopalosiphum padi*). The colonies were reared on their preferable host plants, respectively. *P. nigronervosa* was reared on giant elephant's ear (*Alocasia macrorrhiza*); *A. craccivora* was reared

on snow pea (*Pisum sativum*); *M. persicae* and *L. erysimi* were reared on oilseed rape (*Brassica campestris*); *A. gossypii* was reared on melon (*Cucumis melo*); *R. padi* was reared on corn (*Zea mays*) in a growth chamber at 25°C and 70% relative humidity under a 16hL: 8hD photoperiod.

Calla lily (*Zantedeschia elliottiana* × *Z. pentlandii*) cv. 'Black Magic' weas used in this study because it is the most common cultivar cultivated in Taiwan. Virus-free tissue culture plantlets of calla lily were provided by the Taiwan Seed Improvement and Propagation Station, Council of Agriculture, Taiwan. Plantlets were transferred from tissue culture media to a mixture of peat moss, vermiculite and perlite with the ratio of 2:1:1 in plastic pots (63 mm diameter x 65 mm height) and fertilized weekly. After plantlets grew to three-leaf stage, they were served as healthy test plants for transmission experiments.

KoMV was provided by Dr. Ya-Chun Chang in the Department of Plant Pathology and Microbiology, National Taiwan University. The virus was maintained in calla lily by aphid transmission using *M. persicae* (described below). All virus-infected plants were tested by indirect enzyme-linked immunosorbent assay (I-ELISA, described below) to ensure they were infected

with KoMV. All virus source plants and healthy test plants were grown in growth chambers at 23°C, a photoperiod of 16hL:8hD.

Virus transmission experiments

Six aphid species were examined for their abilities to transmit KoMV in laboratory conditions. Groups of 30 mix-stage indiviuals of *P. nigronervosa*, *A. craccivora*, *M. persicae*, *L. erysimi*, *A. gossypii* and *R. padi* were taken out from their colonies and kept in plastic vials for a 3-hour preacquisition fasting. After fasting, each aphid species was allowed to acquire the virus on a detached leaf of KoMV-infected calla lily. After an acquisition access period (AAP) of 10 minutes, the aphids were immediately transferred from virus source tissues to healthy calla lilies and confined on a leaf with plastic cages (Tsai *et al.*, 2008) with a group of 30 aphids per test plant. After an inoculation access period (IAP) of 24 hours, test plants were treated with an insecticide (Imidacloprid) to kill the remaining aphids and then placed in a growth chamber for symptom development. Test plants were checked with disease symptom and examined by I-ELISA for KoMV infection 8 weeks after IAP. The transmission efficiency of a given aphid species was calculated as percentage of test plants positive for KoMV.

A similar experiment was conducted to examine the transmission efficiency of KoMV by apterous adults of these aphid species. Apterous adults were given a 10-minute AAP and a 24-hour IAP. Ten or twenty potential viruliferous aphids were allowed to inoculate KoMV to each test plant. After IAP, the remaining aphids were killed by an insecticide. Test plants were maintained in a growth chamber for symptom development.

Effect of developmental stage and morph on transmission efficiency

Using the methods described above, aphids with four developmental stages and morphs (early-instar nymphs, late-instar nymphs, alate adults and apterous adults) were allowed to acquire the virus from leaf tissues of KoMV-infected calla lily for a 10-min AAP and followed by a 24-hour IAP on healthy calla lilies. A group of ten *M. persicae* or *A. gossypii* per test plant with different stages and morphs was allowed to inoculate the virus to test plants.

Effect of temperature on transmission efficiency

According to the results of above-mentioned study, late-instar nymphs transmitted KoMV with the highest efficiency, so I chose this morph of aphid for the following experiments. The late-instar nymphs were allowed to acquire KoMV from virus source tissues for a 10-min AAP and followed by a 24-hour

IAP on healthy calla lilies. Because the optimal temperature of culturing calla lilies is between 18°C to 28°C, transmission experiments were conducted at three different temperatures (18°C, 23°C, 28°C) during the period of AAP and IAP. A group of ten *M. persicae* or *A. gossypii* per test plant was allowed to inoculate the virus to test plants at these three different temperatures.

Correlation between virus titer and transmission efficiency

A transmission experiment was designed to determine the relationship between transmission efficiency and virus titer in source tissues. Leaf tissues (0.01 g) were collected KoMV-infected calla lily with different levels of mosaic symptoms. Groups of 10 late-instar nymphs of *M. persicae* were allowed to acquire the virus from virus source tissues for a 10-min AAP followed by a 24-hour IAP on test plants. After AAP, virus source tissues were saved for total RNA extraction with TRIzol reagent (Invitrogen Corp., Carlsbad, CA) following the manufacturer's instructions. Afterwards, five-fold serially diluted RNAs were prepared and then tested by reverse transcription-polymerase chain reaction (RT-PCR) to compare their relative quantity.

All transmission experiments describe above were performed three times,

and five replicate test plants of each treatment were inoculated by aphids in each trial. After IAP, all test plants were treated with an insecticide to kill the remaining aphids and then maintained in growth chambers at 23°C under a 16hL: 8hD photoperiod. Test plants were checked with unwanted aphid infestation by eyes and fertilized weekly until I-ELISA assay. An insecticide was spread on test plants when needed. All transmission experiments had negative controls, calla lilies from the same batch of test plants that were not exposed to aphid vectors. None of the controls was positive for KoMV with I-ELISA assay.

I-ELISA

Test plants were assayed for KoMV infection using a modified version of I-ELISA (Hu *et al.*, 2010). Leaf tissues (0.05-0.1 g according to sample batches) was flash-frozen in liquid nitrogen and ground to powder with mortars and pestles. Tissue powder was immediately added with ISE buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 2% PVP-40, pH 9.6) at 1:50 (w/v). 96-well polystyrene microtiter plates were incubated with 100 µl of the sample lysates overnight at 4°C. The antigen-coated plates were washed with 300 µl of PBST buffer (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.05%

Tween-20, pH 7.4), and then blocked with 200 µl of blocking buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 5% skim milk, pH 9.6) at 37°C for one hour. Subsequently, the plates were washed again with PBST buffer and then inoculated with KoMV polyclonal antibody (1:5,000) at 37°C for one hour. After another wash with PBST buffer, the plate were incubated with an alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1:5,000) at 37°C for one hour. After another wash with PBST buffer, the plate was incubated with p-nitrophenyl phosphate solution (1 mg/ml substrate buffer) in the dark. Absorbance of p-nitrophenyl phosphate solution at 405 nm was measured 60 min after the addition of substrate. Absorbance values that were at least three times more than the standard deviations of the negative controls were considered positive for KoMV. All leaves of each test plant were collected and separately tested for I-ELISA assay to avoid possible problems with uneven distribution of KoMV within plants.

RT-PCR

RT-PCR was carried out with the OneStep RT-PCR kit (Qiagen, Valencia, CA). The reverse transcription was performed at 50°C for 30 min, followed by a PCR activation step at 95°C for 15 min, amplification of 30 cycles at 94°C

for 30 s, 50°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The amplification products were analyzed by electrophoresis on 1% agarose gels and visualized on a UV transilluminator following ethidium bromide staining. Specific primer pair for KoMV was KoF (5’-TGGAGAGGAAGAGAAGGATG-3’) and KoR (5’-ACATCTTCAGCTG TATGTCTC-3’).

RESULTS

Potential aphid vectors

According to the results of field survey, migrating alate adults of *R. padi* and *L. erysimi* were collected in the calla lily fields using yellow water traps. Through active search, there were no aphids found feeding on calla lily. *L. erysimi* and *M. persicae* were found feeding on turnip next to calla lily plots, whereas *P. nigronervosa* also found feeding on giant elephant's ear. *P. nigronervosa, M. persicae, L. erysimi,* and *R. padi* were assumed to be potential vectors of KoMV in calla lily fields.

Because *A. gossypii* is the only known vector of KoMV (Shimoyama *et al.*, 1992), and *A. craccivora* is one of common aphid species in Taiwan, these two aphid species were also included in the screening of potential KoMV vectors. Colonizers and noncolonizers were differentiated by their abilities to live and reproduce on calla lily under laboratory conditions. *P. nigronervosa* was the only one colonizer species of calla lily because it fed and produced offspring on calla lily persistently. *A. craccivora, M. persicae, L. erysimi, A. gossypii* and *R. padi* were non-colonizer species of calla lily because they could not reproduce on calla lily even though *A. gossypii* and *M. persicae* lived on calla

lily for few generations.

Six aphid species were screened for their potentials to be vectors of KoMV by transmission experiments using 30 mix-stage aphids per test plant for virus inoculation. *M. persicae* and *A. gossypii* but not others were able to transmit KoMV with transmission rates of 33% and 27%, respectively (Table 1). There was no significant difference of transmission rate between these two species ($P > 0.05$, χ^2 test).

Table 1. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 30 aphids per plant following a 10-min acquisition access period and a 24-h inoculation access period

Aphid species	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
M. persicae	2/5[1]	1/5	2/5	5/15	33 %
A. gossypii	3/5	1/5	0/5	4/15	27 %
R. padi	0/5	0/5	0/5	0/15	0 %
L. erysimi	0/5	0/5	0/5	0/15	0 %
A. craccivora	0/5	0/5	0/5	0/15	0 %
P. nigronervosa	0/5	0/5	0/5	0/15	0 %

[1] Number of plants positive for KoMV/number of plants tested.

In another experiment, ten apterous adults per test plant were used for virus inoculation to compare the transmission abilities of these aphid species.

Similarly, only *M. persicae* and *A. gossypii* transmitted KoMV under labortary conditions. About 13% and 7% of test plants were infected with KoMV after virus inoculation by *M. persicae* and *A. gossypii*, respectively, and symptom development (Table 2). There was no significant difference of transmission rate between these two aphid species ($P > 0.05$, χ^2 test).

Table 2. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 10 apterous adults of aphids per plant following a 10-min acquisition access period and a 24-h inoculation access period

Aphid species	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
M. persicae	1/5[1]	1/5	0/5	2/15	13 %
A. gossypii	0/5	0/5	1/5	1/15	7 %
R. padi	0/5	0/5	0/5	0/15	0 %
L. erysimi	0/5	0/5	0/5	0/15	0 %
A. craccivora	0/5	0/5	0/5	0/15	0 %
P. nigronervosa	0/5	0/5	0/5	0/15	0 %

[1] Number of plants positive for KoMV/number of plants tested.

The transmission rates of KoMV transmitted by *M. persicae* and *A. gossypii* were 7% and 13%, reapectly, when the number of aphids used for virus inoculation was increased from 10 to 20 ($P > 0.05$, χ^2 test)(Table 3).

Table 3. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 20 apterous adults of aphids per plant following a 10-min acquisition access period and a 24-h inoculation access period

Aphid species	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
M. persicae	1/5[1]	0/5	0/5	1/15	7 %
A. gossypii	0/5	1/5	1/5	2/15	13 %
R. padi	0/5	0/5	0/5	0/15	0 %
L. erysimi	0/5	0/5	0/5	0/15	0 %
A. craccivora	0/5	0/5	0/5	0/15	0 %
P. nigronervosa	0/5	0/5	0/5	0/15	0 %

[1] Number of plants positive for KoMV/number of plants tested.

Effects of developmental stage and morph on transmission efficiency

The effect of developmental stage and morph on KoMV transmission was tested with four groups of *M. persicae*: early-instar nymphs, late-instar nymphs, apterous adults and alate adults. There was no significant difference of transmission rate of *M. persicae* among four developmental stages and morphs ($P > 0.05$, χ^2 test) (Table 4). Within the experiment, the transmission rate of

replicates 1 and 2 were similar but a higher transmission rate occurred in replicate 3 (Table 4). The same transmission experiment was performed with another aphid species, *A. gossypii*. There was also no significant difference of transmission rate of *A. gossypii* among four developmental stages and morphs ($P > 0.05$, χ^2 test)(Table 5).

Table 4. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 10 *Myzus persicae* with different developmental stages and morphs per plant following a 10-min acquisition access period and a 24-h inoculation access period

Stage and morph	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
Early-instar nymph	1/5[1]	0/5	5/5	6/15	40 %
Late-instar nymph	1/5	1/5	5/5	7/15	47 %
Apterous adult	0/5	0/5	5/5	5/15	33 %
Alate adult	0/5	0/5	4/5	4/15	27 %

[1] Number of plants positive for KoMV/number of plants tested.

Table 5. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 10 *Aphis gossypii* with different developmental stages and morphs per plant following a 10-min acquisition access period and a 24-h inoculation access period

Stage and morph	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
Early-instar nymph	0/5[1]	1/5	3/5	4/15	27 %
Late-instar nymph	2/5	1/5	0/5	3/15	20 %
Apterous adult	0/5	2/5	0/5	2 /15	13 %
Alate adult	0/5	2/5	2/5	4/15	27 %

[1] Number of plants positive for KoMV/number of plants tested.

Effects of temperature on transmission efficiency

Aphids acquired and inoculated KoMV at 18°C, 23°C and 28°C, and the transmission efficiency of KoMV was compared among these three temperatures. The results showed that late-instar nymphs of *M. persicae* transmitted KoMV at 23°C and 28°C, but not 18°C (Table 6). There was no significant difference for KoMV transmission rate between 23°C and 28°C ($P > 0.05$, χ^2 test). The results showed that late-instar nymphs of *A. gossypi* transmitted KoMV at 18°C, 23°C and 28°C, but the transmission rates were not significantly different among three temperatures (Table 7) ($P > 0.05$, χ^2 test).

Table 6. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 10 apterous *Myzus persicae* per plant following a 10-min acquisition access period and a 24-h inoculation access period under different temperatures

Temperature	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
18˚C	0/5[1]	0/5	0/5	0/15	0 %
23˚C	1/5	0/5	1/5	2/15	13 %
28˚C	0/5	1/5	0/5	1/15	7 %

[1] Number of plants positive for KoMV/number of plants tested.

Table 7. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 10 apterous *Aphis gossypii* per plant following a 10-min acquisition access period and a 24-h inoculation access period under different temperatures

Temperature	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
18˚C	1/5[1]	0/5	0/5	1/15	7 %
23˚C	1/5	1/5	0/5	2/15	13 %
28˚C	0/5	0/5	1/5	1/15	7 %

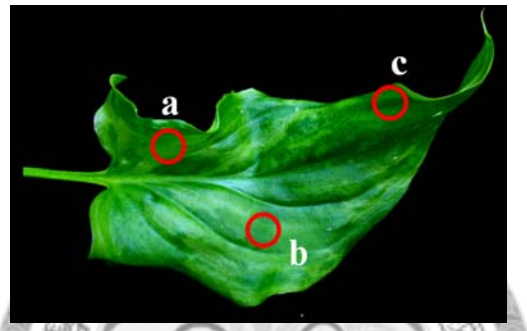
[1] Number of plants positive for KoMV/number of plants tested.

Correlation between virus titer and transmission efficiency

Virus populations in virus source tissues were compared through semi-quantitative RT-PCR. Tissues without mosaic symptom had higher virus population than tissues with severe and mild mosaic symptoms (Fig. 1). From the results of aphid transmission experiments, only 1 of 15 test plants was

positive for KoMV when aphids acquired viruses from severe mosaic tissues (Table 8). Although aphids acquired KoMV from tissues with the highest virus population, the virus transmission rate was not correlated to virus titer in virus source tissues.

A



B

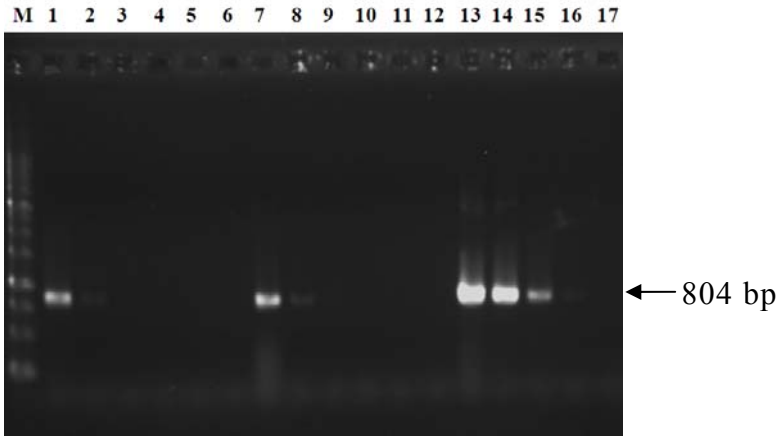


Fig. 1. Semi-quantitative estimate of *Konjac mosaic virus* (KoMV) populations in calla lily tissues with different levels of mosaic symptom. (A) Tissue sampled areas of a KoMV-infected leaf: a, severe mosaic; b, mild mosaic; c, no mosaic symptom. (B) RT-PCR results of serially diluted total RNAs from tissues with different levels of mosaic symptom. M, 1 kb DNA ladder; lanes 1-5, severe mosaic; lanes 7-11, mild mosaic; lanes 13-17, no mosaic symptom.

Table 8. Transmission rate of *Konjac mosaic virus* (KoMV) by groups of 10 apterous *Myzus persicae* per plant following a 10-min acquisition on KoMV-infected calla lily tissues with different levels of mosaic symptom and a 24-h inoculation access period

Mosaic level	Experiment 1	Experiment 2	Experiment 3	Total transmission rate	
Severe mosaic	0/5[1]	0/5	1/5	1/15	7 %
Little mosaic	0/5	0/5	0/5	0/15	0 %
No mosaic	0/5	0/5	0/5	0/15	0 %

[1] Number of plants positive for KoMV/number of plants tested.

DISCUSSION

The cultivation of deciduous calla lily is limited by bacterial soft rot and viral diseases in Taiwan. KoMV is one of the high prevalence viruses in calla lily fields (Chen *et al.*, 2006; Huang *et al.*, 2007). KoMV, a potyvirus, is mainly transmitted by insect and vegetative propagation (e.g. tubers) (Shimoyama *et al.*, 1992; Nishiguchi *et al.*, 2006). However, there is no insect vector identified in Taiwan, and no studies investigating its transmission ecology in details. In this study, two non-colonizing aphids, *M. persicae* and *A. gossypii* were demonstrated to be the vectors of KoMV in calla lily under laboratory conditions. The transmission ecology was further studied by the effects of insect developmental stage, morph and environmental temperature on the transmission efficiency of KoMV by these two candidate vectors. Knowing key vectors of a viral disease is the first step to study epidemiology. To control KoMV epidemic in calla lily fields, more parameters of the transmission ecology of KoMV by aphids are needed to be investigated.

According to the field survey in this study, there is no colonizing aphid found in calla lily fields. *P. nigronervosa*, a colonizing aphid, was caught from aroid weeds near the calla lily plots, but it was not found feeding on calla lily

even though *P. nigronervosa* feeds and reproduces on calla lily with a non-choice test in the laboratory. Other five aphid species tested in this study are non-colonizers for calla lily. For nonpersistently transmitted viruses (e.g. potyviruses), non-colonizing aphids cause primary infection and colonizing aphids are responsible for secondary spread within the field (Atiri *et al.*, 1986). In Taiwan, agriculture authority has encouraged farmers to use virus-free tissue culture plantlets as propagative materials for many years. Taiwan Seed Improvement and Propagation Station, Council of Agriculture, Taiwan provides these virus-free plantlets with a low price. However, the prevalence of KoMV and other calla lily-infecting viruses are still high in the fields. Possible reasons would be aphid transmission or that farmers do not use virus-free propagative materials. *M. persicae* and *A. gossypii* are identified as vectors of KoMV in this study, and *A. gossypii* also proved as a vector of KoMV in Japan (Shimoyama *et al.*, 1992). Both aphid species are metropolitan pests worldwide and infest many crops and weeds (Cutler *et al.*, 2009; Najar-Rodriguez *et al.*, 2009). Cultivation of calla lily is suggested to be aware of these two aphid species migrating from crops and weeds in neighborhood fields.

There are many biotic and abiotic factors affecting the transmission of

plant viruses by insect vectors. Clone, developmental stage and morph of vector and strain, virulence and host plant of virus are these biotic factors (Sadeghi *et al.*, 1997; Bourdin *et al.*, 1998; Srinivasan and Alvarez, 2007; Van Emden and Harrington, 2007; Anhalt and Almeida, 2008). Developmental stage and morph of aphid vector do not affect the transmission efficiency of KoMV by *M. persicae* and *A. gossypii* (Tables 4, 5). Kanavaki *et al.* (2006) also reported the transmission rates of PVY^N by *M. persicae* with no significant difference between alate adults and apterous adults. Different outcomes of the effect of developmental stage and morph on vector transmission of viruses have been reported. *A. craccivora* and *Acyrthosiphon pisum* nymphs transmit *Faba bean necrotic yellow virus* (*Nanoviridae*) more efficiently than adults (Franz *et al.*, 1998). The adult of *P. nigronervosa* is a poor vector of *Banana bunchy top virus* (*Nanoviridae*) compared with the nymphs (Anhalt and Almeida, 2008)

Abiotic factors inducing temperature, relative humidity, light, rainfall and wind also impact the transmission efficiency of vector-borne viruses (Singh *et al.*, 1988; Smyrnioudis *et al.*, 2001). Temperature plays the most important environmental factor for vector transmission of viruses (Gray and Gildow, 2003). Transmission efficiency of KoMV by *M. persicae* was affected by the

environmental temperature when it acquires and inoculates the virus. However, the low temperature (18°C) halts the transmission of KoMV by *M. persicae* (Table 6). Virus transmission is also largely dependent on vector probing behavior (Manoussopoulos, 2000; Chang *et al.*, 2001; Dedryver *et al.*, 2005). Low temperature may affect the transmission efficiency of KoMV indirectly through modifying feeding behavior of *M. persicae*. Low temperature may delay the timing that aphids start to feed or shorten feeding period (Tu and Ford, 1971). The transmission rate of *Zucchini yellow mosaic virus* by *M. persicae* is more efficient at 21°C than at 8°C (Fereres *et al.*, 1992). *R. padi* is unable to transmit *Barley yellow dwarf virus* at 16°C but transmit the virus at 25°C (Lucio-Zavaleta *et al.*, 2001). Syller (1987) found that *Potato leafroll virus* (PLRV) is transmitted by *M. persicae* more efficiently at 26 °C than at 12°C. Robert and Rouze-Jouan (1971) compared the transmission of PLRV by aphids *Aulacorthum solani*, *M. euphorbiae* and *M. persicae* at 6°C, 15°C and 24°C. There is a low transmission rate at 6°C, and the optimal temperature for virus transmission is 24°C (Robert and Rouze-Jouan, 1971). The influence of temperature on virus transmission by aphids is complex, and the effect may act on virus, aphid or host plant.

KoMV is unevenly distributed in an infected leaf so green island and mosaic tissues have different titers of the virus (Fig. 1). Pham *et al.* (2002) also demonstrated that KoMV is not equally distributed in the shoots and tubers when calla lily is infected with the virus. New developing leaves have higher detection rate of virus infection in the late growth season after flowing (Pham *et al.*, 2002). Surprisingly, the results showed KoMV was transmitted when aphids acquired the virus from the tissues with low titer but not with high titer (Table 8). This suggests KoMV must be transmitted when aphids acquired the virus from the high-titer tissues, but the virus is still in a latent infection phase that is not detectable. According to my observation, KoMV-infected plants may become virus-negative in the following growth season and vice versa. This would be strong evidence that the plants were infected with KoMV, but the virus is in a latent infection phase. In the experiments studying the effect of developmental stage and morph of *M. persicae*, the third trial had high transmission rates. It is possible that aphids acquired the virus from low-titer tissues or the test plants infected with the virus but in a latent infection phase in the first and second trials.

Compared to other aphid-borne potyviruses, *M. persicae* and *A. gossypii*

transmit KoMV with low transmission rates. As discussion above, this may be due to uneven distribution or latency of the virus. Even the study shows low transmission efficiency of KoMV by two major aphid vectors, but insect vectors may still play an important role in KoMV epidemic. In calla lily fields, KoMV-infected plants may be symptomless or undetectable because of latency. On the one hand, these plants will become virus sources in the following growth seasons. Farmers prefer years-old tubers than tissue culture plantlets because the bigger tubers produce more flowers. Once these old tubers infected with KoMV, these plants will act as a primary virus source in the fields. On the other hand, tissue culture calla lily may be infected with the virus before flower setting during growth seasons (1-2 years). *A. gossypii* and *M. persicae* have high reproductive rates and occur whole year-round in Taiwan so the potentials to transmit the virus are still very high to cause the KoMV epidemic in calla lily fields.

Chapter 2

A rapid method of RNA extraction for RT-PCR detection of potyviruses in mucilaginous plants

INTRODUCTION

Outbreaks of plant viral diseases that resulted in enormous cases of crop yield losses have been reported worldwide (Jones, 2006; Canto *et al.*, 2009; Jones, 2009). The pathogen diagnosis is crucial for plant epidemiology studies and disease management. Observation of symptom is a simple method to detect the viral infection in a given plant; however, disease symptoms are heavily dependent on virus virulence, host susceptibility, and environment conditions. Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR)-based techniques are widely used to diagnose the infection of plant viruses (Eni *et al.*, 2008). Many variants of ELISA-based techniques have been developed to detect viruses (Chen *et al.*, 2006; Eni *et al.*, 2008; Belliure *et al.*, 2009). ELISA is based on the specific binding of virus particles to antiserum. Advantages of ELISA are simplicity, low cost, and readily be used to in laboratories without special provisions being made. Routine virus detection and disease survey are mainly performed by ELISA (Eni *et al.*, 2010; Peng *et al.*, 2011). PCR and reverse transcriptase (RT)-PCR are more sensitive techniques for the detection and identification of DNA and RNA plant viruses (Garcia *et al.*, 1997; Hu *et al.*, 2010; Kumar *et al.*, 2011b). Different from ELISA,

PCR-based detections are specific amplification of a target sequence in viral genome. PCR-based assay is a rapid, more specific and sensitive diagnostic tool, but careful sample preparation and personnel training are prerequisites. PCR-based assay are usually more costly than ELISA. With special design of primers and protocol, multiplex PCR and specific identification of a virus genus, species or strain are possible (Langeveld *et al.*, 1991; Walsh *et al.*, 2001; Kumar *et al.*, 2011b). PCR-based assay and DNA sequencing have replaced the use of serological techniques for the detection or identification of plant viruses. Moreover, more sensitive and reliable methods (e.g. real-time RT-PCR) for the detection and quantification of plant viruses have been reported (Papayiannis *et al.*, 2011; Tsai *et al.*, 2011).

Presence of inhibitory plant compounds, uneven viral distribution and low virus titer in the tissues of plant hosts do not allow easy detection of viruses by RT-PCR from woody or mucilaginous plants (Demeke and Adams, 1992; Nassuth *et al.*, 2000). Cellular components, polysaccharides (e.g. starch) and reactive secondary metabolites (e.g. polyphenols) of plant extracts may inhibit the enzyme reaction of RT-PCR assay (Wilson, 1997; Demeke and Jenkins, 2010). Many aroids and root corps contain large amount of mucilage, a highly

viscous secondary metabolite, occurs in nature as exo-polysaccharide. Mucilage and other secondary metabolites inhibit the activity of *Taq* polymerase for PCR directly or indirectly (Fang *et al.*, 1992; Jose and Usha, 2000). Removal of the mucilaginous substances during RNA extraction procedure is very difficult from mucilage-rich plants (Bayer *et al.*, 1999).

Many sample preparation procedures have been developed to circumvent the problem of plant derived PCR inhibitors. Cetyltrimethylammonium bromide (CTAB) is added to extraction buffer to extract RNA from a wide range of polysaccharide- or polyphenol-rich plant tissues (Iandolino *et al.*, 2004). Polyvinyl pyrrolidone (PVP)-40 form complexes with polyphenols and therefore relieve PCR inhibition (Wang and Stegemann, 2010). β-mercaptoethanol acts as an antioxidant, have been incorporated into the extraction procedure to reduce the level of inhibitory (Bellstedt *et al.*, 2010). Many commercial kits employed biochemical methods to extract pure RNA. Phenol-chloroform RNA extraction or silicon binding column-based methods yields high quality RNA, but it is time-consuming, laborious and relatively expensive.

For plant viral disease management, identification of ethiological agent,

estimation of prevalence and screening of alternative hosts play important roles, so it is an urgent need to develop a rapid and simple virus detection assay. My objective was to develop a simple, rapid, inexpensive and sensitive sample preparation method for the detection of plant virus infection in mucilaginous plants by PCR. This study describes an improve, rapid, simple and inexpensive protocol for RNA extraction suitable for further downstream PCR assay. The protocol described in this study has been standardized with calla lily infected with two potyviruses, *Konjac mosaic virus* (KoMV) and *Dasheen mosaic virus* (DsMV), and was validated further with calla lilies and mucilaginous plants infected with four potyviruses. The developed protocol would be useful for epidemiology study and disease management for mucilaginous plants.

MATERIALS AND METHODS

Insects, viruses and plants

A single female colony of the green peach aphid (*Myzus persicae*) was derivate from the field-collected aphids. The colony was reared on oilseed rape (*Brassica campestris*) in a growth chamber at 25°C and 70% relative humidity under a 16hL:8hD photoperiod. DsMV, *Zantedeschia mild mosaic virus* (ZaMMV) and KoMV-infected calla lilies (*Zantedeschia elliottiana* × *Z. pentlandii*) cv. 'Black Magic' were maintained in the laboratory by aphid transmission. Virus-free tissue culture plantlets of calla lily were provided by the Taiwan Seed Improvement and Propagation Station, Council of Agriculture, Taiwan. Aphid transmission by *M. persicae* was performed when the plantlets grew to four-leaf stage (Chapter 1). *Turnip mosaic virus* (TuMV) was maintained in the same cultivar of calla lily by mechanical transmission (Hu *et al.*, 2010). DsMV and KoMV were also inoculated to other mucilaginous plants by mechanical transmission with the same buffer and procedure. Taro (*Colocasia esculenta*), giant elephant's ear (*Alocasia macrorrhiza*) and Chinese yam (*Dioscorea alata*), which used in this research were collected from the fields. All virus-infected plants were tested by indirect-ELISA (I-ELISA) and

RT-PCR (describe later) to ensure they were infected with specific virus.

Pre-PCR sample preparation

Different sample preparation buffers were compared to examine their efficiency for PCR-based virus detection in mucilaginous plants. For pure RNA extraction, leaf tissue (0.05 g) was ground into fine powder by liquid nitrogen with mortars and pestles. Tissue powders were immediately added with 2 ml of TRIzol reagent (Invitrogen Corp., Carlsbad, CA) for homogenization and then proceeded to total RNA extraction protocol following the manufacturer's instructions. Crude RNA extraction methods employed different sample preparation buffers: EGB, YG, TES and CS. EGB buffer was made up with 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 containing 2% PVP-40, 0.2% bovine serum albumin, 0.05% Tween-20 and 1% $Na_2S_2O_5$ (Osman *et al.*, 2007); CS buffer was made up with 100 mM sodium citrate, pH 6 containing 0.5% β-mercaptoethanol (Jose and Usha, 2000); TES buffer was made up with 100 mM Tris–HCl, 10 mM EDTA, 1.4 M NaCl, pH 8 containing 2% CTAB and 0.2% β-marcaptoethanol (Ghosh *et al.*, 2009); YG buffer was made up with 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4 containing 2% PVP-40, 0.05% Tween-20 and 1% Na_2SO_3 (Eni *et al.*, 2008). For crude

RNA extraction, leaf tissue (0.05g) was flash-frozen with liquid nitrogen and macerated in one of above-mentioned sample preparation buffers at 1:100 (w/v). Two microlitres of the lysate was added to 25 µl GES denaturing buffer (100 mM glycine, 50 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) (Osman *et al*., 2007) and then heated at 95°C for 10 min to release the nucleic acid of virus. A microlitre aliquots of the GES homogenate were used as templates in a 10 µl final volume of RT-PCR (described later). All experiments were performed three times.

RT-PCR

RT-PCR was carried out with the OneStep RT-PCR kit (Qiagen, Valencia, CA). The reverse transcription was performed at 50°C for 30 min, followed by a PCR activation step at 95°C for 15 min, amplification of 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. We analyzed amplification products by electrophoresis on 1% agarose gels and visualized on a UV transilluminator following ethidium bromide staining. Specific primer pairs for each calla lily-infecting potyviruses were KoF (5'-TGGAGAGGAAGAGAAGGATG-3') and KoR (5'-ACATCTTCAGCTGTATGTCTC-3') for KoMV; TuCPF1 and TuR0 for

TuMV (Hu *et al.*, 2010); ZUNF2 and ZUNR1 for ZaMMV (Hu *et al.*, 2010); DF2 and DR0 for DsMV (Huang and Chang, 2005).

Optimization for crude RNA extraction

To optimize the amount of crude extract added in GES buffer for RT-PCR, 2, 4, 8, 16 and 32 µl of tissue lysates from KoMV-infected calla lily in YG buffer were added in a 25 µl of GES buffer. The best ratio of tissue lylate to GES buffer was chosen to test the best amount of template sample needed for RT-PCR assay. Range of 1-5 µl of homogenates in GES buffer was used as templates in a 10 µl final volume of RT-PCR. The optimized protocol was used as a standard protocol to detect different viruses in various mucilaginous plants in this study. All experiments were performed three times.

I-ELISA

The protocol was performed as described previously (Chapter 1).

Detection sensitivity of new developed protocol

The detection sensitivity of crude RNA extraction following with RT-PCR was compared with I-ELISA and RNA extraction by a phenol-chloroform method following with RT-PCR. One KoMV-infected calla lily leaf was cut into small pieces and separated to three equal portions to prevent from uneven

distribution of virus in tissues. One portion of leaf tissues was frozen with liquid nitrogen and macerated with YG buffer at 1:100 (w/v). The tissue lysate was then serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} fold with YG buffer. Two microlitres of the lysate was added to 25 μl GES buffer, boiled and then proceed to RT-PCR. Another portion of leaf tissues was also frozen with liquid nitrogen and macerated with TRIzol reagen at 1:40 (w/v). Total RNA of this portion of leaf tissues was extracted following the manufacturer's instructions. Total RNA was diluted with DEPC-treated ddH_2O to make the amount of extracted RNA extraction comparable with YG buffer crude extraction. Last portion of leaf tissues was also frozen with liquid nitrogen, macerated with ISE buffer and then diluted with ISE buffer to make the amount of homogenated tissues comparable with YG buffer and TRIzol extractions. I-ELISA assay was performed as previous described.

RESULTS

Detection efficiency of sample preparation buffers

Two calla lily-infecting viruses, KoMV and DsMV, were used to examine the detection efficiencies of sample preparation buffers by RT-PCR. Leaf tissues from KoMV- and DsMV-infected calla lilies macerated with EGB, YG and CS sample preparation buffers successfully extracted RNA, and the crude extracted RNAs were served as RT-PCR templates to yield 804 bp and 457 bp amplicons, respectively, as expected after RT-PCR. Only TES sample preparation buffer failed to extract RNA for RT-PCR assay. Detection efficiencies of these four sample preparation buffers were compared by 10-fold serial dilutions of tissue lysates for RT-PCR assay. YG buffer was the most efficient one used for PCR-based potyvirus detection in calla lilies, and EGB sample preparation buffer was the second (Table 9). The detection sensitivity of YG buffer was about ten times higher than EGB buffer and about a hundred times higher than CS buffer. According to this result, we chose YG buffer as the sample preparation buffer to develop a standard protocol for potyvirus detection in mucilaginous plants.

Table 9. Detection threshold of various sample preparation buffers used for detection of *Konjac mosaic virus* (KoMV) and *Dasheen mosaic virus* (DsMV) in calla lilies, samples were serially diluted ten-fold

	KoMV				DsMV			
	EGB	YG	TES	CS	EGB	YG	TES	CS
Exp. 1	10^{-2}	10^{-2}	-	10^{-1}	10^{-3}	10^{-3}	-	1
Exp. 2	10^{-1}	10^{-2}	-	1	10^{-2}	10^{-4}	-	10^{-1}
Exp. 3	10^{-2}	10^{-2}	-	10^{-2}	10^{-3}	10^{-4}	-	10^{-3}

-: decetion failed

Optimization of crude RNA extraction procedure

Various volumes of tissue lysates from KoMV-infected calla lily in YG buffer were added in a 25 ul GES buffer used as RT-PCR templates, and the result showed that there was no difference when using more or less amounts of tissue lysates within a range of 2-32 ul (Fig.2). Therefore, I decided to add 2 ul of YG tissue lysates into GES buffer as a standard protocol. Similarly, various volumes of homogenates in GES buffer were added in a 10 ul final volume of RT-PCR. The result showed that only 1 ul of homogenate in GES buffer yielded RT-PCR product (Fig. 3). Therefore, I decided to add 1 ul of homogenate in GES buffer as a standard protocol. The optimized protocol was used as a standard protocol for the following experiments.

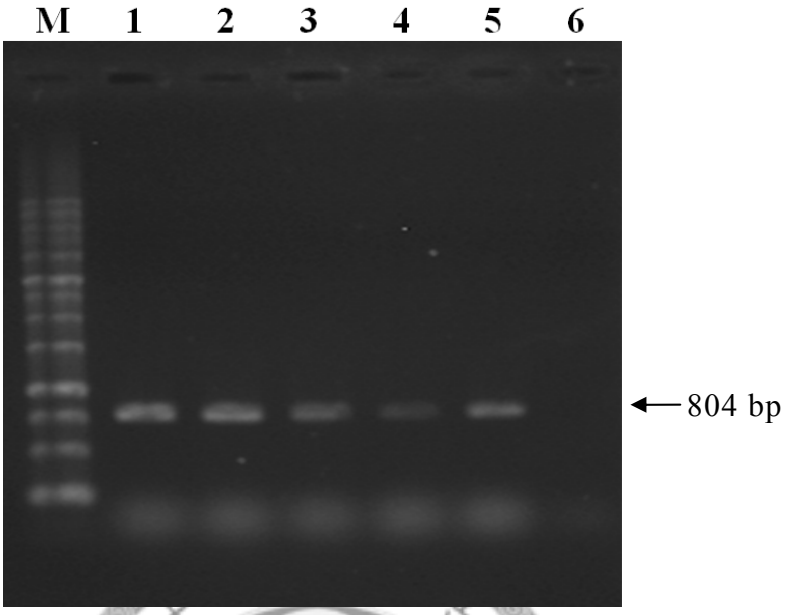


Fig. 2. Agarose gel electrophoresis analysis of the effects of tissue lysate volume on the detection efficiency of *Konjac mosaic virus*-infected calla lily. M, 1 kb DNA ladder; lane 1, 2 ul of tissue lysate in YG buffer; lane 2, 4 ul; lane 3, 8 ul; lane 4, 16 ul; lane 5, 32 ul; lane 6, nagetive control. One representive gel image of three replicate experiments is shown.

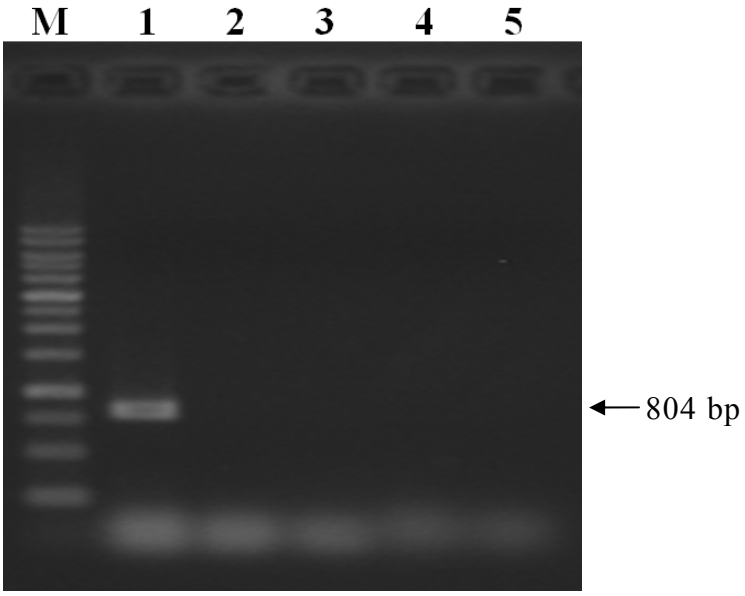


Fig. 3. Agarose gel electrophoresis analysis of the effects of tissue homogenate volume in GES buffer on the detection efficiency of *Konjac mosaic virus*-infected calla lily. M, 1 kb DNA ladder; lanes 1-5, 1, 2, 3, 4 and 5 ul of tissue homogenates in GES buffer. One representive gel image of three replicate experiments is shown.

Detection of four potyviruses by RT-PCR with new developed protocol

Two experiments were designed to examine whether new developed protocol was suitable for potyvirus detection in calla lily and mucilaginous plants. First, I examined whether the protocol could detect calla lily-infecting potyviruses. YG sample preparation buffer successfully extractd KoMV, ZaMMV, DsMV and TuMV viral RNA from virus-infected calla lilies and yielded approximate 804 bp, 792 bp, 457 bp and 1077 bp amplicons by RT-PCR, respectively (Fig. 4). Secondly, I examined whether the protocol could detect DsMV and KoMV in different mucilaginous plants. The results showed that DsMV was detected in four mucilaginous plants, calla lily, Chinese yam, taro and giant elephant's ear, and KoMV was also detected in calla lily, Chinese yam and taro (KoMV-infected giant elephant's ear was not available)(Fig. 5). New developed virus detection protocol worked well not only in calla lilies but also in other mucilaginous plants.

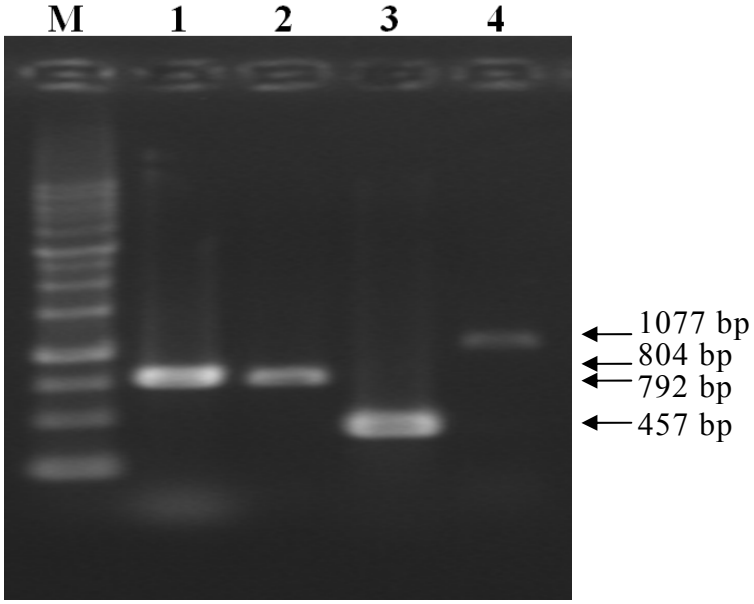


Fig. 4. RT-PCR results of crude RNA extraction by YG sample preparation buffer for the detection of four potyviruses in calla lilies. M, 1 kb DNA ladder; lane 1, *Konjac mosaic virus*; lane 2, *Zantedeschia mild mosaic virus*; lane 3, *Dasheen mosaic virus*; lane 4, *Turnip mosaic virus*. One representive gel image of three replicate experiments is shown.

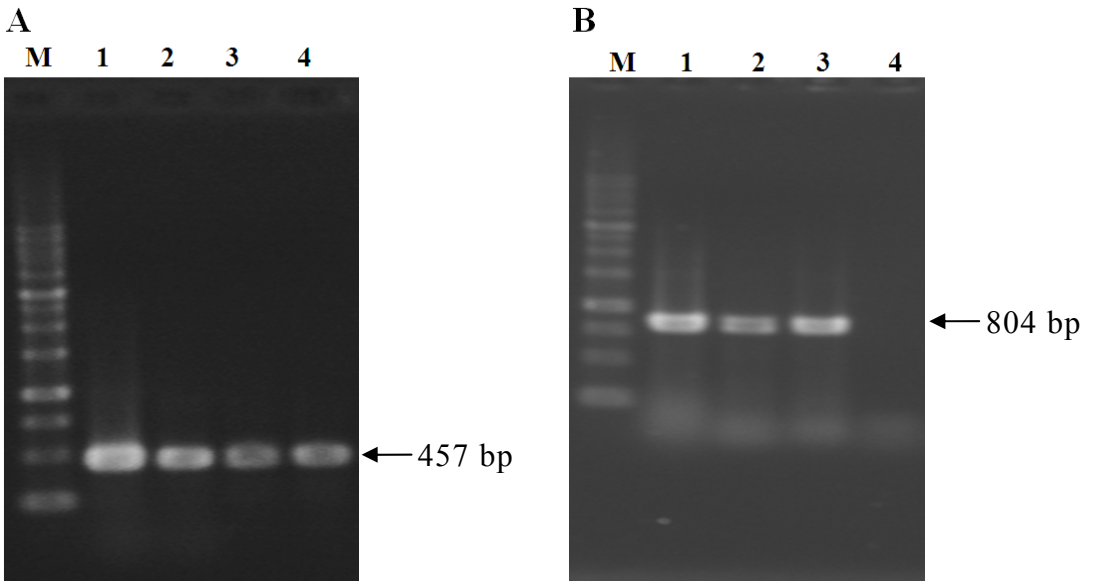


Fig. 5. RT-PCR results of crude RNA extraction by YG sample preparation buffer for the detection of *Dasheen mosaic virus* (A) and *Konjac mosaic virus* (B) in four mucilaginous plants. M, 1 kb DNA ladder; lane 1, calla lily; lane 2, Chinese yam; lane 3, taro; lane 4, giant elephant's ear.

Detection sensitivity of new developed protocol

The detection sensitivity of crude RNA extraction following with RT-PCR was compared with I-ELISA and TRIzol RNA extraction following with RT-PCR. New developed virus detection protocol was a thousand times more sensitive than the RT-PCR detection with TRIzol extracted RNA (Fig. 6). RT-PCR with TRIzol extracted RNA detected KoMV until the first dilution (10^{-1} fold), but RT-PCR with YG RNA extraction detected KoMV until the fourth dilution (10^{-4} fold). In contrast to RT-PCR, I-ELISA had the lowest

detection sensitivity. I-ELISA only detect KoMV in infected tissues by original lysates without dilution (Fig. 6). The new developed virus detection protocol was the most sensitive method to detect potyviruses in calla lily.

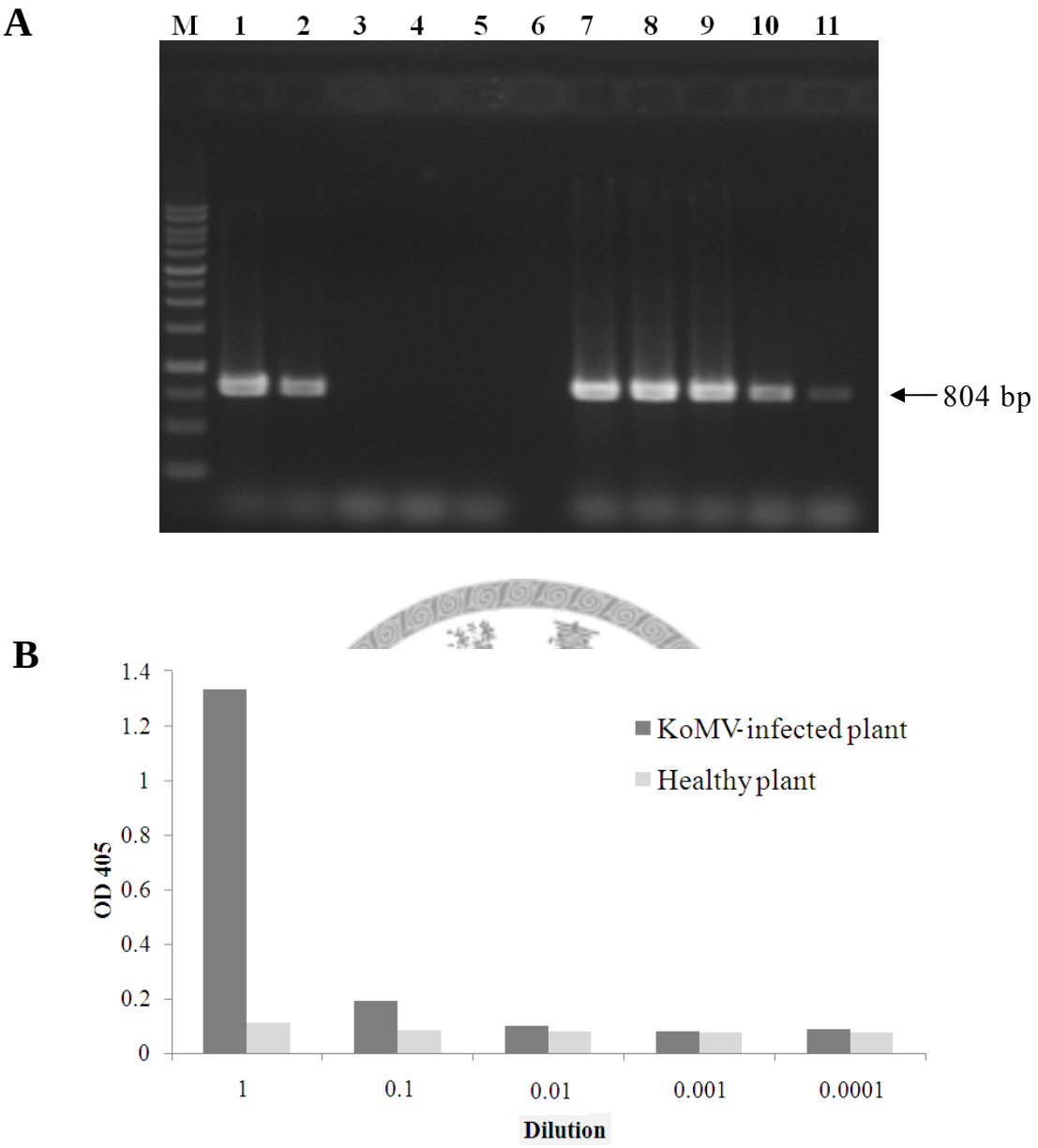


Fig. 6. Sensitivity of three *Konjac mosaic virus* (KoMV) detection assays for calla lily. (A) RT-PCR results of two RNA extraction methods. M, 1 kb DNA ladder; lanes 1-5, Trizol RNA extraction, samples were serially diluted from 1 to 10^{-4}; lanes 7-11,YG buffer crude RNA extraction, samples were serially diluted from 1 to 10^{-4}. One representive gel image of three replicate experiments is shown. (B) Indirect enzyme-linked immunosorbent assay, samples were serially diluted from 1 to 10^{-4}.

DISSCUSSION

A rapid, sensitive and inexpensive RT-PCR-based protocol with an improved sample preparation for the detection of potyviruses in mucilaginous plants was developed. Efficient RNA extraction was made possible by sample preparation buffer with some additives (e.g. PVP-40 and Na_2SO_3) to counter PCR inhibitory substances in tissue lysates. The selected sample preparation buffer (i.e. YG buffer) and optimized protocol could detect potyviruses from Araceae and Dioscoreaceae plants. New developed protocol was more sensitive than I-ELISA and surprisingly even more sensitive than TRIzol RNA extraction following with RT-PCR. The protocol would be very useful to study disease epidemiology and to assist with the control of viral diseases of aroid plants.

The results showed that crude RNA extract from potyvirus-infected mucilaginous plants could be amplified by RT-PCR for virus diagnosis and detection. For sample preparation buffer, YG sample preparation buffer yielded a better sensitivity than EGB, TES and CS sample preparation buffers for potyvirus detection in calla lily. CTAB is one of the frequently used additives for crude RNA extraction to eliminate polysaccharide and phenolic compounds in plant tissues, but CTAB-based RNA extraction failed to yield RT-PCR

product with our test samples. The results suggest that the additives in YG sample preparation buffer are more efficient to relieve RNA inhibitory present in leaf tissues of calla lily. New developed protocol used YG buffer to perform crude RNA extraction and RT-PCR to amplify the viral RNA in the tissue lysates of potyvirus-infected mucilaginous plants. The crude RNA extraction, TRIzol RNA extraction and I-ELISA assays were compared with serial dilution of test samples to evaluate the sensityvity of these methods for virus detection. New developed protocol showed the best sensitivity for potyvirus detection in mucilaginous plants. The results indicate the quality of YG RNA extraction was comparable, even better than that of RNA extracted by a phenol-chloroform method. According to our observation, TRIzol reagent did not remove all inhibitory substances from mucilaginous plant tissues because there were a lot of glutinous substances supposed to be polysaccharides coprecipitated with RNA. Phenol-chloroform method dose not efficiently remove all polysaccharides in leaf tissues (Gasic *et al.*, 2004). These inhibitors coprecipitated with RNA resulted in the reduction of detection sensitivity when compared with YG crude RNA extraction. YG crude RNA extraction was a

rapid method to prepare pre-PCR samples, and additives in YG sample preparation efficiency relieve the inhibitory for following RT-PCR.

Diagnostic PCR is a rapid, specific, and sensitive molecular tool for the detection of microbial infections and genetic diseases (Kumar *et al.*, 2011b). However, the sensitivity and kinetics of PCR may be dramatically reduced when biological samples are directly used as templates in PCR. PCR inhibitory substances presents in blood, feces, soil and plant materials have been identified (Demeke and Jenkins, 2010). To improve diagnostic PCR for routine analysis, the pre-PCR processing of biological sample is crucial for the robustness and reliability of the diagnostic method. Woody plants contain rich polyphenols, whereas aroid plants are rich with starch, glycogen and calcium oxalate crystal (Maga, 1992). Phenolic compounds are readily oxidized to form covalently linked quinines and avidly bond nucleic acids which caused irreversible damage to RNA (Gonzalez-Mendoza *et al.*, 2008). Polyphenols, starch and calcium are found to be PCR inhibitory substances in biological samples (Wadowsky *et al.*, 1994; Wiedbrauk *et al.*, 1995; Demeke and Jenkins, 2010). Many sample preparation methods have been developed to exclude PCR-inhibitory substances and produce more homogeneous samples for PCR

assay to ensure reproducibility of the test (Radstrom *et al.*, 2004). PVP-40 compete RNA form complexes with polyphenols to free the RNA (Kumar *et al.*, 2011a). β-mercaptoetanol is used to inhibit RNase activity, prevent sample oxidation and oxidize phenolic compounds that damage RNA (Zhen *et al.*, 2011). Additives in YG buffer remove the PCR inhibitors and/or reduced oxidative reactions.

New developed protocol is rapid, simple, inexpensive and more reliable compared with TRIzol RNA extraction following with RT-PCR detecting potyviruses in mucilaginous plants. Sample preparation time is largely shorten when using YG crude RNA extraction to replace TRIzol RNA extraction. TRIzol RNA extraction is laborious, time consuming and expensive and even does not provide the desired quality of RNA template for RT-PCR. In contrast, new developed protocol eliminates laborious RNA extraction, and still keeps the high sensitivity of RT-PCR assay. New developed protocol is also time-saving and more sensitive compared with ELISA. RT-PCR after YG crude RNA extraction is done within two hours, but ELISA takes morethan a day to finish all steps. The protocol developed in this study dose not require

hazardous chemicals and many incubation and centrifugation steps, so it is a rapid, simple, sensitive and inexpensive protocol.

In summary, new developed protocol is rapid, reliable, sensitive, inexpensive, easy to perform and applicable to screen large numbers of field samples. To study virus epidemiology, it is crucial to know the prevalence of a virus in the field and alternative hosts of the virus and vector populations. The improved sample preparation method following with RT-PCR developed in this study not only detects major viral diseases in calla lily fields, but also detects viruses in crops and weeds (e.g. taro , giant elephant's ear and Chinese yam) in neighborhood.

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