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Molecular Characterization and Phylogenetic Analysis of *Turnip mosaic virus* Collected from Calla Lily

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ABSTRACT

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The full-length genomic sequences of two isolates of *Turnip mosaic virus* (TuMV) from calla lily (*Zantedeschia* spp., *Araceae*) , RC4 and YC5, were determined from 5'-RACE and overlapped viral cDNA clones derived from reverse-transcription polymerase chain reaction (RT-PCR) using TuMV-specific primers. Both TuMV-RC4 and TuMV-YC5 showed similar genomic features. When compared with TuMV isolates available in GenBank, sequences coding for P1, P3 and 6K2 proteins were identified as the most varible regions. Based on the variablilty of amino acid sequences at the amino acids positions of 471-490 in CIP region, TuMV isolates were divided into two groups. Among 42 TuMV isolates compared, only Q-Ca (Acc. No. D10927) and C1-isolate (Acc. No. AF394601) have a sequence of GSQPVxMxDxVxMxKIGVTL, while RC4, YC5, and other 38 isolates with a sequence of WLTAEYARLGANVEDRRDV. The protease recognition tetrapeptides for P1 protease and nuclear inclusion a protease (NIa-Pro) are variable among TuMV isolates, but both of RC4 and YC5 are identical. The phylogenetic analyses based on the comparison of the deduced amino acid sequences of the complete polyproteins with those of other 30 TuMV isolates, and the coat protein regions with those of other 59 TuMV isolates revealed that RC4 and YC5 are both classified in the group that contains isolates belonging to the *Brassica* pathotype.

Key words: *Turnip mosaic virus*, complete genomic sequence, phylogenetic analysis

INTRODUCTION

Turnip mosaic virus (TuMV), a member of the genus *Potyvirus*, is the most important virus infecting field-grown crucifers worldwide [2, 26], with a wide host range including 318 species in 156 genera of 43 families [3]. Up to date, complete genomic sequences of six isolates of TuMV have been determined, including 1J (Acc. No. D83184) [14], Q-Ca (Acc. No. D10927) [13], UK1 (Acc. No. AF169561 = NC-002509) [9], CHN12 (Acc. No. AY090660) [10], Tu-2R1 (Acc. No. AB105135) [22], and Tu-3 (Acc. No. AB105134) [22]. Moreover, thirty-four isolates of TuMV with nearly complete genomic sequences, most are collected from cruciferous hosts, have also been filed in the GenBank, with the sequences of the 5'-terminal regions lacking [9, 25, 27]. An infectious clone for the UK1 isolate of

TuMV has been established [18]. Sources of resistance to TuMV have been found in the host of *Arabidopsis thaliana* (L.) Heynh. by this infectious clone system [12]. In our previous study [1], two isolates, RC4 and YC5, were collected from different cultivars of calla lily (*Zantedeschia* spp.) with foliar symptoms of yellow spots and stripes. Calla lily is one of the monocotyledonous host of TuMV in addition to *Allium* sp. and *Calanthe* sp. (Orchidaceae) [2, 5]. In this investigation, complete nucleotide sequences of genomic RNAs of RC4 and YC5 isolates were determined, and used to compare with those of TuMV isolates available in Genbank. Phylogenetic analyses based on the deduced amino acid sequences of the polyproteins and the coat proteins indicated that the two calla lily isolates cluster with TuMV isolates belong to the *Brassica* pathotype.

MATERIALS AND METHODS

Virus isolates

Two isolates of *Turnip mosaic virus* (TuMV), RC4 and YC5 [1], individually collected from diseased bulbs of calla lily with yellow spot and stripe on leaves were used for this study. Cultures of YC5 and RC4 were maintained in plants of mustard (*Brassica juncea* L.) by mechanical transfer. Leaf tissues of RC4- or YC5-infested mustard were stored in 50% (vol/vol) glycerol at -20℃ for long-term preservation.

Viral RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)

Virus particles were purified from diseased mustard leaves based on a described method [1]. The viral RNA was extracted from the purified virions by the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany). First strand of cDNA was synthesized from the viral RNAs by the reverse transcription (RT) reagents provided from the MasterAmp™ High Fidelity RT-PCR kit (Epicentre, Madison, WI), with an oligo-d $(T)_{14}$ primer with a *Not*I site at it's 5'end. Three pairs of primers, specific to TuMV designed according to the complete nucleotide sequences of 1J (Acc. No. D83184) and Q-Ca (Acc. No. D10927) isolates, were used for amplifying the full-length sequences of RC4 and YC5 by polymerase-chain reaction (PCR) as manufacturer's instructions. The primer pairs included U3-u (5'-TAATCACGCGAGATGTGT)/ Oligo-d $(T)_{14}$, PH-u/ Tu6260d (5'- TTGATCAAAGGGGCCACAGCGACAG/ 5'-TAGTAGGCCTGAATTGTCTTA), and 5R-u/HC-d (5'-AAAAATATAAAAACTCAACACAACAT/5'-CCTTCC TTCGCTATGTACA), which were used for amplifying the genomic regions of the two isolates corresponding to nts 5798-9850, nts 1636 to 6280, and nts 1-2240, respectively.

PCR amplification was performed with 21 cycles (Perkin Elmer GeneAmp System 2400) using the following program: denaturing at 94℃ for 1 min, annealing at 50℃for 45 s, and synthesis at 72℃ for 1.5 min. An elongation step at 72℃ for 6 min was conducted at the last cycle. Amplified products were analyzed by electrophoresis in a 1.2 % agarose gel. Subsequently, RT-PCR amplified DNA fragments longer than 3 kbp were cloned into the pCR-XL-TOPO vector (Invitrogen, California, USA), while those of DNA fragments equal to or less than 3 kbp were cloned into the pCRII-TOPO vector (Invitrogen), according to manufacturer's instructions. Clones with expected DNA inserts were selected for sequencing.

Cloning of the 5'-end of TuMV genome

Nucleotide sequences corresponding to the 5'-extreme for the RC4 and YC5 were determined by cloning and sequencing the cDNA fragments corresponding to the 5'-terminal region using the 5'-RACE System kit (Gibco BRL, Life Technologies, Inc. USA) and the primers, PH-d1 and PH-d2, designed from the known sequences of the clone that contained the DNA fragment amplified by RT-PCR with the primer pair 5R-u/HC-d (Fig. 1A). First strand cDNA was synthesized by the reagents provided from the kit and the PH-d1 primer (5'-TCCTCCC CTGTCGCTGTGGC). The synthesized first strand cDNA was used as template for amplification of the DNA fragments spanning the 5'-extreme to nt 1518 in 50 μl of PCR reaction containing 5 μl of cDNA template, 5 μl of 10x buffer, 1.5 U of Taq-pol-XL polymerase (Protech Technology, Taipei, Taiwan), 0.2 mM of dNTP, 0.8 μM of PH-d2 primer (5'-ACCTATCTAGTATCTGCACT), and 0.4 μM of abriged anchor primer provided from the kit. Amplified DNA fragment was cloned into the pCRII-TOPO vector for nucleotide sequencing.

Analysis of nucleotide sequences

DNA sequencing of selected clones was done by an automatic sequencer (ABI PRISM 377, Perkin-Elmer, CA, USA). Nucleotide sequences were determined from three to four independent clones. The assembled sequences were compared with those of known TuMV isolates using the program of Vector NTI Suite (InforMax, Inc. Wisconsin, USA).

Phylogenetic analysis

In phylogenetic analysis, the amino acid sequences corresponding to the complete open reading frames of the

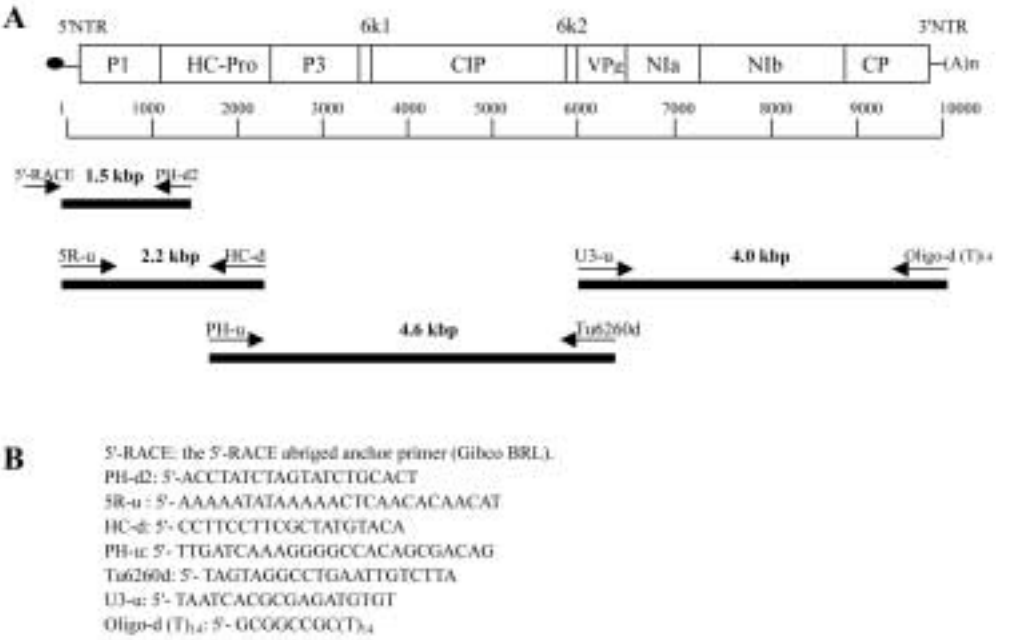

Fig. 1. The strategy for determination of the complete nucleotide sequences of the RNA genomes of two isolates, RC4 and YC5, of *Turnip mosaic virus* (TuMV) collected from calla lily. (A) Schematic representation of the genetic structure of TuMV and the overlapped cDNA fragments amplified from reverse-transcription polymerase chain reaction (RT-PCR) by the TuMV-specific primers. Black bars represent length of amplified DNA fragments. (B) Sequences of the primers used in RT-PCR.

polyproteins and the coat proteins of TuMV isolates were both used as two essential elements. The complete amino acid sequences of the polyproteins of RC4 and YC5 were compared with those of other 30 TuMV isolates used for the phylogenetic analysis described by Tomimura et al. [25], while the deduced amino acid sequences of coat proteins of both RC4 and YC5 were compared with those of 59 TuMV isolates described by Sanchez et al. [19] and Tomimura et al. [25] J1 and m strains of *Japanese yam mosaic virus* (JYMV), GenBank accession no. AB016500 and AB027007, respectively, were used as outgroup members.

Analyzed sequences were first aligned using CLUSTAL X version 1.8 [7]. Their phylogenetic relsationships were determinated by the neighbour-joining (NJ) algorithm with the bootstrap resampling method [4, 24], using PAUP 4.0 [23]. One thousand random resamplings were used to calculate the bootstrap values. The calculated trees were displayed by the TreeView program [16].

RESULTS

Characteriztion of the complete genomes of RC4 and YC5

The full-length RNA genomic sequences of the RC4 and YC5 isolates of TuMV were determined from three-overlapped viral cDNA clones derived from RT-PCR (Fig. 1A), and the 5'-extreme nucleotide sequences were determined from the 5'-RACE clones. Both 5'-ends of RC4 and YC5 were found containing four adenines, different from the known isolates that contain five (including 1J, Q-Ca, CHN12, Tu-2R1, and Tu-3 isolates) or six (UK1 isolate) adenines. The complete genomic sequences of RC4 and YC5 were filed in the GenBank with the accession numbers AY134473 and AF530055, respectively. Both of them showed similar genomic features, containing 9832 nts in length (excluding a 3'-terminal polyA tail), and encoding a large polyprotein of 3164 amino acids. An AUG triplet at nts 129-131 was predicted as the initiation codon for the translation of polyprotein. The deduced amino acid sequences of the individual functional proteins [17] predicted from RC4 and YC5 were compared to those of other 40 TuMV isolates (six isolates containing the complete nucleotide sequences and the other thirty-four isolates containing only the full-length polyprotein) available in GenBank. Amino acid identities of individual viral proteins were found between 73-95% for P1, 92-99% for HC-Pro, 83-97% for P3, 94-100% for 6k1, 92-99% for CIP, 75-98% for 6k2, 88-98% for VPg-Pro, 94-100% for NIa-Pro, 95-99% for NIb, and 94-99% for CP.

Fig. 2. Comparison of deduced amino acid sequences of the aa positions 471-490 in the coding region of cytoplasmic inclusion protein (CIP) among different isolates of *Turnip mosaic virus* (TuMV). The identical deduced amino acid sequence of the isolates RC4, YC5, UK1 and 1J is shown above the dashed line. The corresponding position with a significantly different sequence from the isolates of C1 and Q-Ca is shown under the dashed line. Asterisks in the dashed line indicates the nucleotide gaps between upper and lower coding sequence clusters.

Sequence variability in CIP region

According to the comparison of RC4 and YC5 to the other 40 TuMV isolates available in GenBank, TuMV isolates could be divided into two groups based on the variablilty of amino acid sequences at the amino acid positions of 471-490 in CIP region. Based on the known sequence data, only Q-Ca and C1 (Acc. No. AF394601) isolates have a similar structure, with the sequence of GSQPVxMxDxVxMxKIGVTL in that region; while RC4, YC5, and other 38 isolates contain a uniform sequence of WLTAEYARLGANVEDRRDV in the corresponding region (Fig. 2).

Protease recognition sites of TuMV

In addition to RC4 and YC5, the complete sequences of 40 isolates of TuMV with full-length polyproteins in Genbank were used to summarize the tetrapeptides of TuMV protease recognition sites as following: VxxF/S (29 out of 42 strains) or VxxY/S (13 out of 42 strains) for P1 protease; identical YRVG/G for HC-Pro protease; VxxQ/A(V)(40 out of 42 strain are Q/A), VxxQ/T(A) (37 out of 42 strain are Q/T) and VxxQ/N(S) (32 out of 42 strain are Q/N) for nuclear inclusion a protease (NIa-Pro) at the junctions of P3/6k1, 6k1/CIP, and CIP/6k2, respectively. Although the protease recognition sites are variable among TuMV isolates, both of TuMV-RC4 and -YC5 were identified as VxxF/S for P1; YRVG/G for HC-Pro protease; VxxQ/A, VxxQ/T, and VxxQ/N for the NIa-Pro recognition sites at the junctions of P3/6k1, 6k1/CIP, and CIP/6k2, respectively. Other recognition sites of NIa-Pro at 6k2/VPg, VPg/NIa, NIa/NIb and NIb/CP were identified as VxxE/A, VxxE/S, VxxQ/T, and VxxQ/A, respectively, which are consistent with all other TuMV isolates compared.

Phylogenetic analysis

The phylogenetic tree resulted from the analysis of complete amino acid sequence of the polypoteins of 32 TuMV isolates (Fig.3) was found similar to the phylogenetic clusters reported by Tomimura et al.[25] The RC4 and YC5 isolates were clusterred in the "World-B" group that contains isolates from all continents, and most are the Brassica pathotype as defined by Ohshima et al.[15] In detail, RC4 is closer to those isolates from the countries of China (isolate CHN12), Japan (isolate C42J), and Taiwan, R.O.C. (isolate Tw); YC5 is closer to those isolates from the countries such as United Kingdom (isolate UK1), Kenya (isolate KEN1) and United State (isolate USA1)(Fig. 3).

The phylogenetic tree based on the deduced amino acid sequences of the coat proteins of 61 TuMV isolates (Fig. 4), including RC4, YC5, and the other 59 TuMV isolates described by Sanchez et al.[19] and Tomimura et al.[25],

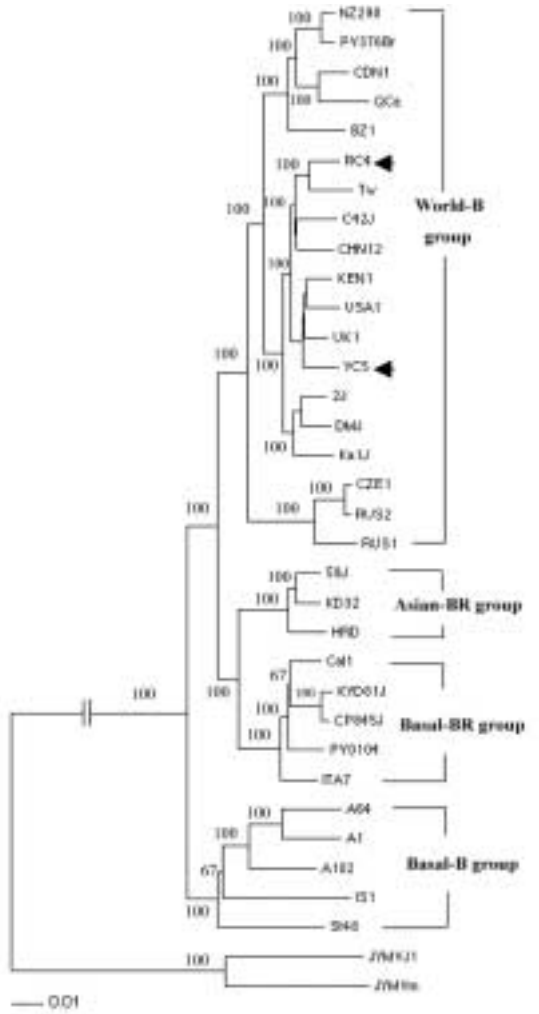

Fig. 3. A phylogram derived from the analysis of genetic distances based on the amino acid sequences of complete polyprotein of 32 isolates of TuMV. The tree was constructed using the neighbour-joining (NJ) algorithm with the bootstrap resampling method (1000 random resamplings) [4, 24], using PAUP 4.0 [23]. Numbers at each node indicate the percentages of bootstrap samples. Horizontal branch length is drawn according to the scale bar indicating 1 substitution per 100 amino acid positions. The accession numbers of TuMV isolates were from GenBank or referred to Tomimura et al. [25] J1 and m strains of *Japanese yam mosaic virus* (JYMV, GenBank accession no. AB016500 and AB027007, respectively) were used as outgroup elements. The genetic groups of basal-B, basal-BR, asian-BR, and world-B were defined according to Tomimura et al. [25]

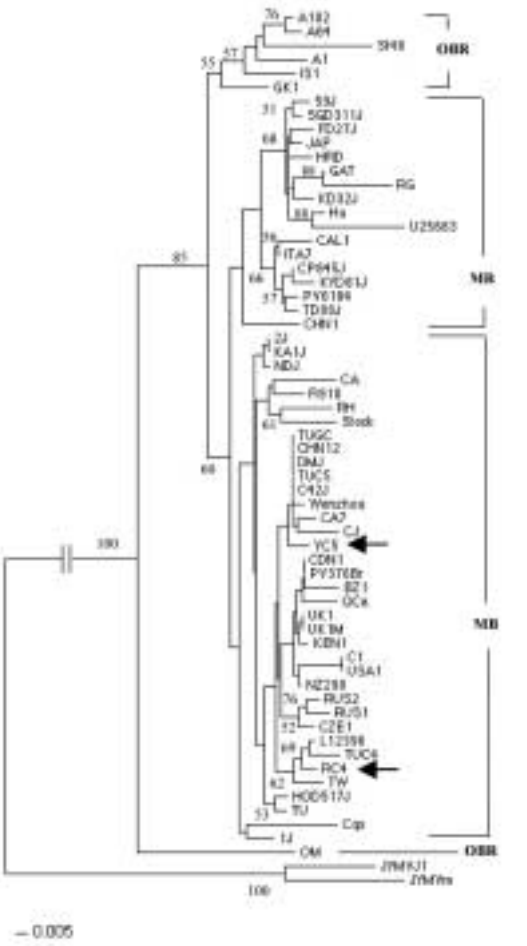

Fig. 4. A phylogram derived from the analysis of genetic distances based on the amino acid sequences of the coat proteins of 61 isolates of TuMV. The tree was constructed using the neighbour-joining (NJ) algorithm with the bootstrap resampling method (1000 random resamplings) [4, 24], using PAUP 4.0 [23]. Numbers at each node indicate the percentages of bootstrap samples (only values ≧ 50 are shown). Horizontal branch length is drawn according to the scale bar indicating 5 substitutions per 1000 amino acid positions. The accession numbers of TuMV isolates were from GenBank or referred to Sanchez et al. [19] and Tomimura et al. [25] J1 and m strains of *Japanese yam mosaic virus* (JYMV, GenBank accession no. AB016500 and AB027007, respectively) were used as outgroup elements. The genetic groups of MB (Most Brassica clusters), MR (Most Radish clusters) and OBR (Outside the Brassica and Radish clusters) were defined according to Sanchez et al. [19]

showed that isolates collected not from *Brassica* spp. or radishes, such as A102, A64, St48, A1, IS1, GK1, and OM, are grouped in OBR cluster, that is obviously outside the genetic groups of MB (a cluster containing most *Brassica* isolates) and MR (a cluster containing most radish isolates) as defined by Sanchez et al. [19] RC4 and YC5 were both classified in the MB group, however, RC4 was clusterred more closely to the strains of TW, TUC4 and L12396, which are genetically separated from the cluster of YC5 (Fig. 4).

DISCUSSION

TuMV occurs worldwide and infects a large number of economical crops and wild plants [3]. It is a highly variable potyvirus by biological and serological criteria [6, 8, 11, 21]. Sequence data of TuMV isolates available in GenBank are constantly increasing in number, most of them are collected from cruciferous crops. Although many nearly full-length genomic sequences were available in GenBank, most are lacking the 5'-noncoding region [25].

The complete nucleotides of the genomic RNA and the amino acid sequences of the polyproteins of the two TuMV isolates, RC4 and YC5, collected from calla lily that is an aroid crop in addition to other monocotyledons hosts of TuMV [2, 5], were determined and filed in GenBank. Our results showed that the genomic features of the two calla lily isolates are not significantly different from other known TuMV isolates. Our results indicated that P1, P3 and 6k2 proteins are the most variable gene products among TuMV isolates, similar to the results from the comparison of 1J and Q-Ca isolates [14]. Our results also agree with the suggestion by Shukla et al. that P1 and P3 were the most variable proteins among distinct potyvirus strains [20].

The variabilily of amino acid sequence at the region of 471-490 in amino acids of CIP region between 1J and Q-Ca isolates was first reported by Oshima et al. in 1996 [14]. According to our results from summarizing the 42 isolates of TuMV with complete polyprotein sequences revealed that only C1 and Q-Ca isolates contain a sequence of GSQPVxMxDxVxMxKIGVTL in the corresponding region (Fig. 2), which was caused by a nucleotide insertion or deletion according to the description by Oshima et al. [14]

TuMV are genetically clustered to different groups based on their complete genomes [25] or deduced amino acid sequences of coat proteins [15, 19]. According to the phylogenetic analysis by Tomimura et al. [25], four groups (basal-B, basal-BR, asian-BR and world-B) were classified by considering the original host plants and countries that a particular TuMV isolate was collected. Moreover, a strain was particularly defined by its pathogenicity to Brassica (B), Raphanus (R), or Brassica-Raphanus (BR) plants. Based on the phylogenetic analysis by Sanchez et al. [19], four genetic clusters were defined: MB, a cluster containing most brassica isolates; MR, a cluster containing most radish isolates; IBR, a intermediated cluster containing brassica and radish isolates; and OBR, outside the genetic groups of MB and MR.

Based on the amino acid sequences of the complete polyprotein and the coat protein region, the results of phylogenetic trees indicated that both of RC4 and YC5 isolates are clusterred in the same genetic groups, and are not significantly different from those strains of TuMV collected from *Brassica* spp., although they were isolated from calla lily. This is also reflected by the biological properties of RC4 and YC5 that induce systemic mosaic symptoms on various cruciferous plants including *Brassica compestris* L. var. *chinese*, *B. compestris* L. var. *ching-geeng*, *B. compestris* L. var. *pekinensis*, *B. juncea* L., *B. oleracea* var. *botrytis*, *B. oleracea* var. *capitata*, and *Raphanus sativas* [1]. The molecular chatacterization further proved that our previous presumption that RC4 and YC5 might be disseminated from TuMV-infected cruciferous plants by alate aphids [1]. Although RC4 and YC5 are clusterred in the same groups (Fig. 3, 4), they are genetically separated from each other. The difference in coat protein clusters might be due to the fact that they were collected from different cultivars of calla lily, RC4 from cultivar Majestic-Red and YC5 from cultivar Black-Magic.

Our results indicated that RC4 is genetically closer to those isolates from Japan and Taiwan, but YC5 is closely related to those isolates outside from these areas (Fig. 3). According to our knowledge, RC4 was from calla lily plants cultured in the field for several passages; however, YC5 was from plants of calla lily consistently kept in greenhouse. Therefore, the sources of TuMV, originated from local or foreign brassica plants, apparently influence the results of phylogenetic clusters between RC4 and YC5. Whether the sequence variation of RC4 and YC5 is really correlated to host cultivars and the geographic origins remains to be clarified by comparing more TuMV isolates collected from different cultivars of calla lily and from different geographic areas.

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摘 要

陳金枝[1,2]、張清安[2]、葉錫東[1,3] 2006. 分離自彩色海芋之蕪菁嵌紋病毒之分子特性及其類緣演化分析. 植病會刊15:1-8. ([1] 國立中興大學植物病理系；[2] 行政院農業委員會農業試驗所植物病理組；[3] 聯絡作者，電子郵件：sdyeh@nchu.edu.tw)

蕪菁嵌紋病毒 (*Turnip mosaic virus*，TuMV) 屬於馬鈴薯 Y 屬病毒 (*Potyvirus*)，其寄主範圍相當廣泛。本研究針對兩種分離自彩色海芋 (*Zantedeschia* spp.) 的 TuMV 病毒分離株 RC4 及 YC5，利用 5'-RACE 及 TuMV 之專一性引子進行反轉錄-聚合酶鏈鎖反應，由相互重疊的 cDNA 片段選殖解序後，各別解得基因體 RNA 均含 9832 酶個核苷酸，此二病毒分離株之全長度基因體核酸序列並已登錄於基因庫 (GenBank)，序號分別爲 AY134473 及 AF530055。RC4 及 YC5 之基因體架構相同，可對應轉譯出一含 3164 個氨基酸之大蛋白。 5' 端之序列包含有四個腺嘌呤核苷酸 (adenylated nucteotides)，大蛋白之第一個胺基酸轉譯之起始位 (AUG) 在 5' 端起第 129-131 nts。與登錄於 GenBank 上之 TuMV 分離株比對結果， 發現 P1、P3 及 6k2 等蛋白在 TuMV 分離株間變異性最大。對應 CIP 蛋白之第 471-490 胺基酸出現差異性，在所比對的 42 個分離株中，僅有 Q-Ca (Acc. No. D10927) 及 C1 (Acc. No. AF394601) 分離株呈現 GSQPVxMxDxVxMxKIGVTL 序列，而 RC4、YC5 與其他 38 個分離株則均爲 WLTAEYARLGANVEDRRDV 序列型態。RC4 及 YC5 乃分離自天南星科之海芋分離株，但二者均能感染十字花科且造成植株系統性嵌紋病徵。進一步根據與其他 30 個具有全長度大蛋白胺基酸序列之 TuMV 分離株，以及與其他 59 個 TuMV 分離株之鞘蛋白胺基酸序列所得之遺傳演化分析結果顯示，RC4 與 YC5 乃同列於大部分由分離自十字花科白菜屬 (*Brassica* spp.) 之 TuMV 所組成的群組，然而 RC4 與 YC5 之間仍有一段遺傳距離。此種遺傳距離的差異性是否因 RC4 及 YC5 乃分離自不同的海芋品系及地理源頭所影響，值得進一步探討。

關鍵詞：蕪菁嵌紋病毒、全長度核酸序列、演化分析