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海芋 potyvirus 之基因體序列分析、快速檢測及應用 RNAi 防治其感染之研究

Studies on genome sequence, rapid detection and RNAi-mediated control for calla lily potyviruses

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目錄

中文摘要

海芋為天南星科，馬蹄蓮屬之多年生球根花卉。為廣受喜愛之觀賞花卉，因此也成為國際間重要之經濟花卉作物。海芋原產非洲，引進台灣已超過十年，除了組織栽培技術已開發成熟外，也培育出許多新品系。然而病毒病害防治仍為彩色海芋培育之重點。目前被報導過可感染海芋之植物病毒已有 9 屬 18 種。其中長絲狀病毒之 *Potyyvirus* 是最早被報導，且已知可感染之病毒種類較多的一屬。*Potyvirus* 為單鏈正意股 RNA 病毒，其基因體大小約 10 kb。而在台灣以 *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TuMV)以及 *Zantedeschia mild mosaic virus* (ZaMMV) 和 Zantedeschia mosaic virus (ZaMV)等四種 potyvirus 為感染海芋之重要病毒。由於 *Potyyvirus* 屬病毒為目前已知數量最多之植物病毒，為能便於檢測及了解其分子特性，因此首先發展能快速選殖及分析 potyvirus 全長度基因體序列之策略。此方法應用 *Potyvirus* 屬之多組廣效性引子對進行 RT-PCR 並配合 terminal transferase 修飾病毒 5'端序列，以 RT-PCR 獲得病毒 5'端序列，經解序及序列分析後可獲得病毒之正確全長度序列。廣效性引子對之設計則是利用目前已知病毒之胺基酸序列進行多序列排併分析，分別選取選取 NIb、CI 及 HC-Pro 基因之高保守性區域設計出 NIbF1、CIF2/CIR2 及 HCF4/HCR4 等引子。以此廣效性引子對分別對七種不同之 potyvirus 進行 RT-PCR 測試，可獲得預期大小之 PCR 產物。利用此策略成功獲得兩種海芋重要病毒 ZaMMV 及 ZaMV 之 5'端及全長序列。經序列分析後發現 ZaMV 與 *Konjac mosaic virus* (KoMV)應為同種病毒。另一方面，為能快速且同時檢測 DsMV、TuMV、ZaMMV 以及 ZaMV 四種病毒，利用病毒專一性引子對研發多引子對 RT-PCR 檢測法，並加入植物之 *nad5* mRNA 之專一性引子對，作為增幅植物樣品的內在對照。經由專一性及靈敏度測試後，發現此方法可在一次反應中成功的偵測出不同病毒，且其靈敏度高於利用抗體進行之酵素連結抗體免疫法(ELISA)二十五倍以上。除檢測方法開發外，

本論文亦希望研究利用基因沉寂(RNA silencing)機制引發植物對入侵病毒的抗性作用。同樣以 DsMV、TuMV、ZaMMV 以及 ZaMV 四種病毒為對象。分析其胺基酸序列中高保守性區域，依照 TuMV 序列設計數種專一性引子對，藉由 PCR 將不同長度之 HC-Pro, NIa 及 CP 基因片段擴增並選殖至 LITMUS 38i 載體，用以產生病毒雙股 RNA (dsRNA)。當以 dsRNA 與 TuMV RNA 共同接種於 *Nicotiana benthamiana* 植物，可得知較短之 dsRNA 片段對於病毒侵入之干擾效果較長片段為差。為進一步在植物上進行分析，將兩段分別位在 HC-Pro 及 NIa 基因，且感染效果較佳之病毒片段，利用農桿菌轉型方式(*Agrobacterium*-mediated infiltration)在植物上表現其雙股之 hairpin RNA (hpRNA)。除了有效的干擾 TuMV 感染外，似乎對於另一種 potyvirus，*Bean yellow mosaic virus* (BYMV)也稍具干擾性。因此將上述兩段含 hpRNA 之載體送入菸草，並獲得轉基因菸草。對於 T0 及 T1 植物初步分析可發現，80%以上的 hpRNA 轉基因菸草皆對 TuMV 感染具有抗性。

Abstract

Calla lily (*Zantedeschia* spp.), belonging to the family Araceae, are perennial bulbous flowers. Because calla lily is a favorite ornamental flower, it becomes an important economic flower crop worldwide. Calla lily original from Africa, and have been introduced into Taiwan more than 10 years. Tissue culture technique has been developed for calla lily propagation and many calla hybrids have been bred in Taiwan. However, the viral disease control is important in calla lily cultivation. There are already 18 viruses have been reported which belonging to 9 genus. *Potyirus* is first reported and mainly calla lily-infecting virus in the field. Potyvirus is a positive sense stranded RNA virus with ~10 kb genome. Four viruseses are important in Taiwan, including *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TuMV), *Zantedeschia mosaic virus* (ZaMV), and *Zantedeschia mild mosaic virus* (ZaMMV). For detection and molecular characterization of potyviruses, the largest plant virus, full-length cloning and sequencing strategy was developed. The RT-PCR-based methods for detection and identification of virus are based on the use of degenerate primers for RT-PCR amplification, combined with modified 5'RACE by using terminal transferase to modify the 5' end sequence of potyviruses genome. The complete sequence would be identified by cloning and sequencing. The degenerate primers were designed from conserved sequences in the viral genome. According to the

sequence alignment, theNIbF1, CIF2/CIR2, and HCF4/HCR4 were designed from potyviral NIb, CI and HC-Pro-coding regions. Expected PCR products were amplified by these primers from seven potyviruses. Complete genome sequences of ZaMMV and ZaMV, were successfully characterized. The sequence analysis reveals that ZaMV is the same with the *Konjac mosaic virus* (KoMV). On the other hand, in order to save time and simultaneous detection of DsMV, TuMV, ZaMV and ZaMMV in field, a multiplex RT-PCR assay was developed for these calla potyviruses. Specific primers for each virus were designed based on the sequences of 3' terminal region of respective viruses. To prevent the false negative results, a primer pair specific to plant mitochondrial *nad*5 mRNA was used as an internal control of RT-PCR. After specific and sensitivity test, the multiplex RT-PCR can rapidly detect multiple targets in one single assay, and the detection sensitivity of multiplex RT-PCR was 25-625 times higher than that of I-ELISA depending on the virus. Furthermore, a control method that prevents the virus infection using the mechanism of RNA silencing was investigated. A dsRNA expression and screening system was used to obtain highly efficient interference fragments from the consensus regions of four calla lily-infecting potyviruses, DsMV, TuMV, ZaMMV and ZaMV. The viruses were chosen for multiple sequence alignment. Several TuMV specific primers were designed within the conserved regions of HC-Pro, NIa and CP genes. Different fragments were PCR

amplified and cloned into LITMUS 38i vector to produce viral dsRNA. Mechanical inoculation of different dsRNA transcripts with target virus on tobacco plants induced different levels of interference with virus infection. Two of the most effective fragments (located in HC-Pro and NIa genes) were further analyzed by using *Agrobacterium tumefaciens* infiltration (agoinfiltration) as transient expression system for hairpinRNA (hpRNA) expression. The successful interferences of TuMV infection were observed in the infiltrated leaves. Besides, *Bean yellow mosaic virus* (BYMV), another calla lily-infecting virus, was interfered by TuMV hpRNAs transiently expressed in plants. Several lines of transgenic *Nicotiana benthamiana* plants transformed with hpHC and hpNIa fragments were obtained. Over 80% of T0 and T1 transgenic plants revealed resistance response to TuMV infection.

Introduction

Brief preface of calla lily

Calla lily is the common name of the genus *Zantedeschia* spp. The genus *Zantedeschia* belongs to the *Aracreae* family. There are seven species and two subspecies of *Zantedeschia*: *Z. aethiopica* (L.) Spreng., *Z. rehmannii* Engl., *Z. jucunda* Letty, *Z. elliottiana* (Watson) Engl., *Z. pentlandii* (Watson) Wittm., *Z. odorata* P. L. Perry, *Z. albomaculata*, *Z. albomaculata* (Hook.) Baill. subsp. Valida Letty (Kuehny, 2000). It is a tropical plant native in Africa. Now the crops are as outdoor garden plants, commercial cut flowers and flowering potted plants. New calla hybrids have been bred for ornamental purpose. The common disease of calla lily is bacterial rot caused by *Erwinia carotovora* subsp. *carotovora* and virus diseases (Chen *et al*., 2003; Kuehny, 2000).

Characterization of Potyvirus

Potyvirus is the one of largest plant virus groups, belonging to the family of *Potyvidiae*, genus of *Potyvirus*. The family *Potyviridae* is classified into six genera including: *Ipomovirus*, *Potyvirus, Macluravirus*, *Rymovirus*, *Tritimovirus*, and *Bymovirus* based on vector transmission and genomic relatedness (Adams *et al*., 2005). Members of the genus *Potyvirus* have particles at least 700 nm in length which

encapsidate a monopartite ssRNA genome 10 kb in length. The genome is characterized by a 5' untranslated region (5'UTR) which is linked to a genome-linked protein (VPg), a single major open reading frame (ORF) and a 3' UTR region terminating in a polyadenylated (polyA) tail (Ha *et al*., 2008). The genome organization is conserved in the genus and contains ten mature proteins: P1, helper component protease (HC-Pro), P3, 6k1, cylindrical inclusion protein (CI), 6k2, small nuclear inclusion protein (NIa; including the VPg and protease (NIa-Pro) domains), large nuclear inclusion protein (NIb) and coat protein (CP) (Adams *et al.*, 2005).

The first protein of the genome, P1, is the least conserved among potyviruses. The function of P1 involved in amplification was supposed to its correlation with HC-Pro because it was able to enhance HC-Pro-mediated suppression of RNA silencing (Pruss *et al*., 2004; Kasschau and Carrington, 1998).

The HC-Pro protein named after its dual features of helper component (HC) for aphid transmission and protease activity is an important viral product that is involved in many functions during the virus life cycle (Urcuqui-Inchima *et al*., 2001). Mutagenesis studies and sequence alignments suggest that HC-Pro can be schematically divided into three regions. N-terminal region of HC-Pro is required for the aphid transmission. Three conserved motifs Ileu-Gly-Asn (IGN), Cys-(Cys/Ser)-Cys (CC/SC) (CCC) and Pro-Thr-Lys (PTK) have been found in

HC-Pro (Cronin *et al*., 1995). Many plant viruses have evolved counterstrategies to knock out post-transcriptional gene-silencing (PTGS) and encode PTGS suppressors. In potyviruses, HC-Pro protein acts as a PTGS suppressor to interfere with plant defense through the suppression of PTGS mechanisms (Kasschau *et al*., 1998).

Among potyviruses, there is a relatively little similarity in P3 proteins compared to other proteins (Urcuqui-Inchima *et al*., 2001). P3 is postulated to participate in replication by direct interacting with NIb which is part of replication complex (Merits *et al*., 1999). The cylindrical inclusion protein (CI) has been proved to have RNA binding and RNA helicase activity (Kadare and Haenni, 1997). Genetic analysis indicated the CI protein is also involved in cell-to-cell passage of viral RNA-protein complexes (Carrington *et al*., 1998). On the other hand, like P3, CI is also regarded as a determinant of virulence to a plant resistance gene (Jenner *et al*., 2002). The potyviral 6K2 protein has been shown to be membrane associated (Restrepo-Hartwig and Carrington, 1994).

NIa is one of the nuclear inclusion proteins and is composed of the N-terminal viral genome-linked protein (VPg) domain and the C-terminal NIa-proteinase (Pro) domain (Schaad *et al*., 1997). NIa is a major proteinase for producing most functional viral proteins from polyprotein (Urcuqui-Inchima *et al*., 2001). NIb also a nuclear inclusion protein is the viral RNA-dependent RNA polymerase (RdRp) responsible

for genome replication of potyviruses, but how the interaction affects viral replication is unknown (Urcuqui-Inchima *et al*., 2001).

The coat protein (CP) is the only structure protein of potyvirus and is also involved in several functions including aphid transmission, viral movement, RNA amplification and encapsidation (Urcuqui-Inchima *et al*., 2001).

The full-length cloning and sequencing strategy of potyviruses

The genome of potyvirus is a single-stranded, positive sense RNA, with a 3' polyA tail, similar to mRNA in plant cells. Therefore, RT-PCR is used for viral genome sequence identification. The complete genome of potyvirus has typically been obtained by constructing cDNA library or by primer walking using primers designed from the polyA sequence and degenerate primers designed on conserved genomic regions (Ha *et al.*, 2008).

For the 5' end sequence acquired, the strategies applied in 5'end mapping of mRNA are used in the RNA virus, including primer extension, cDNA library technique, and rapid amplification of cDNA ends (RACE), etc. The former two methods require relatively large amounts of RNA and often possess difficulty in identifying the 5' end of rare RNAs. Thus, RT-PCR with 5'RCAE is the common method for full-length genome sequencing of potyvirus. The 5'RACE was designed to

amplify the unknown 5' end of a messenger RNA template using a defined internal site. Several alterations of this method were used for different purpose, as anchor RACE which introduced an oligonucleotide into the RNA or first-strand cDNA by RNA ligase; cRACE (circular first-strand cDNA-mediated RACE) were reported (Maruyama *et al*., 1995). Several commercial products were already developed.

PCR-based detection methods for plant RNA viruses

Several detection methods for plant RNA viruses were developed including protein-based enzyme-linked immunosorbent assay (ELISA) which is common usage method for many kinds of viruses. The other methods are PCR-based methods including reverse transcription-polymerase chain reaction (RT-PCR), immunocapture RT-PCR (IC-RT-PCR), multiplex RT-PCR and real-time PCR (Huang *et al*., 2005; Hu *et al*., 2007; Huang *et al*., 2007). This detection method is based on targeting to the viral nucleic acids. Most of these methods are designed for single virus detection in one reaction, except multiplex RT-PCR and TaqMan real-time RT-PCR (Lee and Chang, 2008). The multiplex RT-PCR technique has been applied to many plants for virus detection such as apple, (Menzel et al. 2002), citrus (Roy et al. 2005), olive (Bertolini et al. 2001), potato (Nie and Singh 2000; Du et al. 2006) and orchids (Lee and Chang 2006).

dsRNA triggered-PTGS in plant virus defense

Post-transcriptional gene silencing (PTGS) is a sequence-specific RNA degradation mechanism first discovered in transgenic plants (Napoli *et al*., 1990). It is closely related to RNA interference (RNAi) or RNA silencing in animals and other organisms, as well as it represents an ancient eukaryotic genetic phenomenon, which is also involved in the defense against viruses (Tenllado and Diaz-Ruiz, 2001; Waterhouse *et al*., 2001). The pathway of PTGS is triggered by Dicer-like (DCL, the ribonuclease III-like enzymes) proteins in plants. These proteins play the role to cut dsRNAs from virus into siRNA (small interfering RNA) (Waterhouse and Fusaro, 2006). The dsRNA and hpRNA have been demonstrated that the double-stranded form RNAs can induce PTGS more efficiency than single-stranded RNA in plants (Smith *et al*., 2000). In recent years, by applying dsRNA-derived from viral sequences to induce RNA silencing and confer virus resistance in host plants was reported in various kinds of plant viruses (Tenllado *et al*., 2003; Vargas *et al*., 2008), such as the coat protein genes of different viruses (Wisniewski *et al*., 1990; Kalantidis *et al*., 2002; Vargas *et al*., 2008) and viral suppressor, HC-Pro (Negri-Nicola *et al*., 2005), or viral replicase, 54-kDa protein of *Pepper mild mottle virus* (PMMoV) (Tenllado *et al*., 1995).

Chapter I

Molecular analysis of two potyviruses in *Zantedeschia* spp. plants in Taiwan


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Abstract

The full-length genomic sequences of two potyviruses, *Zantedeschia mosaic virus* (ZaMV) and *Zantedeschia mild mosaic virus* (ZaMMV) from *Zantedeschia* spp. in Taiwan were determined. One universal d(T) primer combined with a potyviral degenerate primer, NIbF1, which was designed from the consensus sequences in NIb gene, were used to amplify the 3' portion (from polyA to 3' portion of NIb gene) of potyvirus genome by RT-PCR. Two pairs of degenerate primers located in the CI (CIF2/CIR2) and HC-Pro (HCF4/HCR4) coding regions were designed according to the conserved sequence and could amplify specific fragments of potyviruses. Virus-specific primers were designed according to the cloned sequences to amplify the intervening sequences. The 5' terminal region was cloned by 5'RACE method that used terminal transferase to add extra oligomers to the end of cDNA and followed by PCR reaction. The complete sequence of ZaMV is 9852 nts and 3096 amino acids. Sequence analysis of ZaMV showed that it has 90% nucleotide sequence identity to the *Konjak mosaic virus* F isolate (KoMV-F), but has 12 more AT-rich nucleotides compared with the 5'UTR sequence of KoMV. However, it indicated that ZaMV and KoMV is the same species of the genus *Potyvirus*. Moreover, the complete genome of ZaMMV is 9973 nts and 3176 amino acids. The sequence analysis reveals that ZaMMV is an individual member of *Potyvirus* group. This RT-PCR method along

with degerate primers was effective to obtain specific fragments from potyvirus member. Combined with the terminal transferase involved 5’ RACE method could easily acquire a full-length sequence of *Potyvirus* species. This strategy has widely appliction in detection and molecular sequencing of potyviruses.

Introduction

The genus *Potyvirus* belonging to the family *Potyviridae* contains over 200 species and more than 1,000 plant species were infected with different potyviruses (Adams *et al.*, 2005). The genome of a potyvirus is positive-sense, single-stranded polyadenylated RNA molecule about 10 kb, covalently linked with a virus-encoded VPg protein at 5' end. The genome contains a large unique open reading frame (ORF), which encodes single polyprotein. Pre-mature polyprotein is processed by three viral proteinases, P1, HC-Pro, and NIa, respectively, into 10 functional proteins: the first N-terminal protein (P1), the helper-component proteinase (HC-Pro), the P3 protein, the cytoplasmic inclusion protein (CI), the nuclear inclusion protein a (NIa) containing VPg and proteinase domains, the nuclear inclusion protein b (NIb) also the RNA-dependent RNA polymerase (RdRp), the coat protein (CP), and two small proteins 6K1 and 6K2 (Adams *et al*., 2005). According to the gene function and multiple sequence analysis, HC-Pro, CI, NIa, NIb, and CP genes showed the conserved characterization intra-genus. On the other hand, the 5'UTR, P1 protein, P3 protein, and 3'UTR region are species specific (Zheng, 2008).

Furthermore, due to the large genome size of potyvirus, complete genome sequence data is not easily acquired. Although *Tobacco etch virus* (TEV) was reported in 1921, the complete genome sequence was not published until 1986 (Allison *et al*.,

1986). Only six species of potyviruses have been completely cloned and sequenced between 1985 and 1992 including TEV, *Tobacco vein mottling virus* (TVMV) (Domier *et al*., 1986), *Plum pox virus* (PPV) (Lain *et al*., 1989; Maiss *et al*., 1989), *Potato virus Y* (PVY) (Robaglia *et al*., 1989) and *Pea seed-borne mosaic virus* (PSbMV) (Johansen *et al*., 1991), *Turnip mosaic virus* (Nicolas and Laliberté, 1992). Although observation of new species of potyvirus constantly reported in resent years, the most complete genome sequences are the new isolates of known potyvirus species. Because of the large RNA genome, reverse-transcription polymerase chain reaction (RT-PCR) method must be used in molecular cloning of potyvirus group. However, to amplify the long PCR fragments, purified viral RNA or large amount total RNA were required. Besides, it is difficult to obtain and handle whole genome or even long cDNA fragment until resent years. Another question is about the 5'UTR, the very diverge region of *Potyvirus*, and an AU-rich conserved 'Poty box' sequence is located at 5'UTR region of most *Potyvirus* species (Chen *et al*., 2001). However, the precisely sequence of the 5'-proximal end is still not easily to identify. This is the problem to acquire full-length genome sequence data of a new potyvirus.

Here we used degenerate primer RT-PCR method to amplify several species of potyviruses. According to the sequence alignments of three conserved gene regions, NIb, CI and HC-Pro, five degenerate primers named PNIbF1, PCIF2, PCIR2, PHCF4,

and PHCR4, respectively were designed to amplify the cDNA fragments of different potyviruses. For identification the 5’ end sequence of potyvirus, a modified rapid amplification of 5' complementary DNA ends (5’RACE) method was developed. This method used terminal transferase for transferring additional deoxynucleotides into the 3’end of cDNA, and followed by PCR amplification which was amplified by specific viral primer and oligo primer such as dT or dC primers, instead of RCAE kit. Using this combined method, there were at last two complete sequences of new reported potyviruses, *Zantedeschia mosaic virus* (ZaMV) and *Zantedeschia mild mosaic virus* (ZaMMV), obtained and used for sequence analysis. The other new reported potyvirus, *Basella rugose mosaic virus* (BaRMV), was also completely sequenced by the same cloning strategy (Jhu, 2005; unpublished data).

Zantedeschia spp., commonly called calla lily, belongs to the family *Araceae* and is originated from Africa. Several cultivars of *Zantedeschia* plants were introduced into Taiwan from New Zealand, the USA and the Netherlands about twenty years ago. Since 1998, a potyvirus was found in calla lilies in Taichung area, the molecular evidence suggested a new species of potyvirus at that time, and thus called Zantedeschia mosaic virus (ZaMV). Several isolates were obtained during 1998 - 2000 in Taiwan, that were named ZaMV-BG, ZaMV-DB, ZaMV-SG, and ZaMV-Zan (Chen, 1998). ZaMV could cause mosaic symptom on *Zantedeschia* spp. and

Philodendron selloum but could not infect other indicator plants such as *Chenopodium amaranticolor* (*Chenopodiaceae*), *Nicotiana benthamiana* (*Solanaceae*), etc. The virus was also detected and reported in South Korea in 2002 (ZaMV-KR) (Kwon *et al*., 2002). In addition, another potyvirus caused mild mosaic symptom of tissue-cultured seedlings of calla lily was observed in 2000 also at Taichung area by Huang and designated as *Zantedeschia mild mosaic virus* (ZaMMV). In addition to *Dasheen mosaic virus* (DsMV), the most common potyvirus in aroid plants, another potyvirus *Turnip mosaic virus* (TuMV), was also reported in Taiwan. According to the virus detection on field survey in 2003-2004 (Huang et al., 2007) and 2005-2006 (data not shown), ZaMV and ZaMMV were widespread within mid-Taiwan. To study the molecular characterization of ZaMV and ZaMMV, the complete genomic sequences of ZaMV-SG isolate (from *Zantedeschia* spp. cv. Super Gold) and ZaMMV-Zun isolate (Huang and Chang, 2005) were obtained using the degenerate primer RT-PCR and modified 5' RACE method described above. The genomes of ZaMV and ZaMMV are 9852 and 9973 nt long, respectively. Sequence analysis with other potyviruses indicated that ZaMV is the same species as *Konjak mosaic virus* (KoMV) (Nishiguchi *et al*., 2006). However, ZaMMV is a new calla lily-infecting potyvirus.

Materials and methods

Virus source and sample collection

The viruses used in this study include *Basella rugose mosaic virus* (BarMV), *Dasheen mosaic virus* (DsMV), *Potato virus Y* (PVY), *Turnip mosaic virus* (TuMV), *Zucchini mosaic virus* (ZYMV), *Zantedeschia mild mosaic virus* (ZaMMV) and *Zantedeschia mosaic virus* (ZaMV). PVY and TuMV were maintained in *Nicotiana benthamiana* plants. ZYMV was inoculated and maintained in *Zucchini squash*. In addition, DsMV, ZaMMV and ZaMV were maintained in calla lily plants. Inoculated plants were grown in pots at 25^{o}C under a 16/8-h photoperiod with 60% humidity.

Plant total RNA extraction

Total RNA was extracted from 100 mg of infected plant tissue using Plant Total RNA Miniprep System (Viogene, Sunnyvale, CA, USA) according to the manufacturer's protocol. The leaf tissue was ground into powder with liquid nitrogen and mixed with 450 μl of RX or PRX extraction buffer, the lysate was filtrated using a Shearing Tube. And then the filtrate was mixed with 230 μl of absolute ethanol, transferred to a new Plant Total RNA Mini Column. After centrifugation to remove filtrate, the column was then washed once with WF Buffer and twice with WS Buffer. Finally, plant total RNA was eluted with 50 μl of RNase-free ddH_2O. The amount and

quality of total RNA was analyzed by 1% agarose gel electrophoresis. Plant total RNA was used directly for RT-PCR or stored at -20^{o}C for further use.

Degenerate primers

Degenerate primers were designed based on amino acid sequence alignment of the conserved regions in NIb, CI, and HC-Pro genes of potyviruses. The NIb degenerate primer, NIbF1, corresponds to the 'GNNSGQP' motif. The CIF2 and CIR2 primers are corresponding to the 'TRPLAEN' and 'IENGVDLT' motifs within the CI genes. HCF4 and HCR4 degenerate primers are located in the 'PSLCDNQ' and 'KxFTKMVR' motifs in the HC-Pro gene. The sequence and corresponding motifs of universal and degenerate primers used in the genome amplification of potyvirus groups are list in Table 1.

First-strand cDNA synthesis and viral genome cDNA cloning strategy

For first-strand cDNA synthesis, 8.5 µl of plant total RNA (0.4 µg/µl) and 2 µl of 10 µM dT-Bam primer (Hu and Chang, 2004) were mixed and denatured at 65^{o}C for 10 minutes and then placed on ice for 5 minutes. For 25 µl reverse transcription (RT) reaction, 1.0 µl of AMV reverse transcriptase (10 U/µl) (Promega, Madison, WI, USA), 5 µl of AMV RT 5X reaction buffer (Promega, Madison, WI, USA), 1 µl of 10

mM dNTPs, 0.5 µl of rRNasin (40 U/µl) (Promega, Madison, WI, USA) and 7 µl of sterile distilled water were added to the previous mixture and incubated at 42 ^{o}C for 1 hour.

Double-stranded cDNA fragments were produced by polymerase-chain reaction (PCR) methods. Different strategies were used to clone viral genome from the cDNA templates. First, three degenerate primer pairs, PNIbF1/dT-Bam, PCIF2/PCIR2, and PHCF4/PHCR4, were used to amplify the conserved domains of the potyvirus NIb, CI and HC-Pro gene respectively. Second, specific primers were designed based on the nucleotide sequences of above fragments in order to amplify the intervening genomic fragments between them (Fig. 1A). PCR reactions were performed using 0.5 µl of DyNAzyme™ II DNA polymerase (2 U/µl, Finnzymes Inc., Espoo, Finland) in total 20 µl PCR mixture, containing 1 µl of 10 mM dNTPs, 1 µl each of 5 mM sense and antisense primers, 2 µl RT product, 2 µl of 10X DyNAzyme™ II DNA polymerase buffer and 12.5 µl sterile distilled water. The amplification protocol involved one cycle of 94^{o}C for 5 min; 26~28 cycles of 94^{o}C for 30 sec, 55^{o}C for 35 sec; 72^{o}C for 2 min; and a final 72^{o}C extension for 7 min. The annealing temperature and extension time of individual PCR reaction depended on the primer pair used. PCR products were analyzed by 1% agarose gel electrophoresis, and expected amplification products were eluted by GFX™ PCR DNA and Gel Band Purification Kit (GE

Healthcare, Buckinghamshire, UK). The purified PCR fragments were ligated into the pGEM-T® Easy Vector (Promega, Madison, WI, USA) or pCR®II-TOPO® Vector (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol, and transformed into *E. coli* DH5α. After blue white selection and the colony PCR screening, plasmid DNAs were purified by Mini-M™ plasmid DNA Extraction System (Viogene, Sunnyvale, CA, USA) and checked by the restriction enzyme digestion to get correct clones for advanced sequencing.

5' RACE method

Sequence of 5'-terminal of virus genome was determined using 5' rapid amplification of cDNA ends (RACE) procedure. The 5'RACE method added extra nucleotides to the first-strand cDNA-end by terminal transferase. A virus specific primer was used to prime first-strand cDNA synthesis as described above except the dT-Bam primer was replaced by viral specific primers near the 5' end. After RT reaction, the cDNA was precipitated with ethanol and resuspended in one third volume of distilled water. To add homopolymeric dC or dG tail to the cDNA 3' end, 10 µl of condensed RT product, 0.5 µl Terminal Transferase (20 U/µl, NEB, Beverly, MA, USA), 5 µl of 10X NEB Buffer 4, 5 µl of 2.5 mM $CoCl_2$, 1 µl of 10 mM dCTP (or dGTP) and 28.5 µl distilled water were mixed and incubated at 37^{o}C for half hour.

The enzyme was then inactivated by incubating the reaction solution at 70^{o}C for 20 minutes. dG-Not (to complement the dC oligo-mer), dC-T7 (complementary of dG oligomer) and virus-specific primers were used for PCR reaction. To obtain authentic 5' terminal sequence, modified RACE was performed with dG-Not or dC-T7 primer and another specific primer closer to the 5' end of virus genome than the primer used for first-strand cDNA synthesis.

Sequencing and sequence comparison

The cDNA clones were sequenced using universal primers or virus specific primers with ABI PRISM® BigDye™ terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Automated sequencing was done on ABI PRISM™ 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, CA, USA). Each nucleotide of the viral genome was determined by sequencing at least two independent clones. Sequence analyses and assembly were done using programs from the Wisconsin (GCG) package. The sequence comparisons of ZaMV and ZaMMV to known viral sequences using BLAST program at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov/NCBI). The entire ORFs comparisons with other potyviruses were performed using program Align X in Vector NTI Suit 9.0

(Invitrogen) and ClustalX (Thompson *et al*., 1997). The alignment results were exported by GeneDoc program (Nicholas *et al*., 1997).

.

Results

Conserved degenerate primers designation

Amino acid sequences of potyviruses were aligned and used for degenerate primer design. The first primer set, HCF4/HCR4, corresponding to the 'PSLCDNQ' motif in the central region and 'KxFTKMVR' motif in C-terminal of HC-Pro gene were designed to amplify an approximately 0.6-kb product within HC-Pro coding region. The second pair of degenerate primers, CIF2/CIR2, were designed based on the sequence of 'TRPLAEN' and 'IENGVDLT' motifs in mid-region of CI gene and were able to amplify about 0.6-kb fragment from potyvirus groups. For the 3' terminal region cloning, the 'GNNSGQP' conserved motif of NIb (RNA dependent RNA polymerase) gene was selected for NIbF1 primer (Table 1).

Degenerate primer RT-PCR amplification assay

To evaluate the specificity of the degenerate primers, seven potyviruses species were tested by RT-PCR using dT-Bam/NIbF1, CIF2/CIR2 and HCF4/HCR4 primer pairs. In the RT reaction, dT-Bam primer reacted with the poly-A tail of viral RNA present in total RNAs of infected plants. A 1.6 ~ 1.8-kb cDNA fragments were then amplified by PCR using NIbF1 and dT-Bam primers from BarMV, DsMV, PYV, TuMV, ZaMMV, ZaMV, and ZYMV-infected plant samples (Fig. 2A). The

amplification efficiency varied in different virus-infected samples. It could be due to either the variation of the virus titer in infected plants or the non-optimized RT-PCR reaction, etc. The CIF2/CIR2 and HCF4/HCR4 primers amplified fragments of expected ~0.6-kb products from the same total RNA samples of seven potyviruses (Fig. 2B, and 2C). The results of RT-PCR suggested that degenerate primer-based PCR method is useful in amplification of several members of *Potyvirus* species. It indicated the application of these primers not only in cloning of specific fragments but also in potyvirus detection.

Amplification and sequencing of the complete genomes of ZaMMV and ZaMV

According to the degenerate primer and RT-PCR-based strategy, three expected size fragments which were amplified from HC-Pro, CI, and NIb regions of ZaMV and ZaMMV-infected samples were cloned into pGEM-T® Easy Vector. After colony PCR selection, three independent clones were sequenced. Sequencing data showed that the dT-Bam/NIbF1 amplified DNAs contained nucleotide sequences corresponding to the partial NIb gene, complete CP gene and 3' UTR followed by a poly-A tail. Three 0.6-kb fragments amplified by CIF2 and CIR2 clones were proved to contain part of CI gene. And other three clones derived from HCF4 and HCR4 were confirmed to include the HC-Pro fragment. The subsequent RT-PCR using

virus-specific primers which were designed from previously sequenced fragments was to amplify and obtain the interval sequences (Fig. 1A).

The 5'-terminal sequence of viral genomic RNA including 5'UTR was resolved by using the 5'RACE method. Specific primer located at 5'-end of sequenced HC-Pro gene was used to initiate the synthesis of first-strand cDNA of 5'-terminal sequence of viral RNA. After dC (ZaMMV) or dG (ZaMV) tailing of cDNA was performed, PCR was carried out with dG-Not or dC-T7 and virus-specific primers. PCR products were analyzed by agarose gel and about 2 ~ 3-kb cDNA fragments were eluted from the agarose gel and cloned into vector as before. The 5'-terminal region consists of complete 5'UTR, P1 gene and part of HC-Pro gene. To confirm the sequence of the 5'-terminal region of the viral genome, 5'UTR was amplified again by PCR using dG-Not (dC-T7) and specific primer near the 5' end of P1 gene with the same cDNA template tailing with oligo-nucleotids (data not shown).

The full-length of ZaMMV is 9973 nts excluding polyA tails, and encoded an open reading frame of 3176 amino acids. The genome of ZaMV is 9852 nts and encodes a putative polyprotein of 3096 amino acids. The 5'UTR of ZaMV and ZaMMV were A/T-rich with terminated in several A residues, typical of other potyviruses (Fig. 3A). The 5'UTRs also contained sequences similar to the potybox-like blocks: "TGAACAG" in ZaMMV and "TCAACAG" in ZaMV (Fig.3A)

(Chen *et al*., 2001; Ha *et al*., 2008).

Sequence comparisons

The entire ORF of ZaMV and ZaMV were compared with other potyviruses using BLAST program. The result revealed that ZaMMV is most similar to *Soybean mosaic virus* (SMV) (68% amino acid sequence similarity of polyprotein). The P1 protein of ZaMMV had 50% sequence similarity to *Bean common mosaic virus* (BCMV-NL1). The amino acid sequence comparisons of polyprotein confirmed the phylogenetic analysis data of CP sequence, that ZaMMV belongs to the BCMV subgroup (Huang and Chang, 2005). Comparison of the putative amino acid sequence of HC-Pro among DsMV, TuMV, ZaMMV and ZaMV revealed the similarity is ranging from 60% ~77%, and HC-Pro gene of ZaMMV is closer to DsMV (77% similarity) than TuMV and ZaMV. Sequence comparisons also revealed several functional motifs of HC-Pro present in ZaMMV and ZaMV genomes such as PTK motif involved in aphid transmission, and CCC, the systemic movement functional motif (data not shown) (Cronin *et al*., 1995).

However, the sequence comparison of the full-length sequence of ZaMV and KoMV revealed high homology (96% amino acid sequence similarity, 90% nucleotide sequence identity) (Table 2). It indicated that ZaMV is the same species as

KoMV. Interestingly, the 5'UTR sequence of KoMV is 152 nt but ZaMV is 167 nt. The sequence identity between KoMV and ZaMV is 81% in 5'UTR region, which is lower than entire genome sequence. However the 3'UTR alignment shows 93% sequence identity between them (data not shown). In addition, the complete sequence of ZaMV compared with KoMV and other three calla lily-infecting potyviruses including DsMV, TuMV, and ZaMMV indicated these viruses are distinct species, except KoMV and ZaMV (Table 2). Moreover, the 5'UTR comparison revealed 50% sequence identity between DsMV and ZaMV (Table 2). The sequence alignments of DsMV and ZaMV showed there is a highly identical 40-bp sequence at 5'end of 5'UTR between these two viruses (Fig. 3B).

Discussion

In this work, several degenerate primers were designed for potyviruses. The GNNSGQP region of the NIb gene is highly consensus in members of *Potyvirus* group. In 1997, Gibbs and Mackenzie reported a primer that matched the GNNSGQ motif of the NIb and used the primer to amplify cDNA from potyviruses as well as other genera in the family *Potyviridae* (Gibbs and Mackenzie, 1997). A few years later, Chen *et al*. published a degenerate primer which was extended version of the GNNSGQP primer with a few more nucleotides in 2001 (Chen *et al*., 2001). In this study, the NIbF1 primer also corresponds to GNNSGQP motif and is successfully used for RT-PCR amplification of potyviruses.

The CIF2 and CIR2 degenerate primers located on the segment Ia and segment V within the CI gene (Kadare and Haenni, 1997) are different from the CIFor and CIRev degenerate primers which were designed in segment I and V of CI gene (Ha *et al*., 2008). Besides, the fragments amplified by CIFor/CIRev are ~100 bp longer than CIF2/CIR2. The degenerate primers of HC-Pro, HCF4/HCR4, designed to amplify the 3' half region of the HC-Pro gene of potyvirus (Fig. 1A) are also different from the recently published degenerate primers HCFor and HCRev (Ha *et al*., 2008). Using these degenerate primers could easily amplify the central (CI) and near 5' region (HC-Pro) of potyviral genome.

For complete genome sequencing of potyvirus, 5'RACE is a useful method. To improve the anchoring efficiency of the first strand cDNA, the terminal transferase was used for transferring the homopolymer into cDNA end instead of adaptor ligation or oligomer annealing of other RACE method. Because of the A residues often in the first 1 ~ 4 nt of 5'UTR region of potyviruses, dC or dG was chosen for modification the cDNA end. The advantages of this strategy include: 1) easily amplifying the genome fragments of unknown sequence of new potyvirus species and 2) efficiently obtaining the variable 5' region of potyviruses. Besides the application to complete sequencing of potyvirus genome, the concept of this strategy is simple and suitable for other plant viruses as long as the conserved degenerate primers exist.

Using the degenerate primers in combination with genome-specific primers and modified 5'RACE. We characterized the complete genomes of two calla lily-infecting potyviruses. Through the sequence analysis, ZaMMV is proved a distinct species of potyviruses as previously reported (Huang and Chang, 2005). Although the sequence analysis indicated ZaMV should be an isolate or strain of KoMV, the biological characterization of these viruses is not similar. The major difference of these two viruses is the host range. KoMV F was isolated from konjak plants with mosaic symptoms but also showed a wide range of host plants including *Aizoacea*, *Araceae*, *Chenopodiacea* and *Solanacea* plants (Nishiguchi *et al*., 2006). In contrast to KoMV,

ZaMV has a narrow host range only in *Araceae* plants (Chang *et al*., 2001; Kwon *et al*., 2002). The other difference is the 5'UTR sequence between ZaMV and KoMV. When compared the first 50 nt of 5'UTR region of ZaMV and KoMV with other eight potyviruses, all show AAA(A/T)TAAA at the first eight residues except KoMV (Fig. 3A). Because the biological features are so different in these two viruses, we have no information about the infectivity of the ZaMV isolated from Taiwan or Korea to konjak (*Amorphophallus konjac* K. Koch, family *Aracea*) plant and vice verse. This means more molecular and biological information are needed before we can understand the relationship of ZaMV and KoMV.

The full-length cloning and sequencing strategy developed in this study successfully detected numerous of potyviruses including a new potyvirus, BarMV (Huang and Chang, 2006) and several other viruses: DsMV, PVY, TuMV, and ZYMV. Due to the efficiency and convenience, using this method should promote the molecular characterization of potyvirus, the important group of plant RNA viruses.

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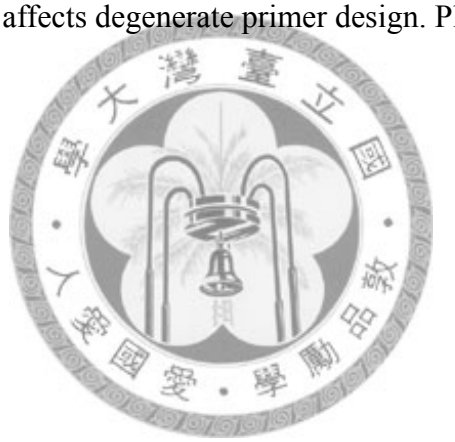

Table 1. Universal and degenerate primers used in this study

Primer	Sequence (5'- 3')[a]	Conserved motif	Usage
dT-Bam	AGCTGGATCCTTTTTTTTTTTTTTTTT		3' first-strand primer
dC-T7	TAATACGACTCACTATAGGGCCCCCCCCCC		5'RACE PCR primer
dG-Not	GATGCGGCCGCGGGGGGGGGGGGGGGGG		5'RACE PCR primer
PNIbF1	GGBAAYAATAGTGGNCAACC	GNNSGQP	Potyvirus NIb gene primer
PCIF2	ACCCGGCCCCTGGCNGARAAYGT	TRPLAEN	Potyvirus CI gene primer
PCIR2	GTCCAGGGTCACGCCRTTYTCDAT	IENGVDLT	Potyvirus CI gene primer
PHCF4	CCTCCCTGCTGTGCGATAATCAGCT	PSLCDNQ	Potyvirus HC-Pro gene primer
PHCR4	CGCGCACCATCTTGGTRAARTCYTT	KxFTKMVR	Potyvirus HC-Pro gene primer

a Nucleotide at degenerate positions are represented by a single letter code; R = A and G; Y = C and T; B = C, G and T; D = A, G and T; N = A, C, G and T.

Table 2. The percentage of amino acid similarity and nucleotide identity of the whole genome and 5'UTR among ZaMV, KoMV and three calla lily-infecting potyviruses

	DsMV	TuMV	ZaMMV	KoMV
nt[a]	24%	25%	25%	**90%**
aa[b]	62%	65%	59%	**96%**
5'UTR	50%	28%	29%	**81%**

[a] the complete nucleotides (nt) sequence identity of ZaMV compared with DsMV (AJ298033), TuMV, ZaMMV (NC_011560), and KoMV (AB219545).

[b] the amino acid (aa) sequence similarity of the polyprotein.

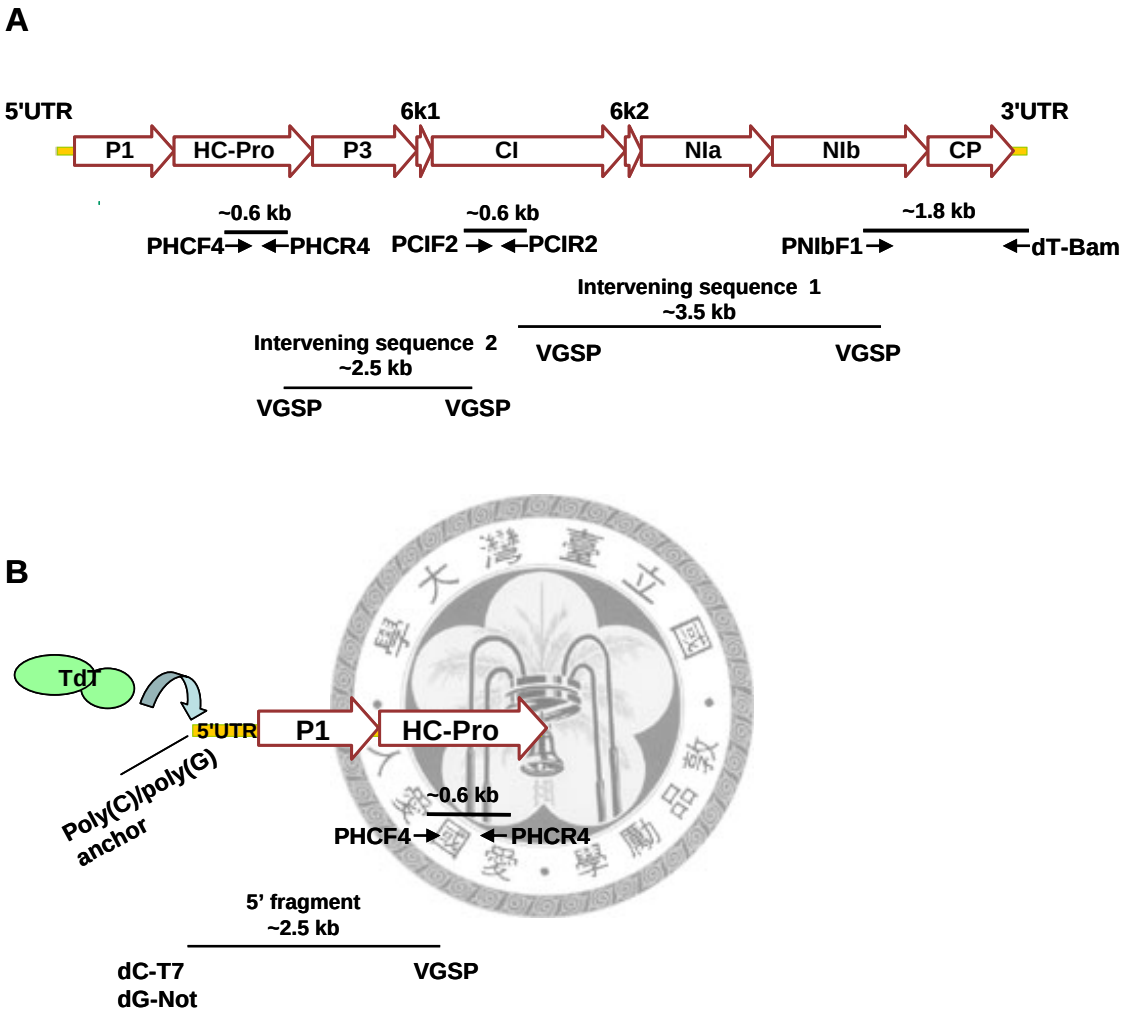

Fig 1. Stragtegy for cloning of the complete genome of potyvirus and the relative positions of the degenerate primers. (A) Genome organization of potyvirus and the cloning strategy of genomic fragments amplified by degenerate and viral gene specific primers (VGSP). (B) 5'RACE strategy by anchoring poly(C) or poly (G) into the 5'end by terminal transferase. The 5' fragments were amplified by VGSP and the universal dC-T7 or dG-Not primer.

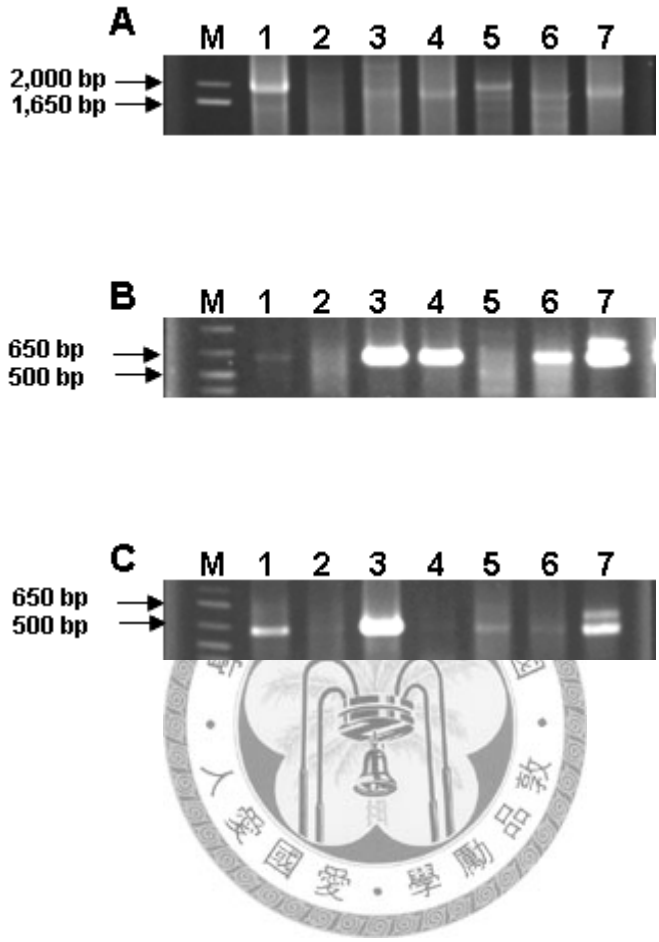

Fig 2. Amplification of potyvirus by reverse transcriptase-PCR from inoculated plant total RNA and analyzed by 1% agarose gel electrophoresis. RT-PCR products amplified by dT-Bam/PNIbF1 (A), PCIF2/PCIR2 (B) and PHCF4/PHCR4 (C) primers, respectively. M, 1 kb plus DNA ladder (Invitrogen), 1. BaRMV, 2. DsMV, 3. PVY, 4. TuMV, 5. ZaMMV, 6. ZaMV, 7. ZYMV. Arrows to the left indicate size of markers.

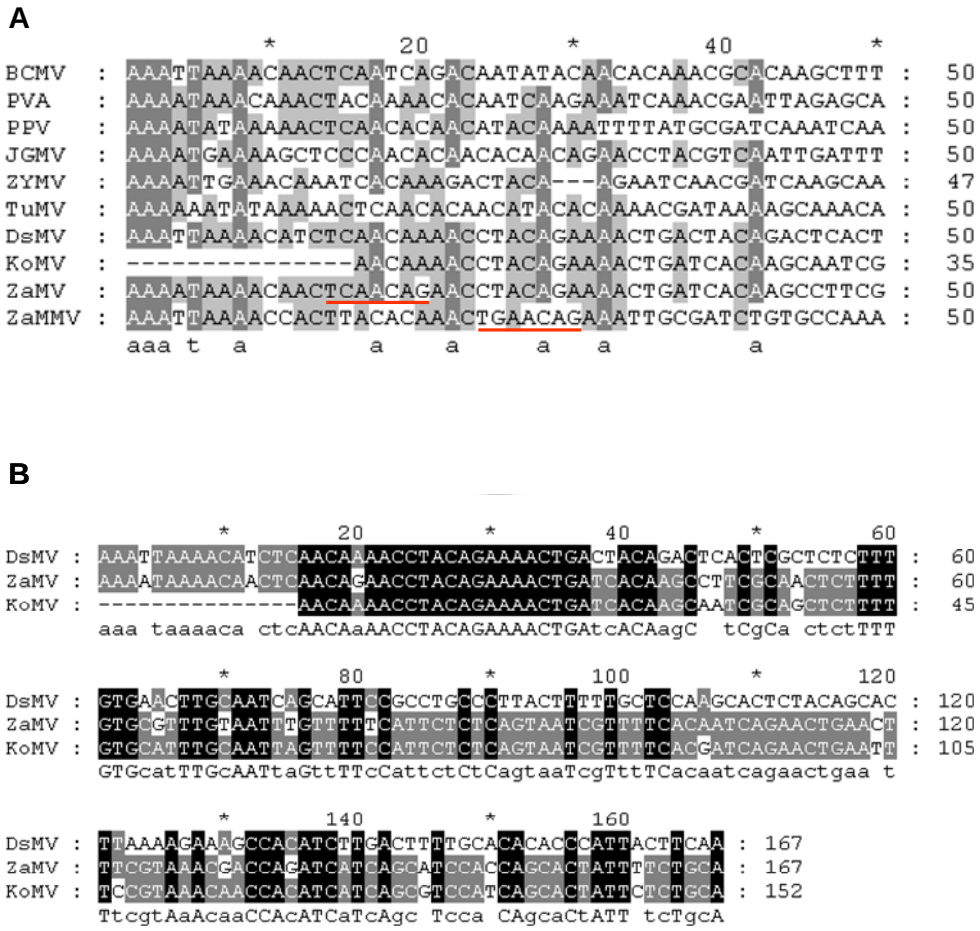

Fig 3. The alignments of the 5'UTR sequence of four calla potyviruses and six potyviruses. (A) The proximal-50 nucleotides in the 5'UTR of six potyviruses: BCMV (U34972), PVA (Z21670), PPV (AJ234957), JGMV (Z26920), ZYMV (L29569), KoMV (AB219545), and four calla lily-infecting viruses: TuMV, DsMV, ZaMV and ZaMMV. the underlines indicated the 'potybox-like blocks'. (B) 5'UTR sequence alignments with DsMV, ZaMV and KoMV.


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Chapter II

Detection of four calla potyviruses by multiplex RT-PCR using *nad5* mRNA as an internal control


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Abstract

Dasheen mosaic virus (DsMV), *Turnip mosaic virus* (TuMV), *Konjac mosaic virus* (KoMV) and *Zantedeschia mild mosaic virus* (ZaMMV) are important potyviruses previously identified in calla lily plants in Taiwan. In order to save time and cost of virus detection, a multiplex RT-PCR assay was developed for these calla potyviruses. Specific primers for each virus were designed based on the sequences of 3' terminal region of respective viruses. To prevent the false negative results, a primer pair specific to plant mitochondrial *nad*5 mRNA was used to produce a 185-bp fragment as an internal control of RT-PCR. The specificities of primers were confirmed by means of simplex and multiplex PCR assays. Optimal primer concentration ratio was identified by multiplex PCR assay. Total RNAs purified from virus-infected plants were used directly or mixed in different combinations, and then tested by multiplex RT-PCR. The result indicated that the expected RT-PCR products could be specifically amplified and identified on the basis of their molecular sizes. The detection sensitivity of multiplex RT-PCR was 25-625 times higher than that of indirect-ELISA (I-ELISA) depending on the virus. When applied to field surveys, multiplex RT-PCR could detect more single as well as mixed infection samples than I-ELISA. Accordingly, our multiplex RT-PCR assay provides a simple, rapid and reliable method for multiple potyvirus detection in calla lily.

Introduction

Calla lily is the common name of the members of *Zantedeschia* spp. which belong to the family *Araceae* and are classified into seven species and two subspecies (Kuehny 2000). As tropical plants native to Africa, calla lilies are now grown as outdoor garden plants, cut flowers and flowering potted plants. Many cultivars of calla lily have been introduced into Taiwan from New Zealand, the Netherlands and the United States for more than 10 years. However, virus diseases are one of the major factors limiting calla lily production (Chen et al. 2003; Huang et al. 2007). Calla lily has been reported as the natural host of various plant viruses, mainly potyviruses (Huang et al. 2007). There are six potyviruses identified in this plant and five of them have been found in Taiwan, including Calla lily latent virus (CLLV) (Chen et al. 2004), *Dasheen mosaic virus* (DsMV) (Chen et al. 1998), *Turnip mosaic virus* (TuMV) (Chen et al. 2003), *Zantedeschia mosaic virus* (ZaMV) (Chang et al. 2001), and *Zantedeschia mild mosaic virus* (ZaMMV) (Huang and Chang 2005). Among them, DsMV is a well-known potyvirus and reported in many kinds of aroid plants (Rana et al. 1983; Zettler and Hartman 1987). TuMV is an important cruciferous plant virus with a broad host range. ZaMV which was recently classified as the isolate of *Konjak mosaic virus* (KoMV) is probably the most prevalent virus infecting calla lily (Chang et al. 2001; Kwon et al. 2002; Nishiguchi et al. 2006; Huang et al. 2007). A newly

identified calla virus, ZaMMV, was found widely spread in the fields in Taiwan and probably in other countries (Huang et al. 2007). The major symptoms of calla lily induced by these four viruses are mosaic, yellow stripe, green island and mild mosaic (Zettler and Hartman 1987, Chang et al. 2001; Pham et al. 2002; Chen et al. 2003; Huang and Chang 2005). CLLV alone does not produce symptoms in calla lilies and may not have a direct impact on the crop (Chen et al. 2004). For that reason we selected DsMV, TuMV, KoMV (ZaMV) and ZaMMV as the detection targets in our study. According to the field surveys in Taiwan, mixed infections by potyviruses are very common in calla lilies (Huang et al. 2007). Severe or mixed infection could cause leaf and flower distortion, stunting, growth reduction and yield loss (Hu et al. 2007).

Tissue culture is an important propagation method for calla lily in addition to tuber production in Taiwan. Viruses can be maintained within the plants during the growing season and storage stage due to systemic viral infection. Therefore, tissue culture together with reliable virus detection methods is essential for production of virus-free plantlets and tubers. Several detection methods for individual calla potyviruses were recently developed such as reverse transcription-polymerase chain reaction (RT-PCR), immunocapture RT-PCR (IC-RT-PCR), dot-blot hybridization, and enzyme-linked immunosorbent assay (ELISA) (Huang et al. 2005; Hu et al. 2007;

Huang et al. 2007). These methods target only single viruses in one reaction. To save time and cost of virus detection, multiplex RT-PCR is chosen because it can rapidly detect multiple targets in one single assay with a small amount of sample. This technique has been successfully applied to many plants for virus detection, including apple (Menzel et al. 2002), citrus (Roy et al. 2005), olive (Bertolini et al. 2001), potato (Nie and Singh 2000; Du et al. 2006), orchids (Lee and Chang 2006), and other crops.

In this study, we established a multiplex RT-PCR system for simultaneous detection of four calla potyviruses in field samples. To rule out false negative results, one primer pair specific to plant mitochondrial NADH dehydrogenase (*nad5*) gene was incorporated to amplify the product of plant *nad5* mRNA as the internal control. This is the first multiplex RT-PCR developed for aroid plants.

Material and methods

Virus isolates and plant materials

Four potyviruses, DsMV, TuMV, ZaMMV and KoMV (ZaMV), were previously isolated from calla lilies. Isolates of DsMV-ZAN (Chen et al. 1998), KoMV (ZaMV-ZAN) (Chang et al. 2001), ZaMMV-ZUN (Huang and Chang 2005) and TuMV-ZAN were separately maintained on tissue culture plantlets of cultivar 'Black Magic' or *Philodendron selloum* by mechanical inoculation (Huang and Chang 2005). TuMV was also maintained in *Nicotiana benthamiana*. These plants were kept at 25^{o}C with 16 h photoperiod in a greenhouse. For the disease survey, field-grown calla lily plants were randomly collected from the Taiwan Seed Improvement and Propagation Station (TSIPS) in Taichung County.

Plant total RNA extraction

Plant tissues collected from healthy, virus-inoculated and field grown plants were used to extract total RNA following the protocol of Plant Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA). In brief, 0.1 g leaf tissue was ground into fine powder in liquid nitrogen and then transferred to a microfuge tube. After being mixed with 450 µl PRX extraction buffer, the lysate was filtered using a Shearing Tube. The filtrate was mixed with 230 µl absolute ethanol, transferred to a

new Plant Total RNA Mini Column, and filtered by centrifugation. This column was then washed once with WF Buffer and twice with WS Buffer. Finally, plant total RNA was eluted with 50 µl RNase-free ddH_2O. The quality of total RNA was analyzed by 1% agarose gel electrophoresis. Plant total RNA was used directly for RT-PCR or stored at -20^oC for further use.

Primer design

Specific primer pairs of four calla potyviruses were designed based on the sequences of 3' terminal region of each individual virus, with the help of the Primer Premier 5 program (Premier Biosoft Int., Palo Alto, CA, USA) to avoid primer dimer formation. The names, targets and sequences of primers and the expected product sizes are shown in Table 1.

Viral and plant *nad5* cDNA clones construction

Plant mitochondrial *nad5* gene (mt) and four viral cDNA fragments were prepared from virus-infected plant total RNA by RT-PCR amplification. The first-strand cDNA was synthesized using dT-Bam (5'-AGCTGGATCC$(T)_{18}$-3') or mtR1 primers. PCR reactions were carried out using dT-Bam and PNIbF1 (5'-GGBAAYAATAGTGGNCAACC-3') primers for potyviruses (Hsu et al. 2005), or

mtR1/mtF2 primers for plant mitochondrial *nad5* gene gene. RT-PCR products were analyzed in 1% agarose gels and the desired cDNA fragments were purified by GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The purified fragments were cloned into pGEM-T® Easy Vector (Promega, Madison, WI, USA). The correct cDNA clones were confirmed by sequencing and then used as templates for PCR experiments.

Multiplex PCR

Five pairs of reverse and forward primers (Table 1) were used in the multiplex PCR reaction. Different primer mixtures containing each primer 10x concentrated were prepared according to the experimental design. The final concentration of every primer was 0.125 μM in original multiplex PCR. To adjust the primer ratio, TuMV primer concentration was decreased to 0.5 and 0.25x and DsMV primer was increased to 1.5x of original concentration (0.125 μM). For multiplex PCR, the 20 μl reaction mix contained 2 μl template mixture (2 ng per cDNA clone), 2 μl 10x primer mixture, 2 μl 10x DyNAzyme™ II DNA polymerase buffer (Finnzymes, Inc., Espoo, Finland), 0.5 μl dNTPs (10 mM), 0.5 μl DyNAzyme™ II DNA polymerase (2 U μl^{-1}, Finnzymes, Inc.) and 13 μl ddH_2O. The amplification was carried out in GeneAmp® PCR system 2400 or 2700 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

PCR program for DNA synthesis was 95°C for 5 min, followed by 30 cycles of 95°C for 35 s, 56°C for 35 s, 72°C for 1 min 30 s, and a final elongation step at 72°C for 7 min. Eight μl of PCR products were analyzed by 1.5% agarose gel electrophoresis in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The DNA fragments were stained with ethidium bromide for 10 min and examined under UV illumination.

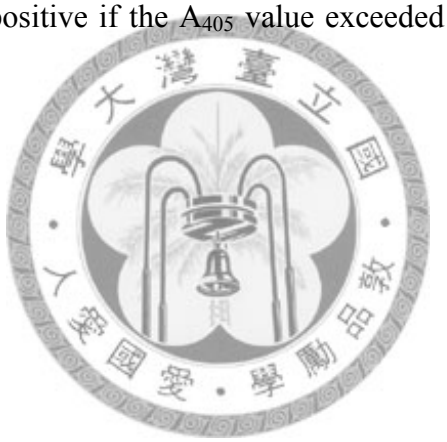
Multiplex RT-PCR

The multiplex RT-PCR protocol has separate RT and PCR steps. Total RNA was extracted as described above. For 25 μl RT reaction, 7 μl plant total RNA together with 2 μl dT-Bam primer (10 μM) and 1 μl mtR1 primer (5 μM) were heated at 65°C for 10 min, cooled at 4°C for 5 min and then 15 μl RT mixture [5 μl 5x first strand buffer (Promega, Madison, WI, USA), 1 μl dNTPs (10 mM), 0.5 μl rRNasin (40 U μl^{-1}, Promega), 1 μl AMV reverse transcriptase (10 U μl^{-1}, Promega) and 7.5 μl ddH_2O] was added. After incubating RT reaction solution at 42°C for 60 min, the multiplex PCR reaction was performed as previously described except that 2 μl RT product was used as template.

Indirect enzyme-linked immunosorbent assay (I-ELISA)

The antisera to DsMV, TuMV, ZaMMV and KoMV were previously prepared in our

laboratory using recombinant capsid proteins as the antigens (Huang et al. 2005; Hu et al. 2007; Huang et al. 2007). I-ELISA was performed according to the protocol of Agdia Inc. (Elkhart, IN, USA) with some modification. One hundred mg of plant tissue was ground in 1 ml of indirect sample extraction buffer [ISE buffer, 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 2% polyvinylpyrrolidone (MW 40,000), pH 9.6]. One hundred μl of the extracts were coated to the 96-well ELISA plate and then assayed as previously described (Lee and Chang 2008). Each sample assayed in triplicate. A sample was regarded as positive if the A_{405} value exceeded twice the mean value of healthy controls.



Results

Specificity of detection primers tested by simplex and multiplex PCR

The performances of the designed primers were first tested using viral and plant *nad5* cDNA clones as templates in simplex and multiplex PCR assays. The final concentration of each primer was 0.125 μM in these PCR reactions. Initially, the specificity of individual primer pairs was analyzed by PCR reaction with single cDNA template. The results of simplex PCR demonstrated that the expected PCR products were successfully generated by each single specific primer pair (Fig. 1a). When individual cDNA clones were tested by the multiplex PCR reaction, the expected fragments were specifically amplified by the primer mixture of TuMV, ZaMMV, DsMV, KoMV and mt control (Fig. 1b). Although a lower yield of the products of DsMV, KoMV and mt were obtained in multiplex PCR compared with simplex PCR, all tested primers were specific to their targets. To further test the specificity of these primers with five templates simultaneously, the same amount of each individual cDNA clone was combined and then used for the multiplex PCR assay. According to the gel analysis, the PCR products of TuMV, ZaMMV, KoMV and mt control were successfully amplified but the DsMV fragment was hardly visible (Fig. 1c) indicating that the multiplex PCR needed modification in order to detect multiple targets.

Optimization of multiplex PCR reaction

To optimize the multiplex PCR for detection of multiple targets, different concentration ratios of primer pairs were tested for amplification efficiency. In previous PCR assays, the same final concentration (0.125 μM) of each primer was used and thus 0.125 μM was assigned as ratio 1x in the primer ratio test. From our preliminary tests, DsMV amplification was hampered when using the same amount of TuMV and DsMV primers (data not shown). Consequently, five primer sets (I-V) with different primer ratios were prepared by decreasing TuMV primer ratio from 1 to 0.5 and 0.25x, and at the same time increasing DsMV primer ratio to 1.5x (Fig. 2). At first individual viral and mt control templates were each tested by five sets of primer mixture, and all expected fragments were produced in multiplex PCR (Fig. 2a-d). Although the size of TuMV amplicon was the largest among five amplified targets, TuMV PCR product yield was reduced only slightly when its primer ratio was lowered to 0.25x (Fig. 2a, lanes 3 and 5). On the other hand, DsMV products increased when the primer ratio was raised to 1.5x (Fig. 2c, lanes 4 and 5). Furthermore, we tested the amplification efficiency of these primer mixtures with five cDNA templates simultaneously. The result clearly indicated that reducing the concentration of TuMV primer to ratio 0.25x allowed a significant increase in DsMV amplification rate and raising the DsMV primer ratio had a similar effect (Fig. 2e,

lanes 3-5). Since primer sets III to V were able to amplify all five targets in multiplex PCR reaction, we chose the primer set V for subsequent RT-PCR experiments.

Specificity of multiplex RT-PCR against total RNAs from virus-infected plants

Purified total RNAs from calla lilies individually infected with TuMV, ZaMMV, DsMV and KoMV were used for cDNA synthesis using dT-Bam and mtR1 primers and multiplex PCR was performed with the primer set V. The correct virus targets were specifically amplified and the mt control fragment was consistently generated in all virus-infected samples (Fig. 3a). However, field-grown calla lilies are frequently infected by mixtures of two or more potyviruses. To test the ability to detect multiple viruses, artificial mixed-infection samples prepared by mixing two, three, or four different virus-infected plant total RNAs were assayed by multiplex RT-PCR. The expected RT-PCR products including virus and mt targets were correctly amplified in all tested virus combinations (Fig. 3b).

Comparison of detection sensitivity between multiplex RT-PCR and I-ELISA

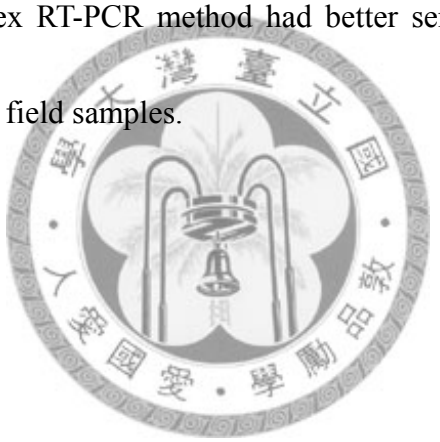
To compare the detection sensitivity, the same amount of virus-infected calla lilies (0.1 g) were used to purify plant total RNA and to prepare original plant extract with 10 volume of ISE buffer. Afterward fivefold serially diluted RNA and sap samples

were prepared with healthy plant total RNA and extract as diluents, and then tested by multiplex RT-PCR and I-ELISA, respectively. The highest dilution at which multiplex RT-PCR showed positive result was 5^{-3} for TuMV, 5^{-4} for DsMV and KoMV, and 5^{-5} for ZaMMV. However, the detection limit of I-ELISA assay was determined as 5^{0} for KoMV, and 5^{-1} for TuMV, ZaMMV and DsMV (Fig. 4). Although KoMV had the lowest ELISA value in this test, the detecting limitation of multiplex RT-PCR still reached to 5^{-4} dilution (Fig. 4d). In summary, the detection sensitivity of multiplex RT-PCR was 25-625 times higher than that of I-ELISA depending on the virus. Although a prozone effect in I-ELISA was noted for TuMV and ZaMMV when 1:10 (w/v) extracts were used compared with 1:100 (w/v) as suggested by AGDIA, use of the former had minimal effect on the results of final assay comparisons (data not shown).

Detection of calla potyviruses in field samples by multiplex RT-PCR and I-ELISA

Fifty full-expanded leaves of calla lily were randomly collected from the field of TSIPS in Taichung County. These samples were used for the detection of calla potyviruses detection by multiplex RT-PCR and I-ELISA assays at the same time. According to the data, 72% (36/50) of calla lily samples tested positive for virus

infection by multiplex RT-PCR, and about two thirds (23/36) of infected samples were mixed infections. By contrast, only 50% of the samples were positive in potyvirus-specific I-ELISA (Table 2). KoMV, found in 68% (34/50) and 44% (22/50) of the samples tested by multiplex RT-PCR and I-ELISA, was the dominant virus in the field. ZaMMV was identified in 44% (22/50) and TuMV was detected in 22% (11/50) of calla lily plants by multiplex RT-PCR. The previously important aroid plant virus, DsMV, was only detected in one sample using multiplex RT-PCR (Table 2). It is apparent that the multiplex RT-PCR method had better sensitivity than I-ELISA to detect calla potyviruses in field samples.



Discussion

This paper describes a multiplex RT-PCR method for simultaneous detection of four potyviruses in calla lily with coamplification of a plant internal control. We selected DsMV, TuMV, ZaMMV and KoMV as the viral targets because these four viruses caused obvious symptoms and yield loss in calla lilies. In multiplex PCR, a DsMV fragment was barely amplified from a multiple-template mix when the final concentration of individual primer was equivalent (Fig. 1c). For unknown reasons, the TuMV primers seemed to interfere with the amplification of DsMV. We solved the problem by increasing the DsMV primer concentrations and decreasing the TuMV primer concentrations as well (Fig. 2). Although several studies indicated that short fragments are amplified more efficiently than longer fragments (Du et al. 2006; Hu et al. 2007), the amplification efficiency of the TuMV fragment was as good as other viral targets in all mixed samples (Figs. 2e and 3b). It might be due to the Tm value of TuMV primers (64 and 68^{o}C) being higher than those of other viral primers, and hence more effectively amplifying the viral target during the PCR process. Even though short fragments were usually selected for multiple target detection, fragment size up to 814 and 942 bp were successfully used for virus detection in citrus trees (Roy et al. 2005). Consequently, large target fragments can still be utilized in multiplex system if the primer design and the PCR condition are optimized.

To avoid the false negative results, different plant internal controls have been introduced into virus detection systems, such as 18S rRNA (Du et al. 2006), chloroplast NADH dehydrogenase ND2 subunit (*ndhB*) mRNA (Thompson et al. 2003) and mitochondrial NADH dehydrogenase (*nad5* and *nad2*) mRNAs (Menzel et al. 2002; Du et al. 2006). Our previously designed mt primers, mtF2 and mtR1, consistently amplified a 185-bp cDNA fragment of *nad5* mRNA from orchid total RNA (Lee and Chang 2006). When the same primers were applied to calla lily, the amplified internal control fragments indicated the success of total RNA extraction and multiplex RT-PCR process (Fig. 3). Nevertheless, the consequences of our preliminary tests showed that the amplification efficiency of the *nad5* fragment was greater than that of the potyvirus targets and sometimes interfered with virus amplification. Therefore, the mt primer was reduced to 1/4 amount of oligo(dT) primer in RT step to lower first-strand cDNA synthesis of *nad5* mRNA; and then the mt primer concentration was also adjusted from 0.25 μM to 0.125 μM in PCR step to improve the results (data not shown). In our opinion, the optimal primer concentration for a plant internal control is influenced by the features of plant species and viral targets, and needs adjustment in every virus detection system.

Due to the characteristic poly(A) tail at the 3' end of potyvirus genome, we chose an oligo(dT) primer to synthesize first-strand cDNA of potyviruses.

Nevertheless, the mtR1 primer was used in addition to the oligo(dT) primer in the RT step to ensure sufficient cDNA synthesis of the plant internal control. In our system, utilization of a universal oligo(dT) primer in the RT step lowered the primer complexity and the PCR conditions could be easily adjusted to facilitate the detection of virus containing a 3'-poly(A) tail. Addition of specific primer pairs could expand the assay to detect calla viruses with and without 3'-poly(A) tail as previously reported in potato virus detection (Nie and Singh 2000).

The detection limit of our multiplex RT-PCR was higher than I-ELISA according to the sensitivity comparison experiment. This was again confirmed by field disease survey, since multiplex RT-PCR identified more single as well as mixed infection samples than I-ELISA. From 50 calla lily plants randomly collected from the field, 36 samples tested positive for potyviruses by multiplex RT-PCR. KoMV and ZaMMV were the two dominant viruses found, in 68% and 44% of the tested samples respectively, and most of them from mixed infections. These data agreed with our investigation in the field survey during 2003-2004 (Huang et al. 2007). According to the prior report, field grown calla lily might be infected by TuMV through random transmission by the vectors from surrounding cruciferous crops (Chen et al. 2003). This might explain why widespread TuMV was found to infect calla lilies at lower (22%) infection rate. On the contrary, DsMV, the previously important virus of aroid

plants, was only identified in one sample by multiplex RT-PCR. It might be due to routine indexing for DsMV in the micropropagation of calla lily by TSIPS and therefore decreased the viral disease incidence (Huang et al. 2007). In addition, we observed the internal control band remained consistent, whereas the intensities of individual viral fragments varied among tested field samples (data not shown). It suggested that the titer or distributions of viruses may be affected in mixed infections (Roy et al. 2005). From the above results, the detection of various combinations of viruses in field samples by the multiplex RT-PCR assay demonstrated that this method will be useful in epidemiological studies of calla potyviruses.

The multiplex RT-PCR technique has been developed for several plant viruses but it is new to calla potyviruses. The sensitivity comparisons with I-ELISA indicated the value of the multiplex RT-PCR protocol. In conclusion, this multiplex RT-PCR assay provides a simple, rapid and reliable method for simultaneous detection of four potyviruses in calla lily. It also demonstrated the feasibility of this detection system in a disease diagnosis and certification program of calla lily.

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Table

Table 1 The targets, names, sequences and the expected product sizes of the primer pair

Target	Primer	Sequence 5'-3'	Product size	Reference
TuMV	TuR0	TGTCCCTTGCATCCTATCAAATGTT	1077 bp	This study
	TuCPF1	GCAGGTGAGACGCTTGATGCAGG		
ZaMMV	ZUNR1	GTCCTTCACGAGTTTAGAGC	792bp	This study
	ZUNF2	GATTCTCGACCTGGCTCATC		
DsMV	DR0	TTGAACACCGTGCACGAAGCATC	457 bp	Huang et al. 2005
	DF2	GACTTCTATGAGGTCAATTC		
KoMV	ZR0	CTCCTATTTAAAAGACATGACTCG	260 bp	Hu et al. 2007
(ZaMV)	ZF3	CCGCCCTGCAAAGCGCAAAC		
nad5	mtR1	ATCTCCAGTCACCAACATTGGCAT	185 bp	Lee and Chang 2006
mRNA	mtF2	GCTTCTTGGGGCTTCTTGTTCGATA		

Table 2 Number of samples (% in parenthesis) of calla lilies collected from the field testing positive by indirect ELISA (I-ELISA) and multiplex RT-PCR (mRT-PCR) methods

Assay result[a]	**I-ELISA**	**mRT-PCR**
ZaMMV	2 (4%)	1 (2%)
KoMV	4 (8%)	12 (24%)
TuMV+ZaMMV	1 (2%)	1 (2%)
TuMV+KoMV	1 (2%)	1 (2%)
ZaMMV+KoMV	10 (20%)	11 (22%)
DsMV+KoMV	0	1 (2%)
TuMV+ZaMMV+KoMV	7 (14%)	9 (18%)
ND	25 (50%)	14 (28 %)
Total	**50 (100%)**	**50 (100%)**

[a] A_{405} nm value greater than twice the mean value of the healthy controls was considered as positive sample. ND: sample did not react to four kinds of antisera or primers including TuMV, DsMV, ZaMMV and KoMV.

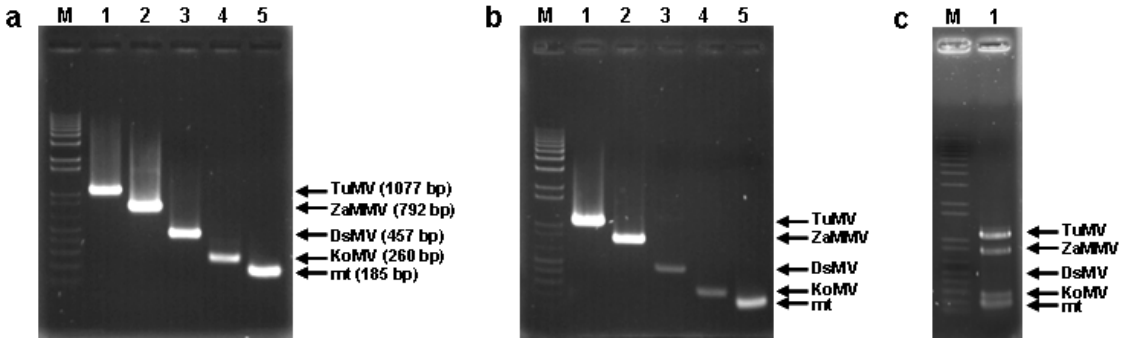

Fig. 1 Primer specificity tested by simplex and multiplex PCR using cDNA clones as templates. (a) The result of simplex PCR using single specific primer pair. (b) The result of multiplex PCR using the primer mixture of TuMV, ZaMMV, DsMV, KoMV and plant mitochondrial *nad5* gene (mt). cDNA clones used for PCR analyses included TuMV (lane 1), ZaMMV (lane 2), DsMV (lane 3), KoMV (lane 4) and mt (lane 5). (c) The result of multiplex PCR with the template mixture and primer mixture of TuMV, ZaMMV, DsMV, KoMV and mt. The final concentration of each primer was 0.125 μM. The PCR products separated by 1.5% agarose gel electrophoresis are indicated by arrows. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

primer	I	II	III	IV	V
TuMV	1	0.5	0.25	0.5	0.25
ZaMMV	1	1	1	1	1
DsMV	1	1	1	1.5	1.5
KoMV	1	1	1	1	1
mt	1	1	1	1	1

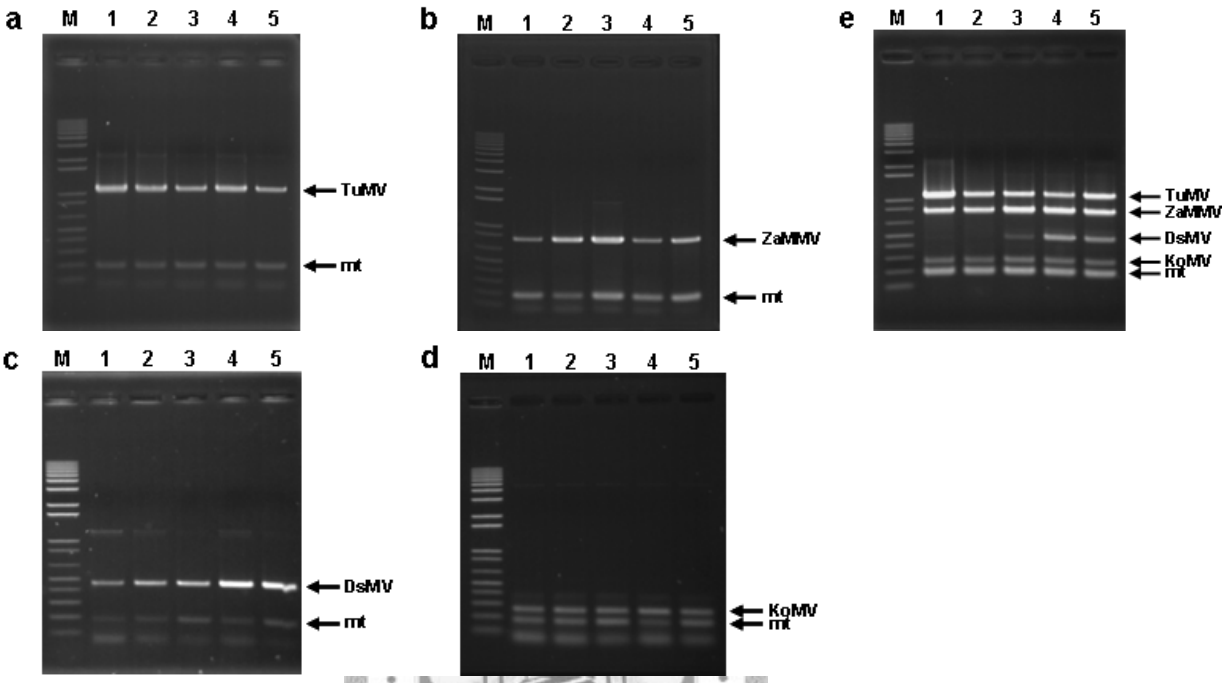

Fig. 2 Optimization of multiplex PCR reaction with different concentration ratios of the primer sets. Five different primer sets were used to test primer amplification efficiency with templates of internal control and single virus or four viruses. Each primer set contains five primer pairs and their primer concentration ratios are listed as table. One represents the final concentration equal to 0.125 μM. Lanes 1-5 indicated the PCR products amplified by primer set I-V, respectively. The results of multiplex PCR with templates of plant mitochondrial *nad5* gene (mt) and TuMV (a), ZaMMV (b), DsMV (c), KoMV (d) or four viruses (e) were analyzed by 1.5% agarose gel electrophoresis. The PCR products are indicated by arrows. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

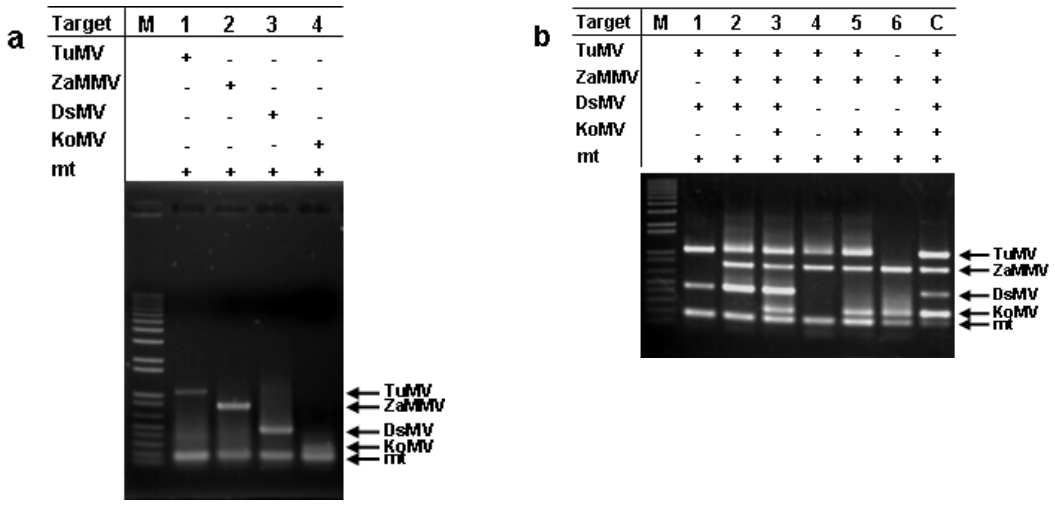

Fig. 3 Specificity of multiplex RT-PCR reaction with the primer set V. (a) Detection of single virus target and plant mitochondrial *nad5* mRNA (mt) by multiplex RT-PCR reaction. Total RNAs derived from TuMV-infected (lane 1), ZaMMV-infected (lane 2), DsMV-infected (lane 3) and KoMV-infected (lane 4) plants were tested. The arrows indicate the RT-PCR product. (b) Detection of multiple virus targets and plant mitochondrial *nad5* mRNA by multiplex RT-PCR reaction. Different combinations of virus-infected total RNAs were shown as table. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA). Lane C: five cDNA clones used as positive control templates. RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and are indicated by arrows.

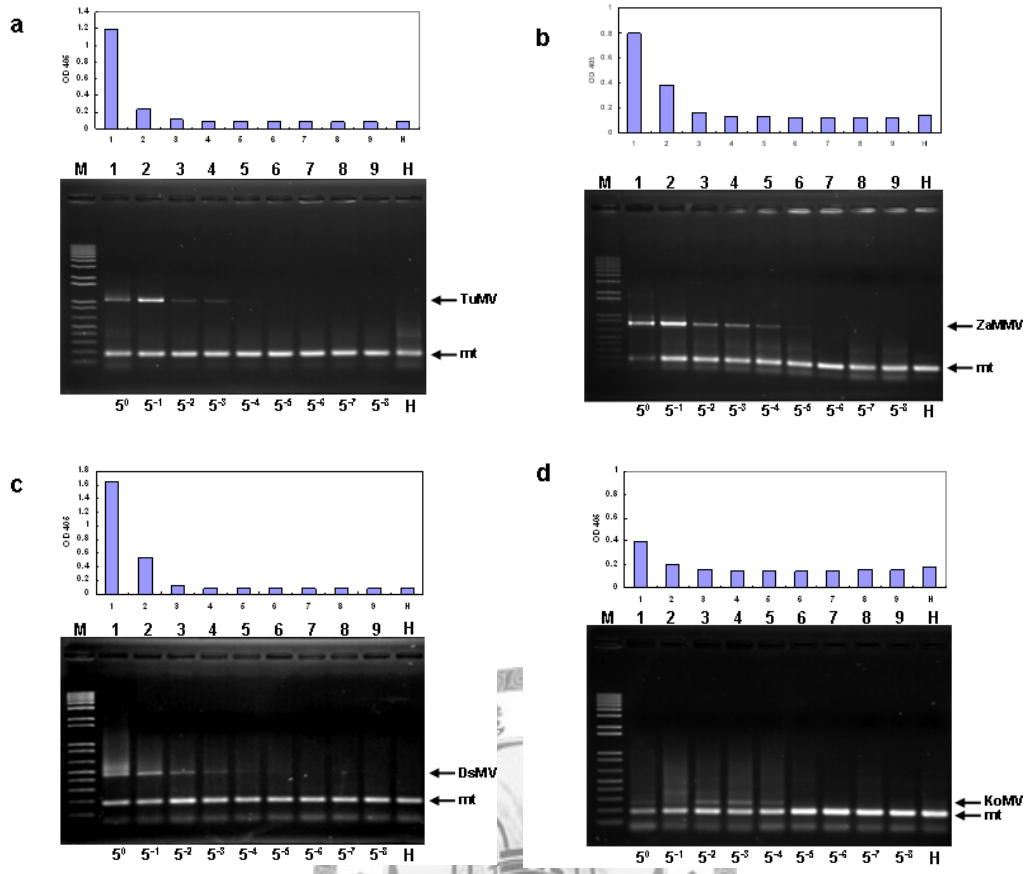

Fig. 4 Comparison of detection sensitivity between multiplex RT-PCR and indirect ELISA (I-ELISA). Calla lily tissues infected by TuMV (a), ZaMMV (b), DsMV(c) or KoMV (d) were used to prepare original plant extracts and samples serially diluted fivefold with healthy leaf sap and then assayed by I-ELISA. Similarly, total RNA purified from TuMV-infected (a), ZaMMV-infected (b), DsMV-infected (c) or KoMV-infected (d) calla lily tissues were used to prepare samples serially diluted fivefold with healthy plant total RNA and then assayed by multiplex RT-PCR. Lanes 1-9 indicated 5^0 - 5^{-8} serial dilutions. Lane M: 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA). Lane H: healthy plant control. RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and are indicated by arrows.


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Chapter III

Application of dsRNAs derived from *Turnip mosaic virus* to interfere infection of calla lily by potyviruses


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Abstract

Homology-dependent gene silencing is a specific defense response of plants against viral infection. Double-stranded RNA (dsRNA) or hairpin RNA (hpRNA) plays a key role in triggering the interference phenomena. A dsRNA expression and screening system was used to obtain highly efficient interference fragments from the consensus regions of HC-Pro, NIa and CP genes of four calla lily-infecting potyviruses including *Dasheen mosaic virus* (DsMV), *Turnip mosaic virus* (TuMV), *Zantedeschia mild mosaic virus* (ZaMMV) and *Zantedeschia mosaic virus* (ZaMV). Eight different dsRNA fragments of TuMV derived from these consensus regions of potyviruses had different levels of interference with TuMV infection. When directly inoculating dsRNA transcripts to the leaves of tobacco plants, the symptoms of TuMV-inoculated plants were completely suppressed by the longer fragments of dsRNAs compared with the shorter ones. It implied that the length of dsRNA may be important for interference with virus infection. Two of the highly interfering dsRNA fragments located in HC-Pro and NIa genes were used for further transient-expression assay by *Agrobacterium*-infiltration in tobacco plants. The successful interferences of TuMV infection were observed in the infiltrated leaves of hypersensitive and systemic host plants which were challenge-inoculated 7 days after infiltration. Moreover, TuMV was inoculated onto the non-infiltrated systemic leaf at 7 dpi after 1st

inoculation of hypersensitive host. The resistance to virus infection was also observed in the inoculated systemic leaves. Besides, *Bean yellow mosaic virus* (BYMV), another calla lily-infecting virus, was interfered by TuMV hpRNAs transiently expressed in plants. Several lines of transgenic *Nicotiana benthamiana* plants transformed with hpHC and hpNIa fragments were obtained. Over 80% of T0 transgenic plants revealed resistance response to TuMV infection. The interference ability of T1 transgenic plants will be tested with potyviruses other than TuMV. In conclusion, the interference assays with different TuMV dsRNA fragments suggested that dsRNAs longer than 400 bp are more efficient in interfering with TuMV infection. The role of viral sequence in triggering RNA silencing response needs further investigation.

Introduction

Plant viral diseases are one of the important biological factors affect plant normal growth. Several methods used in the plant virus diseases control, including virus-free seedlings, sanitation, insect vector prevention, resistant plants, cross-protection, and transgenic plants, etc. Among these methods, cross-protection is one of the effective strategies against plant virus diseases. The unique feature of cross-protection is the prevention of additional infection from a closely related virus or the secondary infection from the same virus (Takeshita *et al*., 2004). For many years, it has been considered that the molecular mechanisms of cross-protection were based on declining the virus infection cycle, such as inhibition of uncoating and/or amplification of the challenge virus, and induction of host resistance to suppress the challenge virus (Hull, 2002). Furthermore, pathogen-derived resistance (PDR) caused by transgenic virus RNA or protein have been studied (Waterhouse *et al.*, 2001). Various models were proposed for the plants transformed by the viral encoding nucleotide sequence showing resistance to the parental viruses (Goldbach *et al.*, 2003). In the past ten years, it has been recognized that posttranscriptional gene silencing (PTGS) plays a key role in viral RNA-mediated resistance in plants (Baulcombe 2002; Goldbach *et al*., 2003).

PTGS was first reported about 20 years ago as an unexpected outcome resulting in

inactivation of the host gene and transgene encoding the same sequences (Napoli *et al*., 1990). PTGS is closely related to RNA interference (RNAi) or RNA silencing in animals and other organisms, represents an ancient eukaryotic genetic phenomenon, which is also involved in the defense against viruses (Tenllado and Diaz-Ruiz, 2001; Waterhouse *et al*., 2001). The RNA-mediated virus resistance is also one phenomenon of the PTGS mechanisms in plants. The viral RNA and the virus-derived transgene mRNA are targets for degradation in plants that display protection against virus infection (Hamilton and Baulcombe, 1999).

The RNA interference mechanism is associated with the production of small interfering RNA (siRNA). siRNA is the small molecules which are composed of 19-25 ribonucleotides original from double-stranded RNA (dsRNA) (Hamilton and Baulcombe, 1999; Ho *et al*., 2006). The pathway of PTGS is triggered by Dicer-like (DCL, the ribonuclease III-like enzymes) proteins in plants. There are four DCL proteins (DCL1 ~DCL4) reported in model plant system, *Arabidopsis thaliana* (Ding and Voinnet, 2007). Among them, DCL2 and DCL4 play the major role to cut dsRNAs from virus. siRNA acts as a guide to incorporate target viral RNAs into RNA-induced silencing complex (RISC) for further degradation (Waterhouse and Fusaro, 2006). siRNA are produced from dsRNAs, which are often synthesized from viruses, repetitive sequences introduced by genetic engineering or endogenously

activated transposons. Thus, siRNA is a multi-function molecule in plants, including antiviral defense, overproduced mRNA silencing, and prevention of the disruption by transposons (Tang, 2005). However, siRNA pathway now is emphasized in the antiviral response, many studies have been devoted to RNA-mediated virus resistance (Vazquez Rover *et al*., 2002, Goldbach *et al*., 2003). In 1997, Voinnet *et al*. indicated that in plants there are nucleic acid-based signal molecules of gene silencing in plants and the signal moleculrs are produced at the initiation site and moves out to other tissues (Voinnet and Baulcombe 1997; Voinnet *et al*., 1998). Therefore, because of the specificity of homologous-sequence degradation and systemic silencing signal spreading, the RNA silencing mechanism is not only used in functional analysis of plant or viral genes but also thought as a new biotechnological tool for plant virus diseases control (Tenllado *et al*., 2004).

On the other hand, contrast part of studies also pointed out that the virus evolved silencing suppressors act as counter-defense reaction against plant RNA silencing pathway (Kasschau and Carrington, 1998). The currently known plant viral suppressors include the p25 movement protein of *Potato virus X* (PVX) which could interfere with the assembly of siRNAs in RISC (Voinnet *et al*., 2000), p19 protein of tombusvirus (Silhavy *et al*., 2002) which was demonstrated to bind siRNAs, thus prevent siRNAs into RISC complex for further degradation process, and HC-Pro

proteinase of potyvirus which is the first reported viral suppressor protein and interfere with the normal function of the RISC complex (Gammelga *et al*., 2007; Kasschau *et al*., 2003; Kasschau and Carrington, 1998.). Many works in PDR have shown that coat protein genes are the first and major sequences used in virus defense (Wisniewski *et al*., 1990; Kalantidis *et al*., 2002; Vargas *et al*., 2008). But other sequences can also work efficiently in resistance, for instance the viral suppressor, HC-Pro (Negri-Nicola *et al*., 2005), or viral replicase, such as 54-kDa protein of *Pepper mild mottle virus* (PMMoV) (Tenllado *et al*., 1995). That means various parts of viral genome are useful in interferring viral infection in a sequence-specific silencing manner. Moreover, expression of the double-stranded or hairpin form RNAs compared with expression of sense or antisense mRNA in transgenic plants could induce high silencing effects (Smith *et al*., 2000; Wang *et al*., 2000). Accordingly, dsRNA- of hpRNA-mediated silencing becomes a popular antiviral strategy.

Calla lily is perennial bulbous flower and has become a popular ornamental in Taiwan. According to several studies in calla lily, virus diseases are one of the major limiting factors of calla lily production (Chen *et al*. 2003; Huang *et al*. 2007). More than 10 virus diseases have been reported, including at last 6 potyviruses. The major calla lily-infecting potyviruses in our country include *Dasheen mosaic virus* (DsMV) (Rana *et al*., 1983), *Zantedeschia mild mosaic virus* (ZaMMV) (Huang and Chang,

2005), *Zantedeschia mosaic virus* (ZaMV) (Chang *et al*., 2001; Kwon *et al*., 2002), and *Turnip mosaic virus* (TuMV) (Chen *et al*., 2003). The tissue culture and tuber production are two major propagation methods of calla lily in Taiwan. Thus, virus indexing of healthy calla lily seedlings is important. Several detection methods have been developed and applied in virus indexing of tissue culture seedlings, such as specific antibodies preparations (Hu *et al*. 2007; Huang *et al*. 2007), reverse transcriptase polymerase chain reaction (RT-PCR), immunocapture RT-PCR (Huang *et al*. 2005; Hu *et al*. 2007; Huang *et al*. 2007), dot-blot hybridization (Hsu *et al*. 2005), and so on. Since the RNA silencing technique is regarded as a new approach for controlling plant virus diseases (Tenllado *et al*., 2004). Many studies have demonstrated it is effectiveness in improving viral resistance of plants, we tried to develop dsRNA-mediated silencing methods against calla lily-infecting potyviruses. To obtain broad spectrum of resistance against potyviruses is the purpose of this study in order to prevent calla lily from mixed infection. *Potyvirus* is the largest plant virus genus, and more than 200 species have been recognized. The number of published full-length sequences of potyviruses increases quickly during these years (Zheng *et al*., 2008). Molecular analysis indicates that there are several conserved sequences within the viral genome, including HC-Pro, CI, NIa, NIb and CP genes. Except CP gene encoding the structural protein, others encode non-structural proteins and all of them

play multiple functions involved in the infection, replication or movement process of potyvirus life cycle. Therefore, the conserved regions of these genes are candidates for interference of multi-virus infection.

In this study, we analyzed the HC-Pro, NIa and CP genes of four calla lily-infecting potyviruses. HC-Pro is the secondary gene product of the potyvirus, with multiple functions including transmission process, proteinase activity, and silencing suppression (Zheng, 2008). NIa protein is composed VPg and proteinase domains, the former is essential for viral replication and host genotype specificity and the latter is the major proteinase responsible for polyprotein cleavage to produce mature viral proteins (Urcuqui-Inchima *et al*., 2001). CP is the caspid protein gene but also involved in several functions including aphid transmission, viral movement, RNA amplification and encapsidation (Urcuqui-Inchima *et al*., 2001). Based on the sequences alignments, eight regions in total were selected for RNA interference assay and TuMV was chosen as the target virus. Eight constructs containing different conserved regions were quickly screening by mechanical inoculation of dsRNA transcripts with target virus on tobacco plants. Most of dsRNAs revealed resistance to the target virus, TuMV, although there was still few fragments shown lower interference efficiency. Two of the most effective fragments (located in HC-Pro and NIa genes with the same length respectively) were further analyzed by using

Agrobacterium tumefaciens infiltration (agoinfiltration) as transient expression system. The hairpin constructs with two inverted repeat fragments separated by an intron were created for encoding self-complementary hpRNAs and the simultaneous dsRNA production in plants. Resistance to TuMV infection was achieved in both viral sequence-expressed constructs. Transgenic plants transformed with the same potyvirus conserved sequences were analyzed. Several lines of *Nicotiana benthamiana* transgenic plants showed resistance to virus infection. Since the viral sequences introduced into plants were partial fragments of viral genes, we assumed this specific resistance is based on the RNA silencing mechanism. By quickly screening using transiently expressed dsRNA and hpRNA, different viral sequences represented special silencing triggers. The interference effects of these sequences still need to be investigated.

Materials and methods

Sequence alignments and conserved region selection for primer design

The full-length genomes of four calla potyviruses, DsMV, TuMV-Zan, ZaMV, and ZaMMV, were sequenced. The amino acid sequences of three consensus genes: HC-Pro, NIa and CP were aligned by pileup program of GCG software (Wisconsin GCG package version 10.3, Accelrys Inc., San Diego, CA, USA). The conserved regions of three genes were chosen for primer design (Fig. 1). To generate TuMV inference constructs, total 12 primers were designed depending on the alignment results and the primer sequences were specific to TuMV-Zan sequence. There are four forward and four reverse primers located in the conserved region of the HC-Pro gene (nt 1697 - 2584); one primer pair is in the conserved region of the NIa gene (nt 6324-6924), and also one primer pair is in the conserved region of the CP gene (nt 9131-9496). The primer sequences and positions are shown in Table 1. In addition, two primers in the CP gene of *Cucumber mosaic virus* (CMV) was used to construct a 580-bp (corresponding to CP nt 1-580) non-homologous dsRNA control (Table 1)

Construction of dsRNA interference clones

Primer designation based on the conserved genes (Fig. 1) and their sequences are specific to TuMV-Zan sequence. The full-length cDNA clone of TuMV-Zan (Wu,

2004) was used for the template of dsRNA interference constructs. Construction of dsRNA interference clones were performed by PCR amplification with specific primers, which were anchored with *EcoR*I, *Hind*III or other suitable restriction sites. The expected PCR fragments were analyzed in agarose gel and then followed by enzyme digestion. The digested fragments were purified by Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and ligated with the same enzyme digested Litmus 38i vector (New England BioLabs Inc., Beverly, MA, USA), the dual T7 promoter plasmid, by T4 DNA ligase (Promega, Madison, WI, USA). After screening with PCR reaction, six correct clones containing different fragments of TuMV HC-Pro gene were named as TuHC1-1 (nt 1697-1919, 223 bp), TuHC2-2 (nt 2188-2572, 385 bp), TuHC (nt 2127-2492, 366 bp), TuHC1-1L (nt 1697-2170, 474 bp), TuHC2-2L (2097-2566 nt, 470 bp), and TuHC1-2 (nt 1697-2572, 876 bp). One clone containing 470 bp NIa fragment was named as TuNIa (Fig. 2C) and the other one clone including 366 bp of CP fragment was named as TuCP (Fig.2D).

In vitro transcription

For dsRNA synthesis, DNA templates were prepared by PCR reaction with T7 promoter primer for eight interference constructs described above. Twenty microliter

PCR reactions contained 1X PCR buffer, 0.25 mM dNTPs, 0.2 uM primer, and 1 U DyNAzyme™ II (Finnzymes, Inc., Espoo, Finland). PCR products were purified using Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Three hundred microgram of the purified PCR products were used as *in vitro* transcription templates for dsRNA production. dsRNA fragments were synthesized by TranscriptAid™ T7 high yield transcription kit (MBI, Fermentas Ltd., NY, USA), following the protocol of manufacturer. The dsRNA transcripts were checked in 1% agarose gel.

Virus source, plant material and dsRNA mechanical inoculation

TuMV-Zan was isolated from *Zantedeschia* spp. cv. Black Magic (Wu, 2004), maintained in *N. benthamiana* plant and used as inoculation source. Interference assays were performed in TuMV systemic (*N. benthamiana*) and hypersensitive (*N. tabacum* var. White Burley) hosts. All plants were grown in greenhouse with day/night temperature of 28/25^{o}C and time period of 16/8 hours. For dsRNA mechanical inoculation, inocula contained 300 ng of TuMV-Zan-infected plant total RNA with or without dsRNA transcripts (the same molar ratio as 150 ng of TuHC1-1 dsRNA transcript). The inocula were diluted in 1X GKP buffer (50 mM glycine, 30 mM K_2HPO_4, 1% bentonite, 1% celite) and mechanically inoculated onto the fourth

and fifth fully expanded leaves of 3-4 week old tobacco plants. For sequential inoculation, dsRNA transcripts or buffer were inoculated to the tobacco leaves 12 hr before challenge-inoculated with TuMV-infected plant total RNA. After 7 days of inoculation, the inoculated plants were photographed and used for further analysis.

Immunoblot analysis

Crude plant extracts were prepared by grinding leaf tissue in liquid nitrogen, and then mixed with one volume of 2X SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 4.4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.025% bromophenol blue). The supernatants were collected after centrifugation at 12,000 rpm for 5 min and used for immunoblot assay. The total proteins of plant tissue were separated by 12% SDS-PAGE in duplicate. One gel was stained with Coomassie brilliant blue and the other one was transferred to PVDF membrane (Osmonics, Westborough, MA, USA). After blocking with 5% non-fat milk in 1X PBS solution, the membrane was incubated in 1X PBS containing anti-TuMV antiserum diluted 1:8000 at room temperature for one hour or 4^{o}C overnight. After washed three times by 1X PBST buffer for 10 min, the membrane was probed with goat anti-rabbit secondary antibody conjugated with alkaline phosphatase and then reacted with the colorimetric substrate, NBT/BCIP (Roche Applied Science, Mannheim, Germany).

Construction of hairpin RNA (hpRNA) expression clones

pBI121, a binary vector completely sequenced in 2003 (Chen *et al.*, 2003), was used to develop constructs expressing the hpRNAs of TuHC2-2L and TuNIa *in planta*. Two hpRNA constructs, pBI-hpHC2-2L and pBI-hpNIa, were designed to contain the corresponding inverted repeat fragments of the TuHC2-2L and TuNIa sequences which were separated by the 85-bp intron of *rolA* gene of *A. rhizogenes* (Margelli *et al*., 1994). For construction of hpRNA expression clones, an intermediated vector, pBS-rolA, was first made by introducing the *rolA* intron into pBluescript SK(+) vector (Stratagene, LaJolla, CA, USA) by invert-PCR with rolAR1 primer : 5'-AAAATAGTAATAATTGAACCTACAACCACACTCACCCCGGGCTGCAGG AATTCGAT-3', and rolAF1 primer : 5'-TGAAGCTGTGTATTTCCCTTTTTCTAATATGCACCTATTTCATGTTTCAG GGATCCACTAGTTCTAGAGCGG-3' (underline corresponding to the *rolA* intron sequence). After self-ligation and screening of transformants, the correct pBS-rolA clone was confirmed by sequencing and used for creating hpRNA constructs The viral sequences, antisense TuHC2-2L fragment with *HindII*I and *Eco*RI was amplified, enzyme digested and introduced into pBS-rolA to create pBS-HC2-2-rolA. Then the sense TuHC2-2L fragment with *BamH*I and *Sac*I restriction sites was cloned into

pBS-HC2-2-rolA to obtain the pBS-hpHC2-2L which incorporates a *rolA* intron as a spacer sequence between the antisense to sense orientations of the TuHC2-2L sequences. Sequencing confirmed the correct ligation of these components. Furthermore, antisense-rolA-sense cassette was excised as a *Kpn*I-*Sac*I fragment and cloned into *Kpn*I and *SacI* digested pBI121 vector to generate pBI-hpHC2-2L (Fig. 6B, top). The other hpRNA construct, pBI-hpNIa, was created by the similar process. The antisense TuNIa fragment was PCR amplified by TuNIaF1/TuNIaStuHR primers (the same primer sequence as TuNIaHinR1 except a *Stu*I site added prior to *Hind*III site) and digested with *EcoR*I and *Hind*III. The fragment was cloned to *EcoR*I-*Hind*III digested pBS-rolA to generate pBS-NIa-rolA. The sense NIa fragment anchored with *BamH*I and *Sac*I sites was amplified, enzyme digested and ligated into similarly digested pBS-NIa-rolA to create the pBS-hpNIa. The orientation of pBS-hpNIa was antisense-rolA-sense. The entire hairpin NIa fragment of pBS-hpNIa was inserted into the *Stu*I and *Sac*I sites of pBI-V vector, a pBI121 with a created *Stu*I site, to create pBI-hpNIa as a NIa hpRNA expression vector (Fig. 6B, bottom).

Agrobacterium infiltration and virus inoculation

A. tumefaciens strain C58C1 (Deblaere *et al*., 1985) was utilized in this study. The binary vector, pBI121, and two hpRNA expression clones, pBI-hpHC2-2L and

pBI-hpNIa, were transformed into C58C1 by a freeze-thaw method. For ago-infiltration, recombinant C58C1 transformants were grown overnight at 28°C in Luria Both supplemented with 50 ppm kanamycin, transferred to 20 ml LB medium with the appropriate antibiotics and 20 μM acetosyringon, and then incubated at 28°C with 200 rpm shaking for 24 hrs. The bacteria cultures were centrifuged at 5,000 g for 15 min at 4°C and pellets were resuspended in MMA buffer (10 mM MES, 100 mM $MgCl_2$, 2 mM acetosyringon) to an optical density (OD_{600}) of 1.0. Both *N. benthamiana* and *N. tabacum* plants were used for agoinfiltration. Two leaves per plant were infiltrated with 0.1 ml bacteria culture. After 1 day or 7 days of infiltration, the infiltrated leaves were inoculated with TuMV-infected plant sap (ground in 50 volumes of 0.05 M sodium phosphate buffer, pH 7.0) and carborundum. At the 7 and 14 dpi of TuMV challenge inoculation, the tested plants were photographed. The local lesions of *N. tabacum* plants were counted, and inoculated and systemic leaves of *N. benthamiana* were analyzed for virus accumulation by ELISA and northern blot assays.

RNA extraction and northern blot hybridization

Plant total RNA extraction followed the protocol of Plant Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA). For TuMV genomic RNA

detection, 3-5 ug total RNA was separated by electrophoresis on 1% agarose gel. Total RNA was transferred to Hybond-N^+ membranes (GE Healthcare) by capillary transfer according to standard procedures (Sambrook *et al*., 1989) and fixed by UV crosslinking (1,200 μJ, Stratalinker, Stratagene, Barcelona, Spain). Northern blot analysis was performed by DIG-labeled DNA probes specific to TuMV 3' UTR (nt 9626–9835). For the hybridization procedure, membrane was prehybridized in 10 ml hybridization solution at 55°C for 2 hours, and 2 μl denatured DIG-labeled TuMV 3' DNA probe was added. Hybridization was performed at 55°C overnight (12-16 hours). After hybridization, membrane was washed twice in 2X SSC and 0.1% SDS for 5 minutes at room temperature, followed by 0.1X SSC and 0.1% SDS wash twice at 65°C for 15 min. The detection of hybridization signals followed the procedure of the manufacturer (Roche Applied Science, Mannheim, Germany).

Indirect ELISA

The indirect ELISA (I-ELISA) was performed according to the protocol of Agdia Inc. (Elkhart, IN, USA) with some modification. One hundred mg of plant tissue was ground in 10 volumes of indirect sample extraction buffer [ISE buffer, 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 2% polyvinylpyrrolidone (MW 40,000), pH 9.6]. One hundred μl of the extracts were coated to the 96-well ELISA plate and then assayed as

previously described (Lee and Chang, 2008). Each sample assayed in triplicate with anti-TuMV antibody or corresponding antiserums. A sample was regarded as positive if the A_{405} value exceeded twice the mean value of healthy controls.

Small RNA accumulation analysis

Total RNA was isolated from the 7 dpi inoculated and systemic leaves of TuMV challenge-inoculated *N. benthamiana* plants by Tri-reagent (Ambion, Austin, TX, USA) according to the manufacturer's protocol. For the detection of siRNAs, 30 μg of total RNA was heat treated at 96°C for 10 min in 8 M urea loading buffer (50 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, 8 M urea) and loaded onto a 15% polyacrylamide gel (acrylamide : bis-acrylamide 19:1) containing 7 M urea and separated at 300V for 1 hr in 0.5 X TBE buffer (50 mM Tris, 41.5 mM boric acid, 0.5 mM EDTA). After electrophoresis the gels were stained in 2.5% HealthView Nucleic Acid Stain (Genomics BioSci & Tech Corp, Taiwan) for 5 min. Nucleic acid was electro-transferred from polyacrylamide gels onto nylon membranes (Hybond N+, GE Healthcare) by semi-dry transfer cell (Bio-Rad, Hercules, CA, USA) and fixed by UV cross-linking. The membrane was hybridized with DIG-labeled TuHC2-2L and TuNIa antisense RNA probes which were prepared from pBS-hpHC2-2L, and pBS-hpNIa by *in vitro* transcription, previously treated with alkaline hydrolysis to generate short

fragments about 50 nt (Hamilton and Baulcombe, 1999).

Transgenic *N. benthamiana* assay

The transformation of *N. benthamiana* was performed by the leaf disc method using *Agrobacterium tumefaciens* (Horsch *et al*., 1985). Virus resistance of the T0 plants were analyzed by whole leaf infection assay and followed by I-ELISA with specific antibodies. Leaf blade of the transgenic lines were taken from plantlet and inoculated with virus-infected plant sap as described above and then incubated on 1.5% water agar plates. Virus accumulation was analyzed by I-ELISA at 7 dpi of virus inoculation. The seeds of transgenic *N. benthamiana* were collected from self-pollination T0 plants as the T1 progeny. Three lines of T1 hpNIa and at last four plants of each line were mechanical inoculation with a 1:10 dilution of plant sap from TuMV-infected *N. benthamiana*. The degree of virus resistance was also evaluated by I-ELISA.

Results

Conserved fragments within the different genes of potyviruses used for dsRNA constructs

Although the identities of nucleotide and amino acid sequence of *Potyvirus* species are ranged between 49% ~ 75% and 42% ~ 81%, respectively (Adams *et al*., 2005), we found several highly amino acid consensus regions within the genomes of potyviruses. The amino acid sequences of HC-Pro, NIa and CP genes of four major calla lily-infecting potyviruses were alignments. There are 60% ~ 77% sequence similarities in HC-Pro gene, 61% ~ 80% similarities in NIa gene, and 50% ~ 71% similarities in CP gene (data not shown). Moreover, when the consensus sequences without variable regions were compared, the amino acid similarity increased to more than 70% in HC-Pro and NIa genes (Fig. 1). The highest homology of these conserved regions is in the CP gene, the sequence similarity is over 85%, and the identity of this region is over 71%. It means that the most conserved area is located within the CP gene. In addition, there are more continuous consensus fragments in the CP gene than in the HC-Pro or NIa genes (Fig. 1C).

As many researches indicated previously, the dsRNA can trigger the antivirus pathway in plants. To construct the dsRNA interference clones, specific primers were designed based on the TuMV-Zan genomic sequence (Table 1), and used to amplify

the consensus fragments we selected (Fig. 1). Afterward the amplified TuMV fragments were introduced into a dual T7 promoter vector, Litmus 38i, and created eight constructs that could synthesize TuMV dsRNAs by *in vitro* transcription (Fig. 2). In addition, as a non-homologous control, another dsRNA construct which contains 580 bp of CMV CP fragment was created. Within these constructs, TuHC1-2 derived from TuHCEcoF1 and TuHCHinR2 primers is the largest one containing 876 bp of TuMV HC-Pro gene. TuHC1-1 derived from TuHCEcoF1 and TuHCHinR1 primers included the shortest 223-bp fragment. There are two sets of constructs located at different genes but with similar length, the first set ranged from 366 - 385 bp including TuHC2-2 (amplified from TuHCEcoF2/TuHCHinR2, 385 bp), TuHC (amplified from TuHCBamF/TuHCSalR, 366 bp), and TuCP (amplified from TuCPBamF/TuCPSalR, 366 bp); the second set is about 470 bp long including TuHC1-1L (amplified by TuHCEcoF1/TuHCHin1-1R, 474 bp), TuHC2-2L (TuHCEco2-2F/TuHCHinR2, 470 bp) and TuNIa (amplified by TuNIaF1/TuNIaHinR1, 470 bp). TuHC1-1L locates at the 5' half of TuHC1-2 fragment and covers the entire TuHC1-1. TuHC2-2L locates at the 3' half of TuHC1-2 fragment and covers the entire TuHC2-2. Total six TuHC dsRNA interference constructs are within the region of TuHC1-2 (Fig. 2).

Interference with TuMV infection by direct dsRNA inoculation

At first, the efficiency of RNAs interference was investigated by mechanical inoculation of virus. In this experiment, dsRNA transcripts mixed with total RNA, which was purified from TuMV-infected plants, were applied onto the hypersensitive host, *N. tabacum* var. White Burley. The mechanical inoculation of TuMV-infected total RNA together with TuMV dsRNA transcripts interfered with the lesions elicited by TuMV infection in *N. tabacum* at 7 dpi. However, coinoculation of TuMV-infected total RNA and CMV dsRNA transcripts did not have any interfering effect on TuMV infectivity (Table 2). Furthermore, three shorter types of dsRNAs, TuHC1-1 (223 bp), TuHC (366 bp) and TuCP (366 bp), did not block lesions formation completely. The interfering activity was slightly reduced as the lesion numbers of these treatments were higher than those of three longer dsRNAs (TuHC1-2, TuHC2-2, and TuNIa). However, the lesions on TuHC1-1, TuHC and TuCP dsRNA-inoculated plants were highly suppressed when compared with inoculated control plants (Table 2).

To evaluate the capability of dsRNA to interfere with TuMV infection in a systemic host, *N. benthamiana*, the plants were inoculated with the mixtures of TuMV-infected plant total RNA plus each of the dsRNA transcripts as described above. Seven days after inoculation, TuMV infection was completely inhibited by

three types of dsRNAs, TuHC1-2, TuHC2-2 and TuNIa (Table 2 and Fig. 3G-I). Whereas the ability to interfere with the virus infection by TuHC1-1, TuHC and TuCP dsRNA transcripts was not so good since there were about 1/3 of plants infected by TuMV (Table 2; Fig. 3D1, E1, and F1). However, still 2/3 of the tested plants did not show symptom at 7 dpi (Fig. 3D2, E2 and F2). These results suggested the interference with TuMV infection by shorter TuMV dsRNA fragments were not as good as the longer ones. The outcomes of interfering capacity of different dsRNAs were summarized in Table 2. The experiments were repeated three times and similar results were observed.

To confirm the infection of TuMV, tested plants were assayed by northern blot and immunoblot. Analyses of total RNA and total protein extracted from the inoculated and systemic leaves of the inoculated control plant at 7 dpi indicated the infection and amplification of TuMV (Fig. 4 and 5, lane 3). CMV dsRNA transcripts could not interfere with TuMV infection (Fig. 4 and 5, lane 10). When homologous dsRNAs were present in the inocula, TuMV amplification was apparently blocked in the inoculated leaves (Fig. 4, lane 4-9) and did not accumulate to detectable level in the non-inoculated systemic leaves (Fig. 5, lane 4-9). Because only about 30% of TuHC1-1, TuHC and TuCP treated plants displayed symptom, the samples used for virus accumulation assays were collected from asymptomatic plants and were not

infected by TuMV (Fig. 4 and 5, lane 4, 7, 9). These protected plants were free of symptoms until their life cycles were completed (data not shown).

According to our experimental results, the lengths of dsRNA fragments with lower interference ability were 223 bp to 366 bp, and the longer ones had better interference with TuMV infection. These data suggested that the dsRNA-mediated resistance has length-dependent effect. However, the complete interference construct, TuHC2-2 (385 bp), is only 20 bp longer than TuHC (366 bp) and contains almost the whole sequence of TuHC with only a slightly shift (Fig. 2B). But these two dsRNA fragments revealed different outcome in virus interference assay. In addition, TuHC1-1 (223 bp) is only about half length of TuHC2-2, and both fragments were located in different parts of HC-Pro conserved region (Fig. 2A) and also displayed varied results in TuMV interference. This raised another question in regard to the importance of length and sequence from viral genome in resistance response.

From the results of coinoculation assays, both the length and sequence might contribute to the interference of virus infection. To further clarify the sequence effect of dsRNA, two constructs, TuHC1-1L and TuHC2-2L, were generated to express 470-bp dsRNA the same as TuNIa. The protective effect against TuMV infection was assayed by sequential inoculation. TuMV was challenge-inoculated on the leaves which were first inoculated with dsRNA transcripts with an interval of 12 hours.

Virus accumulation could be detected in TuMV-inoculated control plant at 7 dpi (Fig. 6A). The systemic leaves of TuHC1-1L and TuHC2-2L inoculated plants did not show symptoms as the mock control (Fig. 6B). The results meant expanding the fragment length of dsRNA could improve the interfering effect. Accordingly, using this mechanical co-inoculation method could quickly screen the highly potential interference sequences.

Agrobacterium-mediated transient expression of dsRNAs in plants

From the results of dsRNA transcripts inoculation assay, the fragment length with the stable interference effect may be up to 400 bp. Agrobacteria infiltration is a method for quickly expression of dsRNAs molecules in plant cells. Thus, two of dsRNA constructs, TuHC2-2L and TuNIa, which revealed better protection capability, were chosen for transient expression of dsRNA in host plants by argoinfiltration. TuHC2-2L sequence is located near 3' part of HC-Pro gene and covers the region of TuHC2-2 (Fig. 2B). From previous tests, TuHC2-2, TuHC2-2L and TuNIa had high interference with virus infection. We believed TuHC2-2L and TuNIa would be more stable and suitable for further analysis since they had longer length than TuHC2-2. For efficient dsRNA expression *in planta*, a *rolA* intron was introduced between two inverted viral sequence fragments and created two hpRNA expression constructs

(pBI-hpHC2-2L and pBI-hpNIa, Fig. 7B). The hypersensitive, *N. tabacum*, and systemic, *N. benthamiana,* hosts of TuMV were used for transient expression assay.

For evaluation the protective effect against virus infection at different times after delivery of hpRNA expression clones into plant, TuMV was inoculated on tobacco plants at 1 and 7 days after infiltration with either pBI-hpHC2-2L, pBI-hpNIa, pBI121 or buffer in the same leaves (Fig. 8 and Fig. 11, transient expression procedures).

Transient expression of hpRNA in hypersensitive host, *N. tabacum* var. White Burley

First, the hypersensitive *N. tabacum* plants were infiltrated with *A. tumefaciens* cultures carrying pBI-hpHC2-2L, pBI-hpNIa or pBI121 vector, and MMA buffer alone. At 1 and 7 days post-infiltration (designated as 1-inf and 7-inf), plants were challenge-inoculated with TuMV on the infiltrated leaves (1st inoculation). TuMV was inoculated onto the non-infiltrated systemic leaf at 7 dpi after 1st inoculation. The lesions were clearly appeared at 7 dpi to 14 dpi on inoculated leaves that had been infiltrated with buffer, pBI12, pBI-hpHC2-2L or pBI-hpNIa 1 day or 7 days before (Fig. 9A). In 7-inf plants, the local lesion symptoms of buffer and pBI121 control plants were more obvious and severe than those of hpRNA transiently expressed plants (Fig. 9A). The virus accumulation level was higher in control plants than in hpRNA expression plants (Fig. 9B). However, the lesion numbers were similar in all

treatments (Fig. 9C). By contrast, the symptoms, lesion numbers, and virus titer were significantly different between controls and hpRNA expression plants on the non-infiltrated systemic leaves (Fig. 10).

Transient expression of dsRNA in systemic host, *N. benthamiana*

The systemic host, *N. benthamiana*, were first infiltrated with buffer or *A. tumefaciens* containing pBI-hpHC2-2L, pBI-hpNIa or pBI121, and was challenge-inoculated by TuMV after 1 or 7 days of infiltration (Fig. 11). The challenge inoculation on 7-inf plants that had been infiltrated with pBI-hpHC2-2L, pBI-hpNIa contracts remained symptomless throughout the entire testing times (about 6~8 weeks), and the plants grew normally as mock control (Fig. 12A, 7-inf). But other inoculated treatments including all 1-inf plants and the 7-inf buffer and pBI121 plants showed systemic symptom at 4-7 dpi of TuMV inoculation (Fig. 12A and B). Northern blot analysis of RNA samples taken from the systemic leaves at 7 days of challenge-inoculation indicated that TuMV could be detected in the plants showing symptoms (Fig. 13A). This data inaccordance with the I-ELISA results of the inoculated and systemic leaves (Fig. 13B and C). These results suggested that only 1 day for hpRNA infiltration and expression is unable to protect plants from virus infection. In addition, the ability of interference with TuMV infection by transient expression of homologous dsRNAs could reach to 7 days. According to these data, it

indicated that the hpRNA-mediated interference is time-dependent.

HC2-2L and NIa fragments were selected from the conserved region of at last four potyvirus species, the interference of these two hpRNA constructs was also tested on *Bean yellow mosaic virus* (BYMV) which was reported as a calla lily-infecting potyvirus (Pham *et al*., 2002). Interference with BYMV infection was assayed by transient expression of hpHC2-2L and hpNIa RNAs on *N. benthamiana*. After challenge-inoculation of BYMV on agro-infiltration plants at 7 dpi, three out of hpHC2-2L and two out of hpNIa expressed plants were symptomless compared with buffer and pBI121 control plants (data not shown). The virus accumulation analyzed by I-ELISA also revealed the same result (Supple. 1). However, two of the three independent experiments showed that hpRNA transiently expressing plants had delayed symptoms at 10 ~ 12 dpi (data not shown). These results indicated that even our hpRNAs were derived from the conserved regions of potyviruses, the efficiency of interference depended on virus species. More virus species need to be tested in order to understand the interference spectrum of pBI-hpHC2-2L and pBI-hpNIa.

Analysis of hpHC2-2L and hpNIa transgenic *N. benthamiana* plants

The hpRNA-expressing transgenic plants were regenerated according to the *A. tumefaciens*-mediated transformation method. The kanamycin resistant T0 transgenic

N. benthamiana plants were transferred to soil and grown in a greenhouse as previously described. Rapid TuMV resistance test was performed by inoculating transgenic leaf tissue with TuMV (1:10 w/v of virus-infected *N. benthamiana* leaf ground in 0.05 M sodium phosphate buffer pH 7.2) and incubated on water agar plate. Virus accumulation was analyzed by I-ELISA at 7 days after inoculation. Among 17 T0 plants (7 lines of hpNIa, and 10 lines of hpHC2-2L), all hpNIa lines and 7 hpHC2-2L lines were not detected with virus (Fig. 14). This rapid screening was a mimic of the whole plant inoculation assay, and used to pre-screen the resistance plant lines. According to this data, the transgenic plants resistant to TuMV infection may be up to 70% ~ 100%. Three T1 lines of hpNIa and hpHC transgenic plants were chosen for further virus inoculation assay. Line 1, 2 and 13 of hpNIa T1 plants were highly resistant to TuMV infection and expressed transgene RNA due to RT-PCR positive response (Supple. 3).

Discussion

Gene silencing has been used to confer protection against plant viruses recently. Control methods against different potyvirus species were developed, including RNA silencing tool. Most of the RNA interference triggered by transient- or transgenic-expressed viral-derived sequence was resistant to single virus resistance. Multi-virus resistance was not easily achieved by only one construct. For example, transgenic *N. benthamiana* with the CP sequence of *Potato virus A* (PVA) was able to inhibit PVA infection but could not protect against *Potato virus Y* (PVY) (Savenkov and Valkonen, 2001). The reasons involved in this interference deficiency might include high level sequence identity requirement, dsRNA expression timing (Tenllado and Diaz-Ruiz, 2001) and various silencing suppressor appearance (Hiroko *et al*., 2009).

This study tried to develop a rapid and simple screening system for dsRNA-mediated virus resistance. Because the multi-virus infection is the common phenomenon in plant, broad-spectrum virus resistance is one of the important issues in plant protection. Conserved genes and consensus segments from published potyvirus sequences became the targets of our experiment.

There are five proteins with lower variability within the genus *Potyvirus*, which are HC-Pro, CI, NIa, NIb and CP. These proteins all play multi-functions and involve

in different regulation level of virus infection cycle (Urcuqui-Inchima *et al*., 2001). HC-Pro, CI, NIa, and NIb have different enzymatic functions involved in protein cleavage, RNA binding, RNA proliferation, etc. According to the functional assay, HC-Pro can be divided into three regions: aphid transmission in N-terminal; proteinase and silencing suppressor activity in C-terminal; silencing suppressor and small RNA binding ability in the central region (Shiboleth *et al.*, 2007). The central region also contains several highly conserved motifs including the FRNK box, which is required for small RNA binding (Shiboleth *et al.*, 2007). The HC-Pro fragments selected for dsRNA silencing in this work were within the central to C-terminal region of TuMV HC-Pro protein (Fig. 2A). Besides FRNK box, other consensus motifs were observed in the four calla lily-infecting potyviruses (Fig. 1A) confirming the regions of silencing suppressor function. This low variable features of HC-Pro sequences indicated the suppressor activity is similar and important in potyvirus infection. Blocking the suppressor seems useful for broad-spectrum resistance. Although the FRNK box located in TuHC1-1, the shortest dsRNA construct, the assay indicated its incomplete interference with TuMV (Table 2 and Fig. 3D1). In addition, other two longer constructs TuHC1-2 and TuHC2-2, with (the former segment) or without FRNK box (the latter one) displayed high interference capability. This meant the dsRNA triggered siRNA silencing response in plant might be length-dependent.

TuHC2-2/TuHC2-2L fragments located in the C-terminal domain which contains suppressor and proteinase functions also revealed high interference ability. Interestingly, the TuHC fragment is a little shift and only 20 bp shorter than TuHC2-2 (Fig. 1A and Fig. 2B) had lower protection ability (Fig. 3F2). Although the TuHC contained almost the same conserved segments of TuHC2-2, maybe suggested that the sequence itself also sufficient for specific interference. However, this point need to been demonstrated by further experiments. It also pointed out that the influence of conserved motifs near the C-terminal of HC-Pro was inhibited HC-Pro function.

NIa consists of C-terminal NIa-Pro proteinase domain and N-terminal genome-linked VPg protein (Germundsson *et al*., 2007). The VPg is indicated importantly in virus-host interactions (Schaad *et al*., 1997). Except P1 and HC-Pro, other mature protein products are processed by NIa-Pro proteinase (Germundsson *et al*., 2007). Therefore, NIa is though to be a suitable target sequence of virus interference. The multiple sequence alignments showed sequence variation in the N-terminal regions, and region (S654GLRDTN; LPGIGYGxxVIT; KDMVLIxMPKD and ERVCMVG3NFQxKSM) in NIa-Pro domains of four calla lily-infecting potyviruses (Fig. 1B). The only fragment including regions described above used for interference of TuMV infection was effective in co-inoculation and transient expression as well as the hairpin transgenic plants in this study. This indicated the NIa

region is a good trigger or target for PTGS. However, different from VPg domain, NIa-Pro domain has not been used for RNA interference experiment (Germundsson *et al*., 2007).

Interestingly, for some unclear reasons, in our analysis CP region was not good enough in interference assay despite that it is highly conserved. Our result is different from that of Vargas, *et al*. (2008). One possibility might be the sequence length. Therefore, extending the CP fragment for interference assay will realize the role of target fragment length in RNA silencing. The various efficiency induced by different viral target regions in interference response were discovered in the study of *Potato virus X* (PVX) (Takahashi et al., 2006). It was reported that hpRNA of TGBp1, the suppressor gene of PVX, had stronger interference effects on PVX infection than that of CP gene.

From the transient expression assay, *N. tabacum* was used for local and systemic silencing assay. Fortunately, the tested plants showed silencing on both TuMV challenge-inoculated leaves which were infiltrated or non-infiltrated systemic leaves (Fig. 9 and Fig. 10). Although the lesion numbers in inoculated leaves of all treatments were similar (Fig. 9C), the virus replication was interfered in the test plants (Fig. 9B). It might be due to lesions on hpHC2-2L and hpNIa-infiltrated leaves quickly restricted by plant defense response compared with control plants. Because

the P1/HC-Pro-expressed transgenic tobacco was proved to enhance SA-dependent resistance and induce PR-1 gene expression (Pruss *et al*., 2004), this finding might explain the lesion size and virus titer was interfered in hpHC2-2L and hpNIa-infiltrated leaves. Expressed the hpRNAs in the plants might induced some effects related plant defense pathway. We considered that the possible reason could be the synergistic effect of the hypersensitive and silencing response. However, the relationship between hpRNA- triggered RNA silencing and plant defense need more investigation and demonstration.

In contrast, the transient-expression induced silencing assay in *N. benthamiana* demonstrated significant silencing effect appeared in 7-inf plants, but not 1-inf test plants (Fig. 12 and 13). This result indicated the agroinfiltration delivered dsRNA triggered silencing in plants is time-dependent as previous studies (Tenllado *et al*., 2003; Vargas *et al*., 2008). On the other hand, in 7-inf plants small RNAs (extracted from systemic leaves) were absent in the symptomless hpHC2-2L and hpNIa-infiltrated plants but were highly accumulated in TuMV-infected plants according to the northern blot hybridized with the RNA probes recognizing the sense strand of TuHC and TuNIa sequences (Supple.2). Ho and colleagues isolated TuMV siRNAs from TuMV-infected *Brassica juncea* and demonstrated these siRNAs were derived from different part of TuMV genome (Ho *et al*., 2007). Moreover, TuMV

siRNAs also revealed hotspot sequence and GC-rich contents. Most siRNAs were mapped to NIb region; however several were located in HC-Pro, NIa-Pro and CP genes in which the siRNAs derived from these genes represented sense strand sequence (Ho *et al*., 2007). In addition, dsRNA with spacer sequence was not detected by RT-qPCR assay in virus immune transgenic tobacco indicating the efficient degradation (Mitter *et al*., 2006). These studies supported our results that the small RNAs were abundant in TuMV challenged control plants but rare in the hpRNA expression construct-infiltrated plants.

Application of dsRNA transcripts or constructs driven by 35S promoter into leaf surface could trigger RNA silencing response to defense plant virus. Instead of expressed functional viral RNA or proteins, the partial conserved sequences were selected for virus interference assay in this study. This strategy is still successfully interference virus infection, with no effect on the plant normal growth. In this work, we provided two different methods for interfering with virus infection: i) mechanical inoculation of dsRNA transcripts with virus-infected total RNA and ii) transient-expression of dsRNAs by agroinfiltration. The former process could quickly screen the efficient sequence for RNA silencing, whereas the later could directly check the effect of the constructs in dsRNA-induced viral defense pathway. The selected sequences can be further applied to generate transgenic crops. According to

the transient expression and transgenic plant assays, introducing the applicable sequence to interfere with the enzyme-function domain of potyviral genes could obtain virus resistance effects.

In TuMV-infected plant, the virus-induced siRNAs were observed and analyzed that there are siRNA hotspots in viral genome (Ho et al., 2007). We still do not understand the roles of the siRNA hotspots, are they the efficient silencing trigger or just the results of degradation? Furthermore, the effect of sequence and fragment length of dsRNA on inducing resistance to virus infection is another interesting issue. Combination of the SA-induced defense pathway and RNA interference pathway, the mechanism of PTGS should be more complex than what is known today (Pruss *et al*., 2004; Alamillo *et al*., 2006). More studies in these issues will help us to understand the RNA silencing pathway in defense of virus infection.

In 2004, Tenllado and colleagues suggested that successful interference with virus infection by RNAi could reduce the use of pesticide in agriculture and also reduce the environmental risks (Tenllado *et al*., 2004). Nevertheless, there still are questions concerning the potential ecological impact of virus-resistance transgenic plants, for instance, the recombination between transgene and infecting viral RNA. Although in this study, TuMV applied to dsRNA-expressed tobacco plant, not only virus itself but also dsRNA were reduced to non-detectable level (Supple. 2) confirmed the

consequence of the detection data from Mitter *et al*. in 2006. Accordingly, the biosafety concern of these transgenic plants is not so critical. However, to achieve the multi-virus defense is still important for many crops including calla lily and other ornamental plants.

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Table 1. Primers for the construction of the TuMV and CMV interference constructs

Corresponding gene	Primer name	Sequence (5' to 3')[a]	Position
TuMV-Zan			
HC-Pro	TuHCEcoF1	GCATGAATTCGAGATAGCACGATACCTGA	1697-1715 nt
	TuHCEcoF2	GCATGAATTCGTACATAGATCTCCCTGAGATCG	2188-2210 nt
	TuHCEco2-2F	GCATGAATTCTGTGTCACAACAGAATCAGGCG	2096-2117 nt
	TuHCBamF	GCATGGATCCTGTCTGAAATTAAAATGCCAACC	2127-2149 nt
	TuHCHin1-1R	TACGAAGCTTGTTACCAATCACTAGGTGGTGTT	2176-2153 nt
	TuHCHinR1	TACGAAGCTTGGGTGTAACCTTTCTTTGGA	1919-1900 nt
	TuHCHinR2	GCGGTAGTGTTTCAAGCTTGAT	2584-2563 nt
	TuHCSalR	GCTAGTCGACACAGTGATCCATATGAATCAAC	2492-2471 nt
NIa-Pro	TuNIaF1	CACCACACATACCTCTCAAAGT	6324-6345 nt
	TuNIaHinR1	TACGAAGCTTCCGTCTTTCGTACTAATCCAGTG	6924-6902 nt
CP	TuCPBamF	GCTAGGATCCACGGAGGACAAAATGCAAATCATTC	9131-9155 nt
	TuCPSalR	GCTAGTCGACCAGTGCTGCTGCTTTCATCTG	9496-9476 nt
CMV			
CP	CMVCP-F1	ATGGACAAATCCGAATCAACCAG	1-23 nt
	CMVCP-R1	GCTAGCGGCCGCAACTGGGAGCACCCCTGATG	654-635 nt

[a] sequence with underline indicates restriction enzyme cutting site

Table 2. Interference with TuMV infection by dsRNA treatments in hypersensitive and systemic hosts

Treatment[a]	*N. tabacum*[c]	*N. benthamiana*[d]
Mock control	**0**	**0/9**
Inoculated control	**48**	**9/9**
TuHC1-1 dsRNA	**1**	**2/9**
TuHC1-2 dsRNA	**0**	**0/9**
TuHC2-2 dsRNA	**0**	**0/9**
TuHC dsRNA	**3**	**2/9**
TuNIa dsRNA	**0**	**0/9**
TuCP dsRNA	**4**	**3/9**
CMV CP dsRNA[b]	**46**	**9/9**

[a] All treatments except mock control were inoculated with TuMV-infected plant total RNA which was mixed with different dsRNA transcripts or 1X transcription buffer (inoculated control).

[b] CMV dsRNA treatment was constructed as the non-homologous dsRNA control.

[c] Total lesion number on nine inoculated *N. tabacum* plants, the hypersensitive host.

[d] Numbers of infected / total inoculated *N. benthamiana* plants, the systemic host.

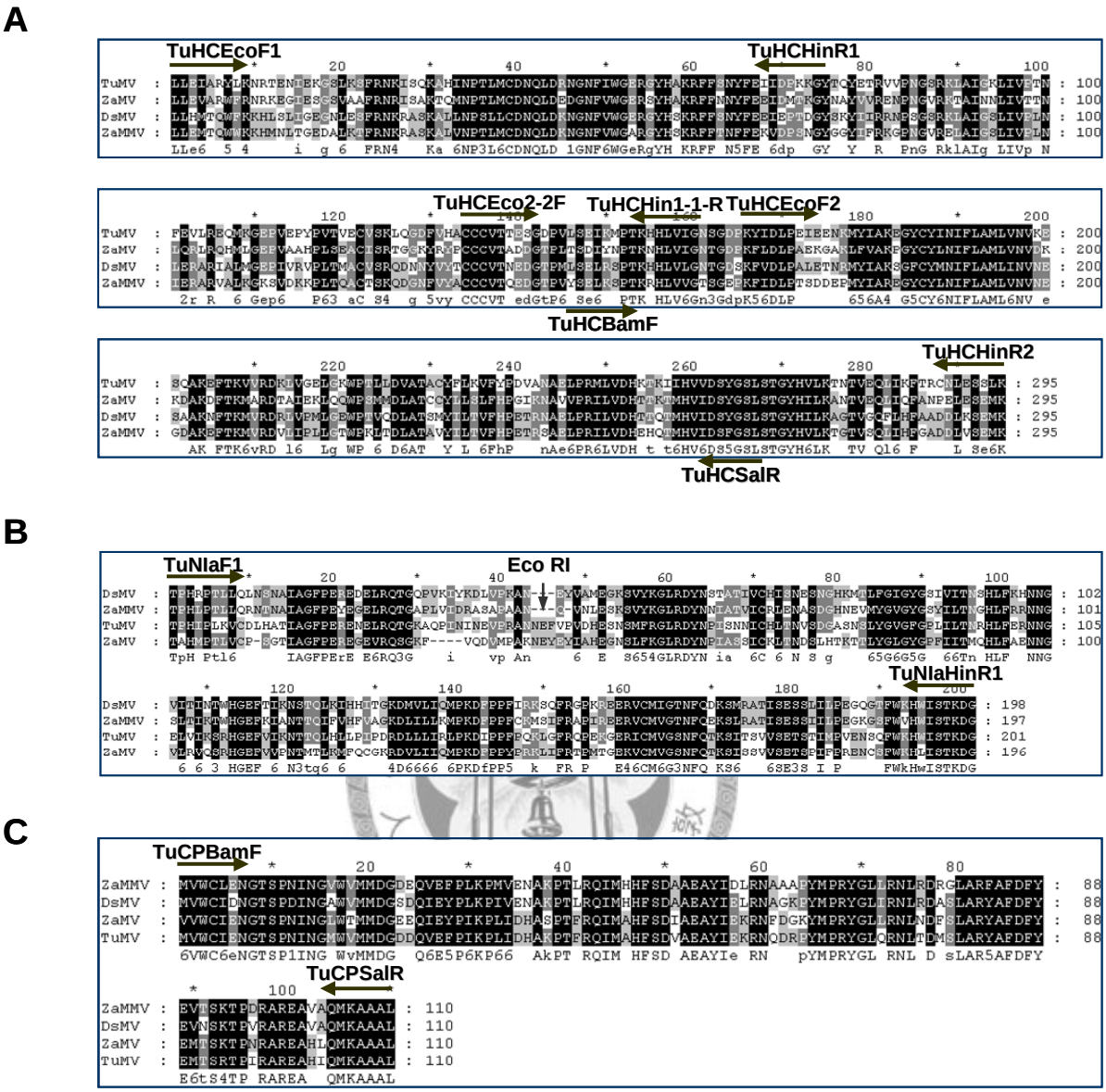


Fig 1. The amino acid sequence alignments of the selected conserved regions in three consensus genes, HC-Pro, NIa and CP genes of four calla lily - infecting potyviruses (TuMV, ZaMV, ZaMMV, DsMV). The range of sequence similarity is 77 ~ 86% in HC-Pro (A), 70 ~ 81% in NIa (B), and 85 ~ 93% in CP (C) genes, respectively**.** The locations of the primers are indicated by arrows.

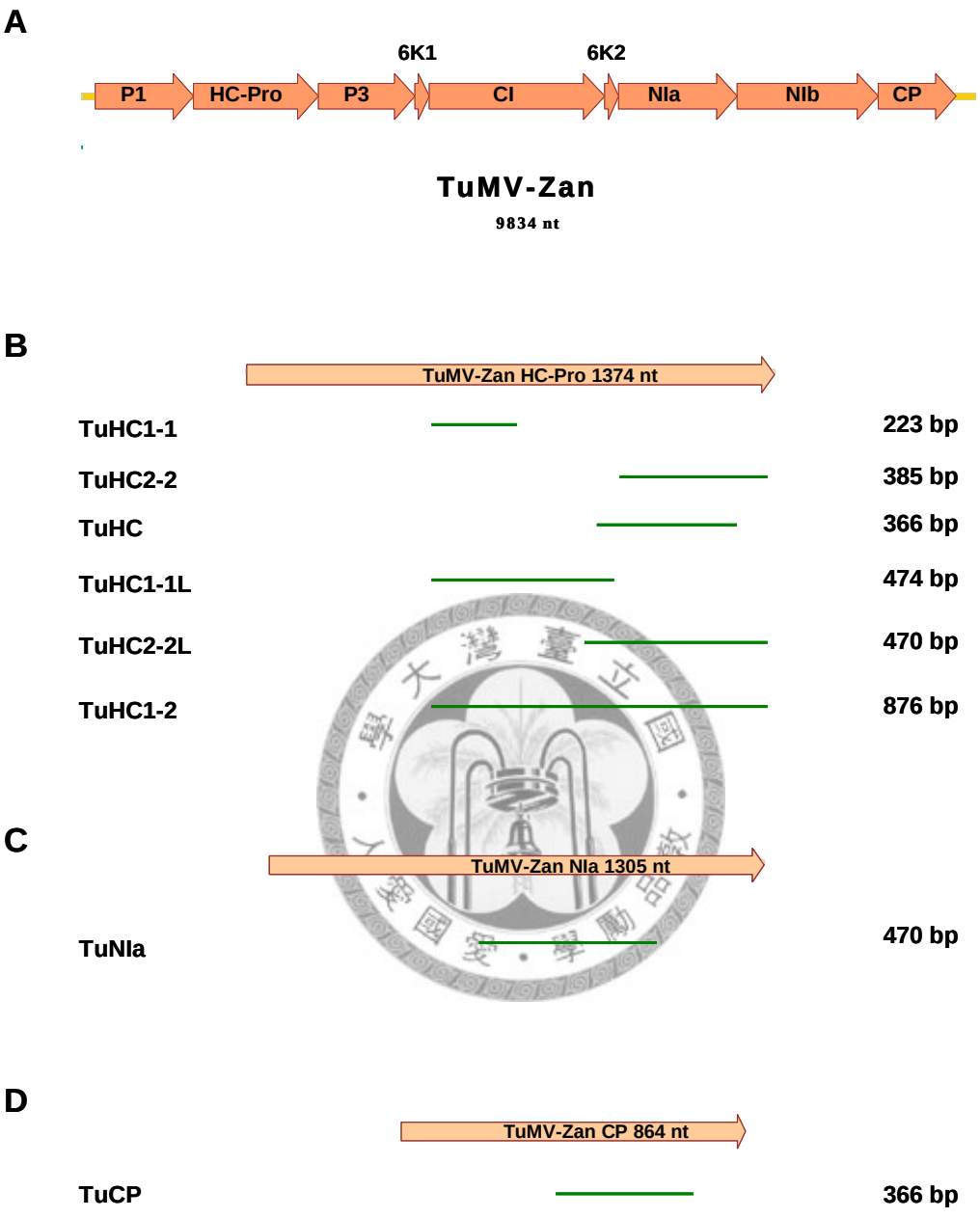


Fig 2. Schematic representation of TuMV-Zan genome, and the dsRNA constructs. (A) TuMV-Zan genome. (B) The locations and sizes of six dsRNA constructs derived from TuMV HC-Pro gene. (C) The location and size of the dsRNA construct derived from NIa gene. (D) The location and size of the dsRNA construct derived from CP gene.

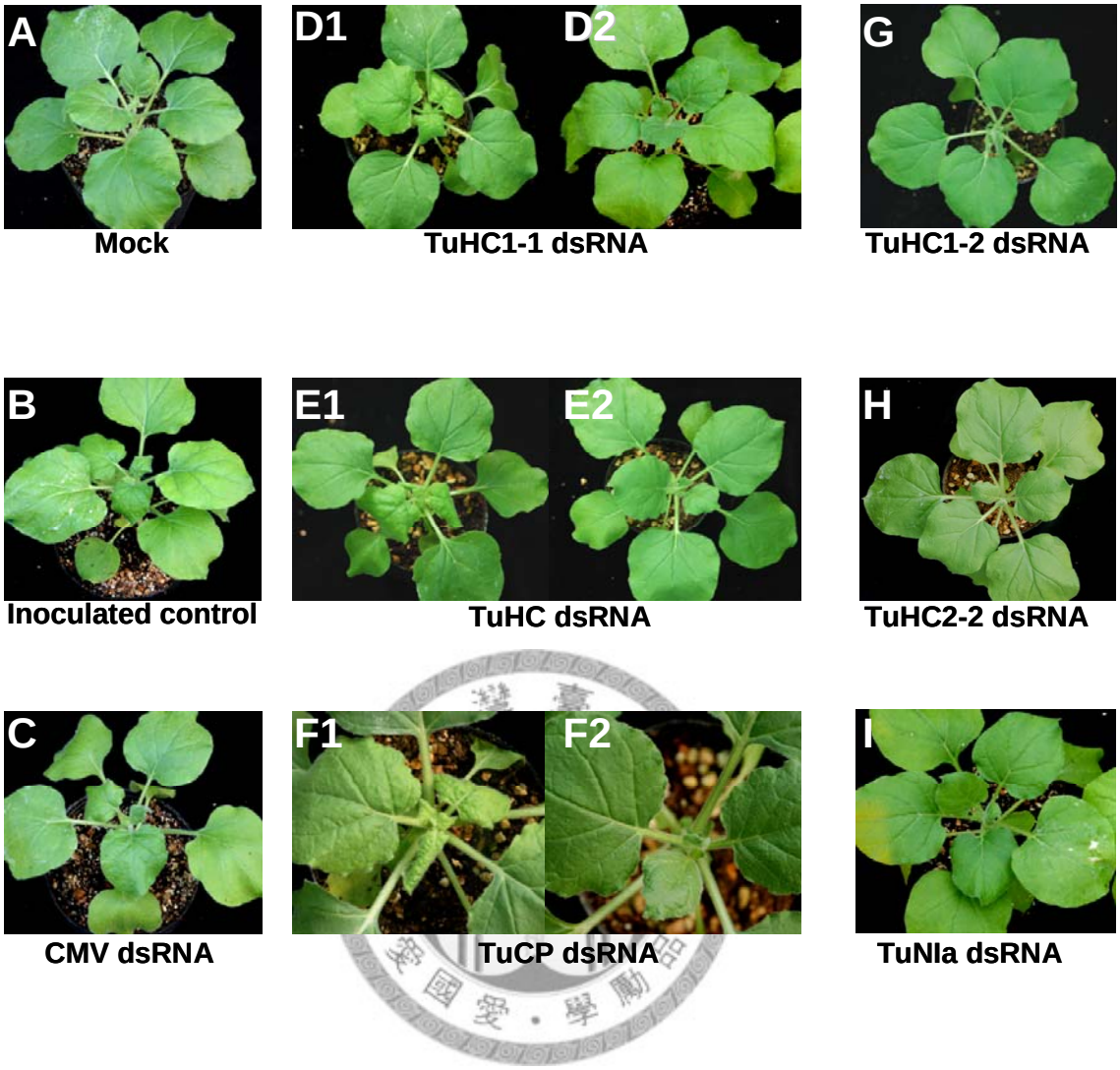


Fig 3. TuMV interference assay by dsRNA in systemic host, *Nicotiana benthamiana*. Plants were inoculated with TuMV-infected plant total RNA plus dsRNA transcripts or buffer. (A) Mock, (B) inoculated and (C) CMV dsRNA controls. TuMV interference assayed by different TuMV dsRNAs including (D) TuHC1-1, (E) TuHC, (F) TuCP, (G) TuHC1-2, (H) TuHC2-2, and (I) TuNIa. Symptomatic (D1, E1, F1) and asymptomatic (D2, E2, F2) plants treated with TuHC1-1, TuHC, and TuCP dsRNAs, resprectively. The dsRNA fragments interference assays were performed in three repeats.

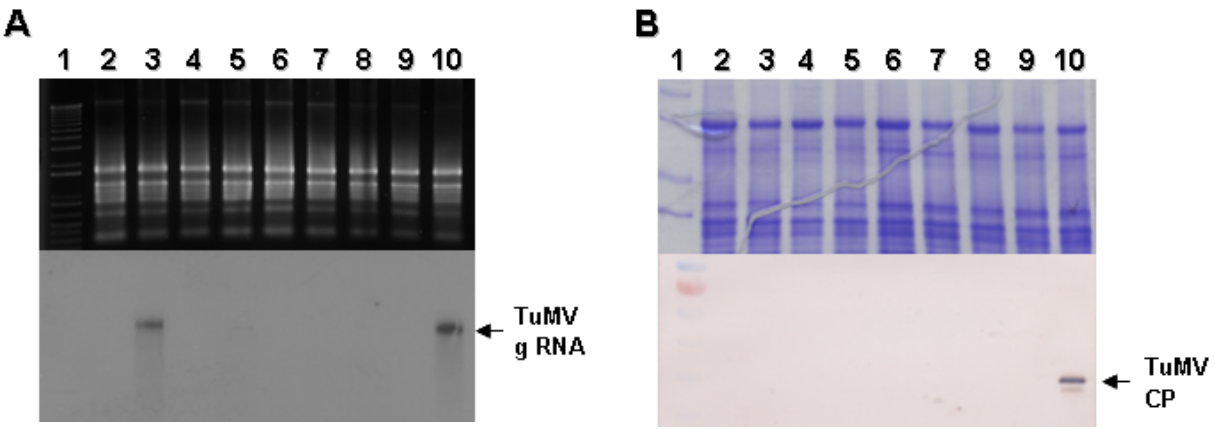


Fig 4. TuMV accumulation in virus and dsRNA-coinoculated leaf of *N. benthamiana*. *N. benthamiana* plants were inoculated with TuMV and dsRNA mixture. Inoculated leaves were harvested at 7 dpi for analysis. (A) Total RNA extracted from inoculated leaf was analyzed in 1% agarose gel (top) and northern blot was hybridized with DIG-labeled TuMV 3' DNA probe (bottom). (B) Total protein crude extracts from inoculated leaf was separated in 12% SDS-PAGE and stained with Coomassie brilliant blue (top). Western blot was detected by TuMV CP antibody (bottom). Lane 1, marker. lane 2, mock, lane 3, inoculated control, lane 4-10, dsRNA plus TuMV-infected total RNA. Lane 4, TuHC1-1, lane 5, TuHC1-2, lane 6, TuHC2-2, lane 7, TuHC, lane 8 TuNIa, lane 9, TuCP, and lane 10, CMV dsRNA treatments, respectively. The arrows indicate TuMV genomic RNA and coat protein.

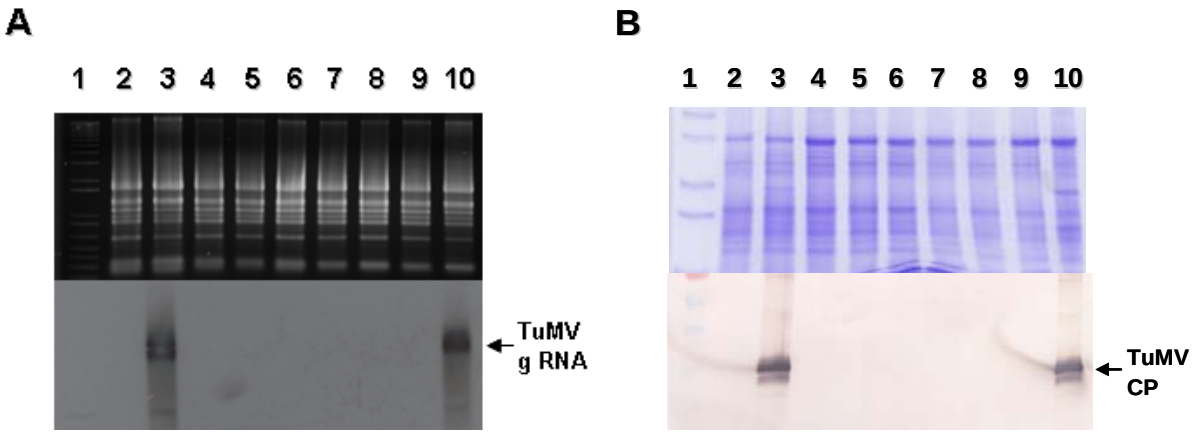


Fig 5. TuMV accumulation in systemic leaf of virus and dsRNA-coinoculated *N. benthamiana*. *N. benthamiana* plants were inoculated with TuMV and dsRNA mixture. Systemic leaves were harvested at 7 dpi for analysis. (A) Total RNA extracted from systemic leaf was analyzed in 1% agarose gel (top) and northern blot hybridized with DIG-labeled TuMV 3' DNA probe (bottom). (B) Total protein crude extracts from systemic leaf was separated by 12% SDS-PAGE and stained with Coomassie brilliant blue (top). Western blot was detected by TuMV CP antibody (bottom). Lane 1, marker. lane 2, mock, lane 3, inoculated control, lane 4-10, dsRNA plus TuMV total RNA interference assays. Lane 4, TuHC1-1, lane 5, TuHC1-2, lane 6, TuHC2-2, lane 7, TuHC, lane 8 TuNIa, lane 9, TuCP, lane 10, CMV dsRNA treatments, respectively. The arrows indicate TuMV genomic RNA and coat protein.

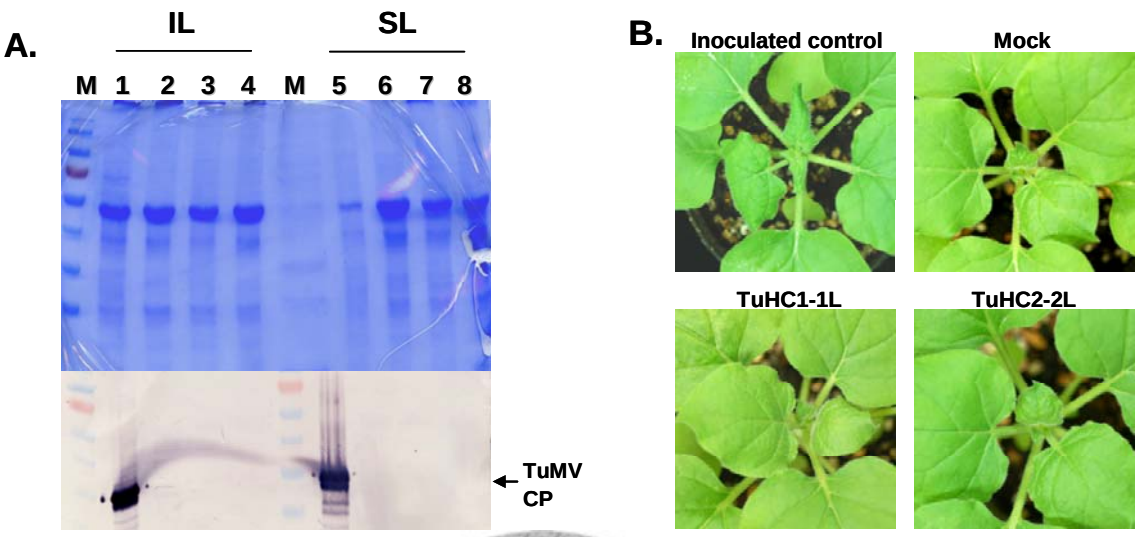


Fig. 6 dsRNA interference with TuMV accumulation by sequential inoculation in systemic host, *N. benthamiana.* *N. benthamiana* plants were inoculated with dsRNA transcripts or transcription buffer, and then challenge-inoculated with TuMV-infected plant total RNA after 12 hr. Inoculated and systemic leaves were harvested at 7 dpi for analysis. (A) Total protein crude extracts were analyzed in 12% SDS-PAGE and stained with Coomassie brilliant blue (top), immunoblot analysis was probed with TuMV CP antibody (bottom). Lane 1-4: crude extract from inoculated leaves (IL), lane 5-8: protein extracted from systemic leaves (SL). M, Marker, lane 1 and 5, TuMV-inoculated control, lane 2 and 6, Mock control, lane 3 and 7, TuHC1-1L dsRNA, lane 4 and 8, TuHC2-2L dsRNA. The arrow indicates TuMV coat protein. (B) The newly emerged leaves of *N. benthamiana* plants at 7 days after TuMV inoculation.

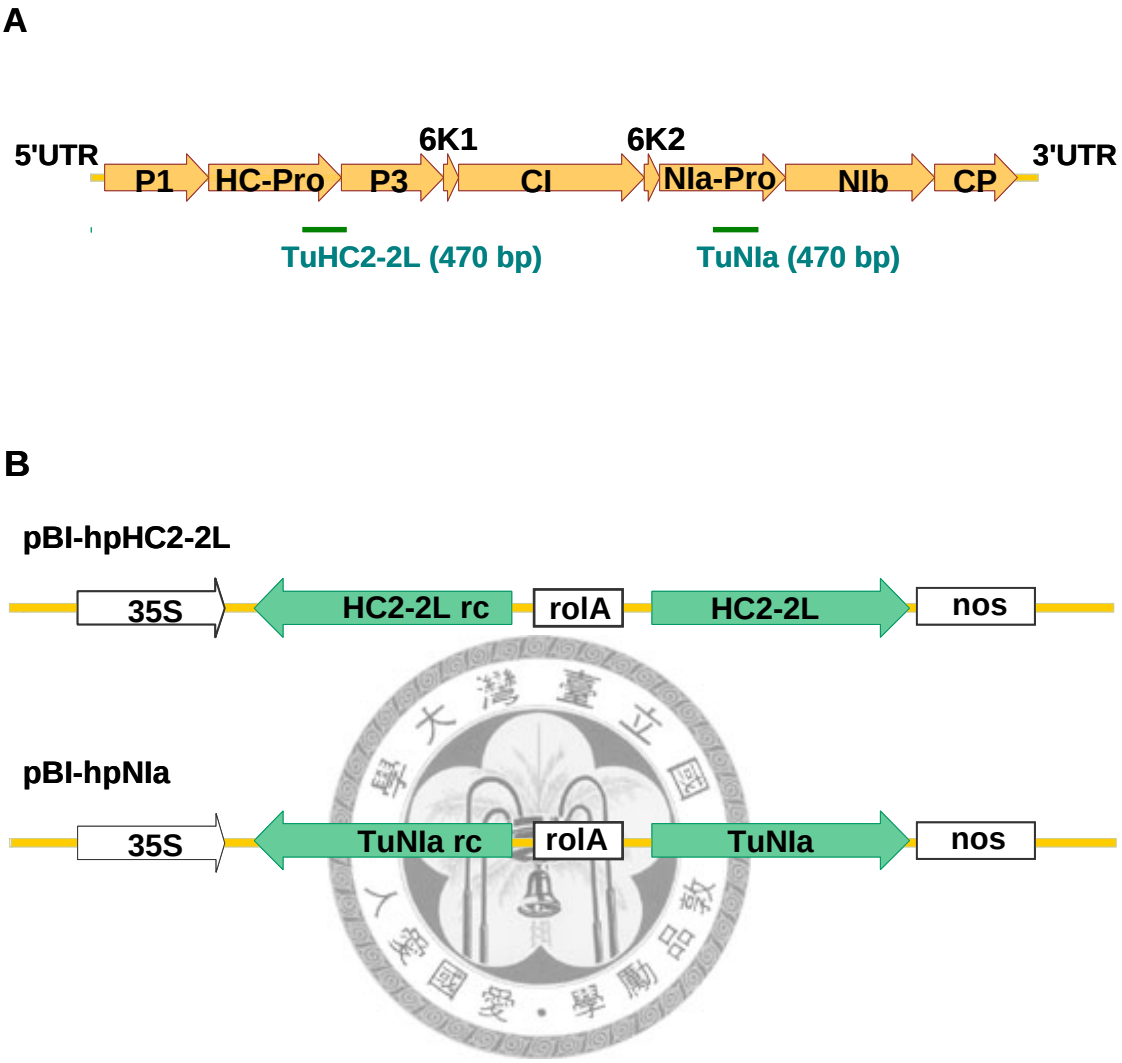


Fig 7. Schematic representation of TuMV genome and the hairpin RNA (hpRNA) constructs used in this study. (A) TuMV genome and the locations of the two fragments for hpRNA constructs are indicated. (B) pBI-hpHC2-2L, the cDNA fragments expressing sense and antisense TuHC2-2L sequence separated by a *rolA* intron (85 bp) were cloned into pBI121 binary vector. pBI-hpNIa, the pBI121 binary vector containing sense and antisense TuNIa cDNA fragments together with a *rolA* intron.

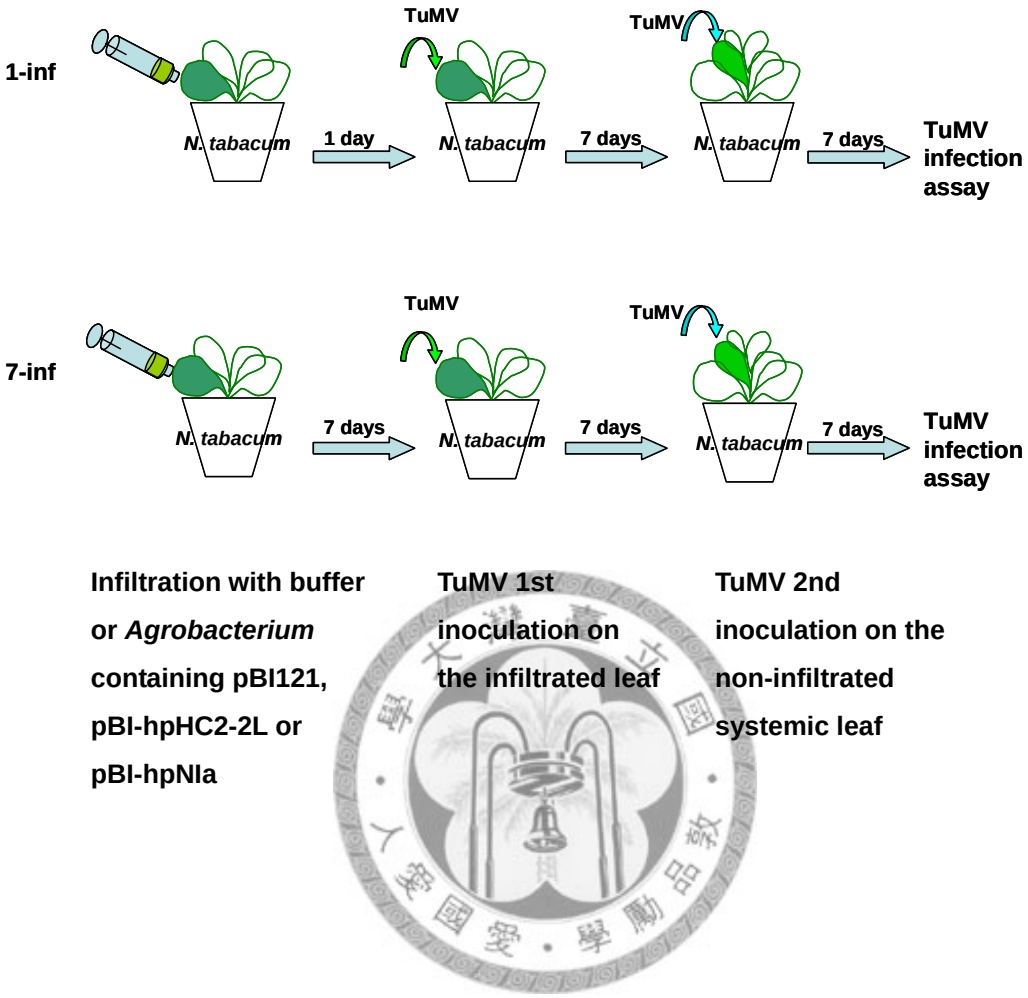


Fig 8. The experimental procedures of *Agrobacterium*-mediated transient expression of the hairpin RNA (hpRNA) in TuMV hypersensitive hosts (*Nicotiana tabacum* var. White Burley*).* Two fully expanded, lower leaves of *N. tabacum* plants were infiltrated with buffer or *A. tumefaciens* containing pBI121, pBI-hpHC2-2L or pBI-hpNIa, respectively. After 1 day or 7 days of agro-infiltration (1-inf or 7-inf), TuMV was challenge-inoculated on the infiltrated leaves of these plants (1st inoculation). At the 7 dpi of first inoculation, secondary challenge inoculation was performed on the non-infiltrated systemic leaves. Accumulation of TuMV was analyzed after 7 days of secondary TuMV inoculation.

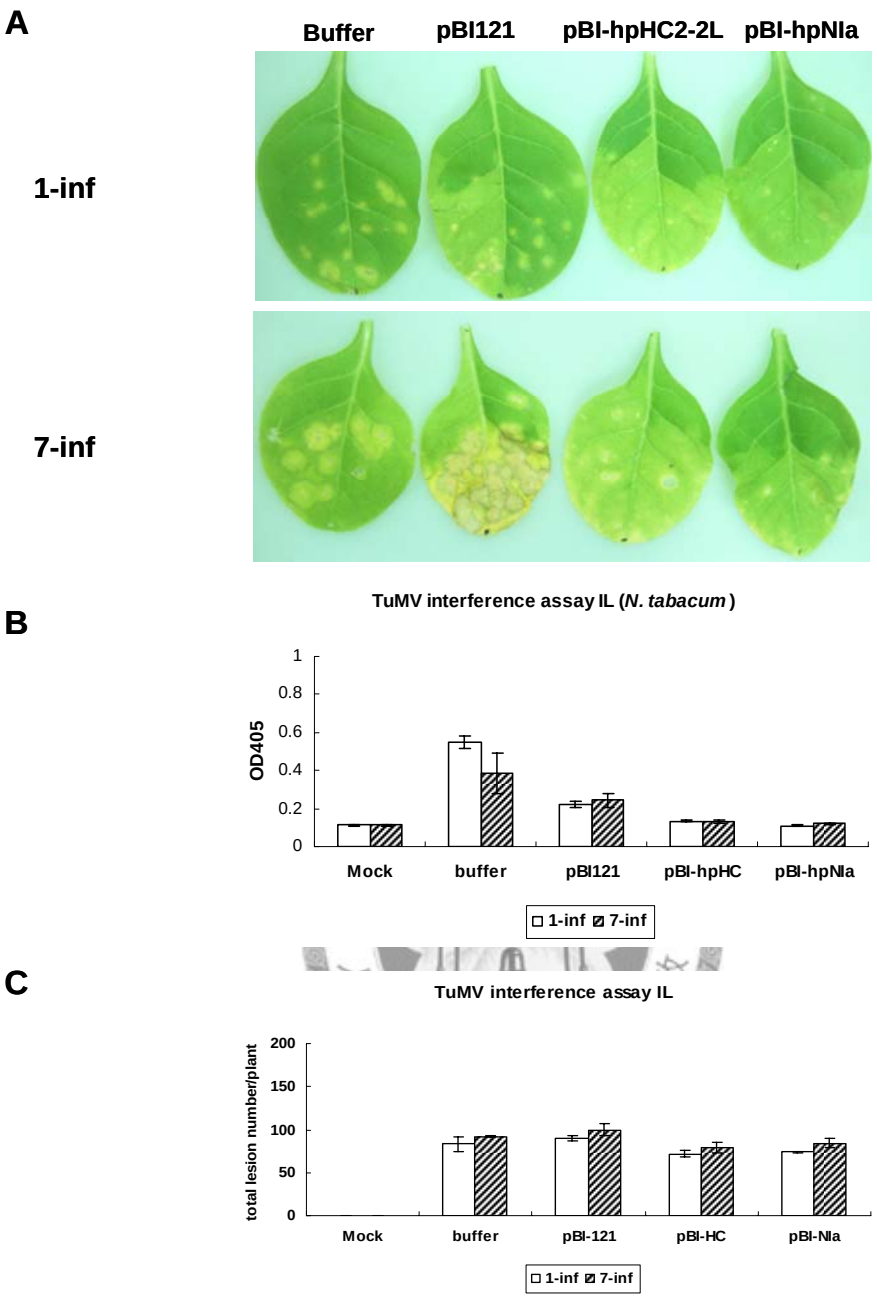


Fig 9. TuMV infection interfered by transient expression of hpRNA in the infiltrated leaves of the hypersensitive host, *N. tabacum*. TuMV 1st challenge inoculation was performed on the infiltrated leaves and analyzed at 14 dpi. (A) Symptoms of TuMV-inoculated leaves of 1-inf (top), or 7-inf (bottom) infiltrated plants with buffer, pBI121, pBI-hpHC2-2L, and pBI-hpNIa. (B) TuMV accumulation in challenge-inoculated leaves were analyzed by I-ELISA with TuMV polyclonal antibody. The ELISA value >2 times of mock control was considered as positive response. (C) Total lesion numbers on the challenge-inoculated leaves per plant. The open bars indicate the infiltrated leaves of 1-inf plants, and the slant bars represent 7-inf plants.

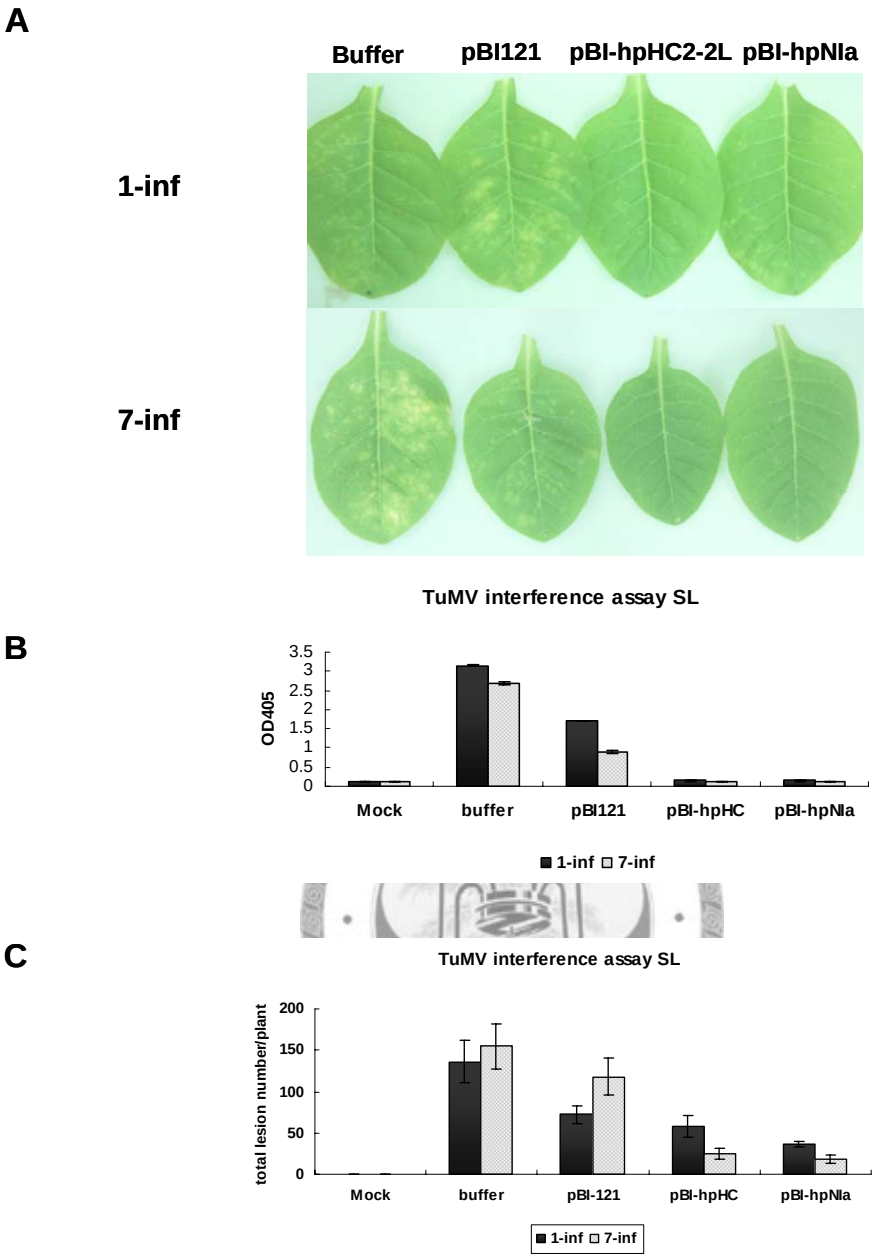


Fig 10. TuMV infection interfered by transient expression of dsRNA in the non-infiltrated, systemic leaves of the hypersensitive, *N. tabacum*. TuMV 2nd challenge inoculation was performed on the non-infiltrated systemic leaves and analyzed at 7 dpi. (A) Symptoms of TuMV-inoculated systemic leaves of 1-inf (top) or 7-inf (bottom) infiltrated plants with buffer, pBI121, pBI-hpHC2-2L, and pBI-hpNIa. (B) Analysis of TuMV accumulation in the challenge-inoculated systemic leaves by I-ELISA. (C) Total lesion number on the challenge-inoculated systemic leaves per plant. Filled bars indicate the systemic leaves of 1-inf plants, dotted bars represent 7-inf plants.

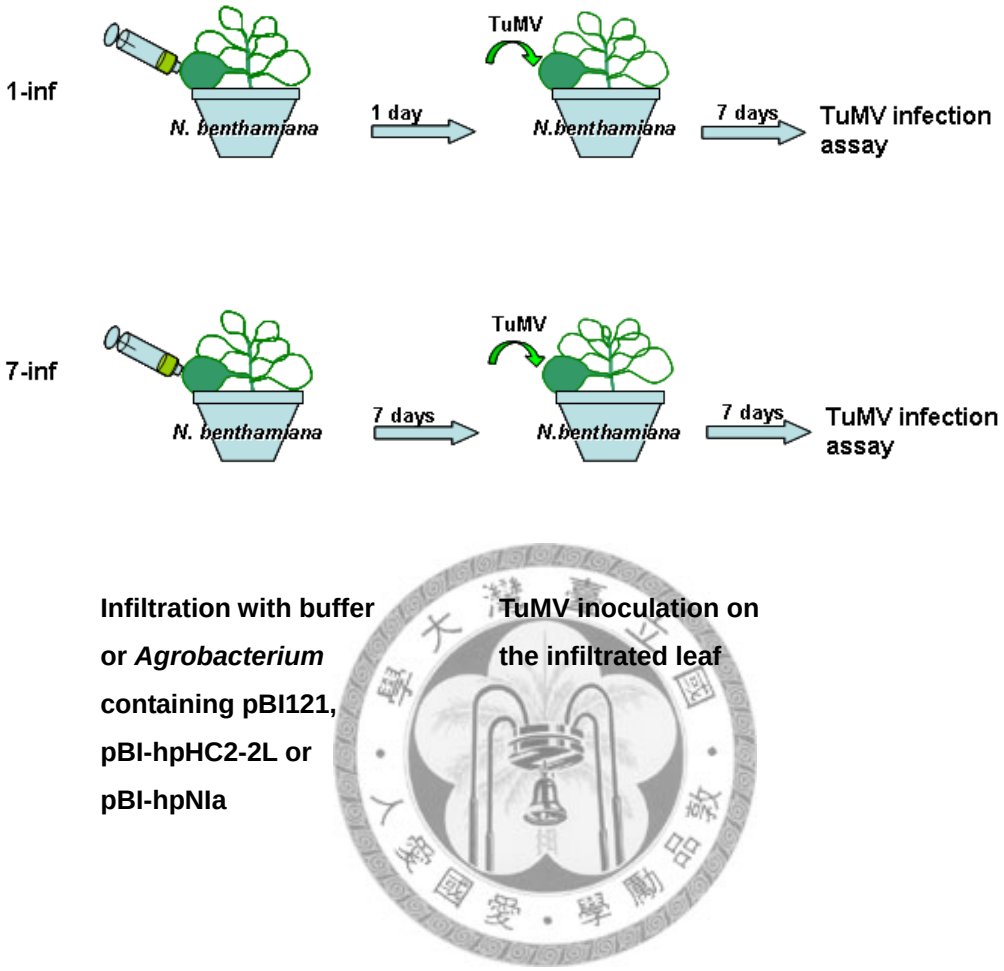
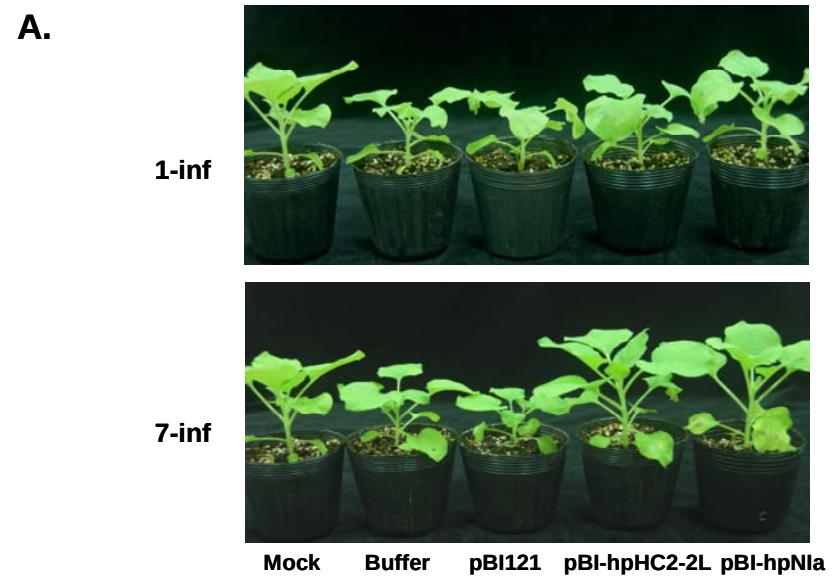


Fig 11. The experimental procedures of *Agrobacterium*-mediated transient expression of the hairpin RNA (hpRNA) in TuMV systemic host, *N. benthamiana*. Fully expanded lower leaves of *N. benthamiana* plants were infiltrated with buffer or *A. tumefaciens* containing pBI121, pBI-hpHC2-2L, or pBI-hpNIa, respectively. After 1 day or 7 days of agro-infiltration (1-inf or 7-inf), TuMV was challenge-inoculated on the infiltrated leaves of these plants. TuMV accumulations in inoculated and non-inoculated systemic leaves were analyzed by indirect-ELISA and northern hybridization at 7 dpi.



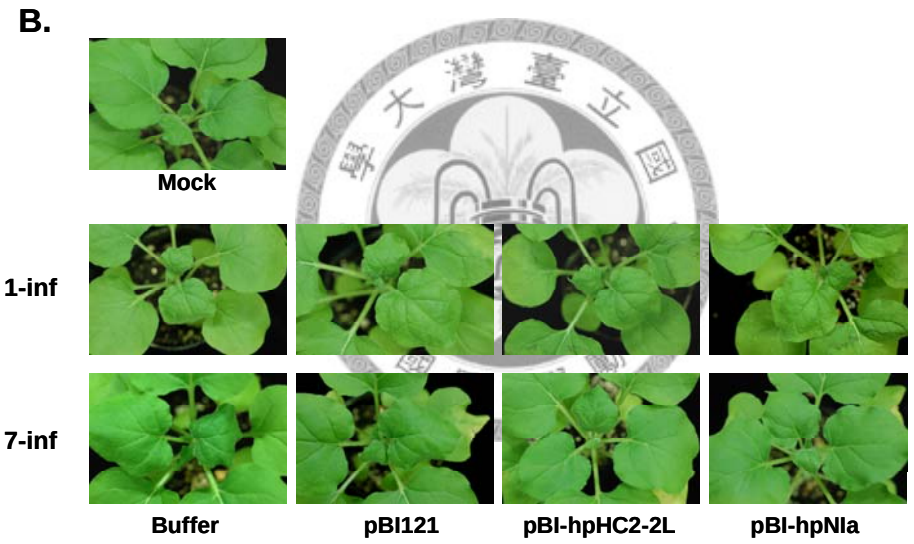


Fig 12. TuMV interference assay by *Agrobacterium*-mediated transient expression of hpRNA in *N. benthamiana*. (A) After 1 day or 7 days of agro-infiltration, TuMV was challenge-inoculated at the infiltrated leaves. The systemic symptoms of the treated plants were observed at 7 dpi. From left to right were mock, buffer, pBI121, hpHC2-2L, and hpNIa-infiltrated plants. (B) The photos revealed the newly grown leaves of the 1-inf and 7-inf plants after 7 days of TuMV challenge inoculation.

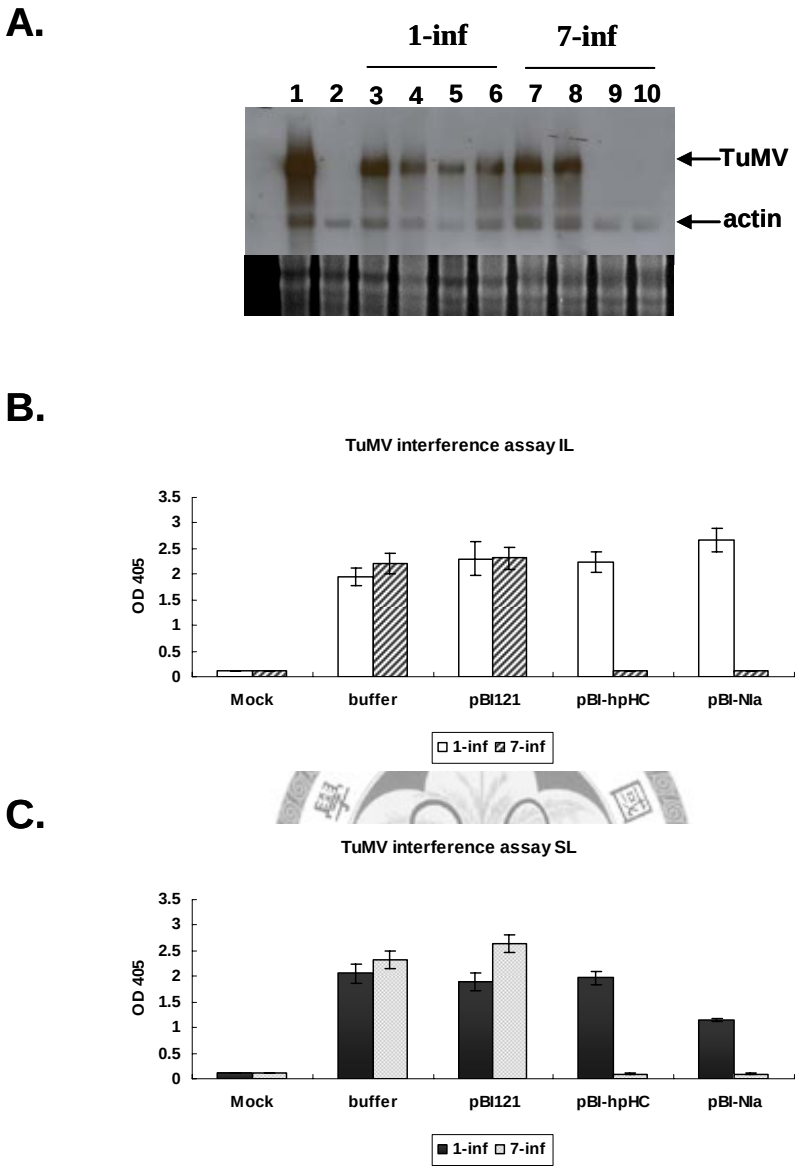
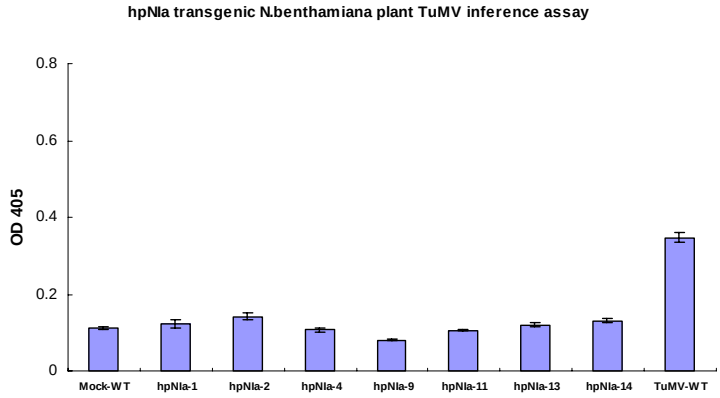


Fig 13. TuMV interfernece assay by *Agrobacterium*-mediated transient expression of hpRNA in *N. benthamiana*. (A) Northern analysis of the TuMV accumulation. Total RNA extracted from systemic leaves of TuMV-inoculated control and hpRNA expressed *N. benthamiana* plants. Lane 1, TuMV-infected control, lane 2, mock control. Lane 3-6 represent 1-inf plants, lane 3, buffer, lane 4, pBI121, lane 5, pBI-hpHC2-2L, lane 6, pBI-hpNIa. Lane 7-10 represent 7-inf plants, lane 7, buffer, lane 8, pBI121, lane 9, pBI-hpHC, lane 10, pBI-hpNIa. The arrows indicate the TuMV and actin signals. (B) TuMV accumulation was assayed by I-ELISA on challenge infiltrated leaves. (C) TuMV accumulation was analyzed by I-ELISA on systemic, non-inoculated leaves.

A.



B.

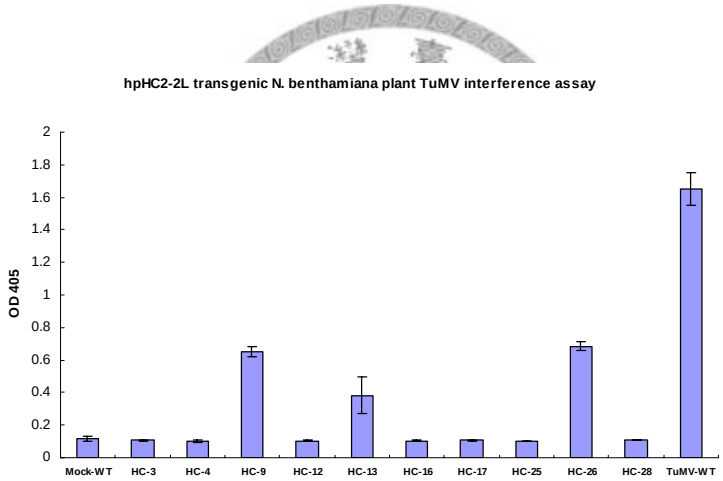
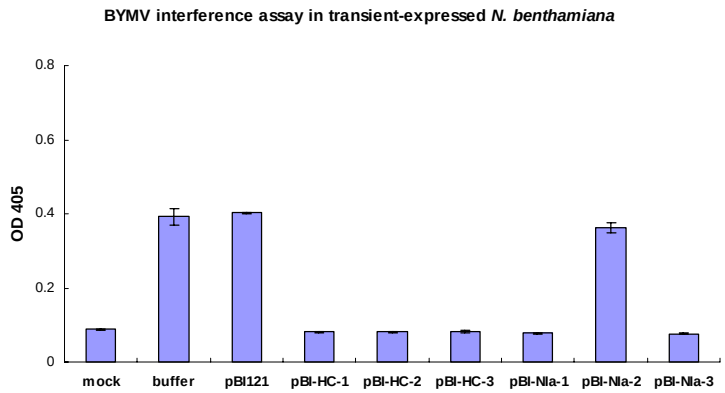
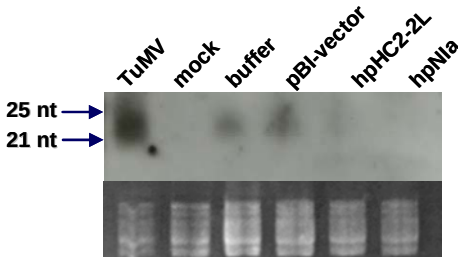


Fig 14. TuMV interference assay on leaves of T0 transgenic *N. benthamiana* plants. (A) Seven hpNIa transgenic lines and (B) ten hpHC2-2L transgenic plants were inoculated with TuMV and then analyzed by I-ELISA at 7 dpi.

Supplemental figures

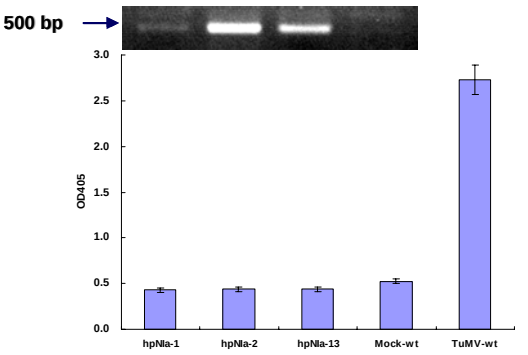


Supple. 1. BYMV interference assay by *Agrobacterium*-mediated transient expression of hpRNA in *N. benthamiana*. *N. benthamiana* plants were infiltrated with buffer or Agrobacterium containing pBI121, pBI-hpHC2-2L, or pBI-hpNIa. After 7 days of agro-infiltration, BYMV was challenge-inoculated on the infiltrated leaves of these plants. I-ELISA was performed at 7 dpi after BYMV inoculation. The mean values of A_{405} > 2 times of mock control was considered as the positive response.



7-inf

Supple. 2. Northern blot analysis of siRNAs accumulation in TuMV challenge-inoculated infiltrated plants. Samples were taken 7 days after inoculation. The blot was hybridized with a mixture of alkali-hydrolyzed DIG-labeled RNA probes specific to the sequences of TuHC2-2L and TuNIa. Equivalent loading of samples was shown by staining the gel with Healthview Nucleic Acid stain (Genomics BioSci & Tech Corp, Taiwan) before transfer. The oligonucleotides of the indicated length are shown to the left.



Supple. 3. Relationship between virus resistance and the inheritance of the hpNIa transgene in transgenic *N. benthamiana* plants. T1 plants of hpNIa lines 1, 2 and 13 were challenge-inoculated with TuMV. Virus accumulation was detected by I-ELISA at 21 days after inoculation. Agarose gel data above presented the detection of the hpNIa trangene mRNA expression by RT-PCR from the same plant samples of I-ELISA.