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用於鑑定番茄斑點萎凋病毒(TSWV)血清群病毒種類引子對組之開發

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摘 要

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*Tospovirus*病毒屬寄主範圍廣泛，為世界性危害重要經濟作物的植物病毒之一。根據核鞘蛋白 (nucleocapsid protein, NP) 的血清學親緣關係，tospoviruses可進一步分類為數個血清群 (serogroups)，大部分的病毒分屬於以西瓜銀斑病毒 (*Watermelon silver mottle virus*, WSMoV) 及番茄斑點萎凋病毒 (*Tomato spotted wilt virus*, TSWV) 命名之兩大血清群。WSMoV血清群病毒發生於亞洲，而TSWV血清群病毒則主要在歐美地區。TSWV血清群的成員尚有水仙百合壞疽條斑病毒 (Alstroemeria necrotic streak virus, ANSV)、菊花莖部壞疽病毒 (Chrysanthemum stem necrosis virus, CSNV)、花生輪斑病毒 (*Groundnut ringspot virus*, GRSV)、鳳仙花壞疽斑點病毒 (*Impatiens necrotic spot virus*, INSV)、甜瓜嚴重嵌紋病毒 (Melon severe mosaic virus, MeSMV)、辣椒壞疽斑點病毒 (Pepper necrotic spot virus, PNSV)、番茄黃化斑點病毒 (*Tomato chlorotic spot virus*, TCSV) 及矮南瓜致死黃化病毒 (*Zucchini lethal chlorosis virus*, ZLCV) 等。本研究利用分析TSWV血清群病毒之N基因序列設計多組上游引子 (forward primers：FT1、FT2、FT3、FT4、FT5) 及下游引子 (reverse primers：RT1、RT2、RT3、RT4、RT5)，彼此互相交叉配對為25組引子對，以ANSV、CSNV、GRSV、INSV、MeSMV、TCSV及TSWV等七種病毒的N基因選殖質體進行聚合酶連鎖反應 (polymerase chain reaction, PCR) 測試。除了編號05: FT1/RT5、10: FT2/RT5、15: FT3/RT5、20: FT4/RT5及25: FT5/RT5等引子，對所有病毒N基因選殖質體完全沒有任何反應外，其他引子對則藉由擴增片段有無的多形性 (polymorphism) 可鑑定出不同的病毒種類。使用不同引子對組合：編號02：FT1/RT2、03: FT1/RT3、04: FT1/RT4、06: FT2/RT1及07: FT2/RT3 等5個引子對，可成功地自感染病毒的菸草 (*Nicotiana benthamiana*) 葉片組織總量RNA中，以反轉錄聚合酶連鎖反應 (reverse transcription-polymerase chain reaction, RT-PCR) 鑑定出TSWV及INSV。研究結果顯示，所設計之引子對組具有鑑定TSWV血清群病毒種類之潛力。

關鍵詞：番茄斑點萎凋病毒、血清群、病毒鑑定

緒 言

*Tospovirus*病毒屬為危害世界性重要經濟作物的植物病毒之一，在寄主作物上主要造成斑點 (spots)、黃化 (chlorosis)、斑駁 (mottle)、矮化 (stunt)、萎凋 (wilt) 及壞疽 (necrosis) 等病徵，寄主範圍廣泛，能感染82科1090種以上的重要經濟作物[63]。回顧過去番茄斑點萎凋病害發展的歷史，1915年首次由Brittlebank於澳洲發現[4]，1927年Pittman證實該病害由纓翅目 (Thysanoptera) 的薊馬類 (thrips) 昆蟲媒介傳播[68]，直至1930年才由Smauel等人證實該病害病原是由病毒所引起[74]。1950年代由於大量殺蟲劑的施用，本病害所造成的經濟損失一直扮演次要病害的角色，並不受到特別的重視，直到1990年代前後，由於西方花薊馬 (*Frankliniella occidentalis* Pergande) 大發生[35, 36, 37, 44, 66, 67, 68, 73]，擴散蔓延，致使英國、北歐及北美等北半球國家的主要及溫室作物蒙受嚴重的經濟損失，迄今此病害已廣泛分佈至全世界[44, 63, 67, 68, 73]。

Tospoviruses的形態為球型或近似球型的顆粒，完整病毒顆粒的直徑大小約80-120 nm，外圍具有雙層套膜 (envelope)，膜表面具有棒狀的小突起[60, 82]，其中包裹L、M及S三條單股的RNA基因體 (ssRNA) [33, 81]。L RNA為負極性 (negative polarity)，具有一個大轉譯架構 (open reading frame, ORF)，可對應產生一個RNA複製酶 (RNA-dependent RNA polymerase, RdRp)[26, 28]。M RNA為雙極性 (ambisense)，具有兩個轉譯架構，病毒股 (viral sense) 表現一非結構性的移動蛋白 (movement protein, NSm)[17, 49, 53]，互補股 (viral complementary sense) 則表現套膜表面突起的Gn和Gc醣蛋白 (glycoprotein)，醣蛋白與媒介昆蟲薊馬傳播病毒有關[47]。S RNA亦為雙極性，其病毒股及互補股分別表現一非結構性NSs蛋白及結構性核鞘蛋白 (nucleocapsid protein, NP)[27, 41, 52, 88]。NSs蛋白在寄主細胞內形成絲狀體結構[45, 48]，為病毒抵禦植物基因沉寂作用 (gene silencing) 之抑制因子 (suppressor)[7, 48, 79]。NP則為核酸結合性蛋白，其血清學及序列親緣關係為*Tospovirus*屬病毒分類之重要依據[63, 82, 81]，NP胺基酸序列相同度 (identity) 達90%以上者，視為同一病毒種 (species)，以此分類規範，目前已知之*Tospovirus*屬病毒共有29種，包括經由國際病毒分類組織 (ICTV) 認定的11個正式病毒種：花生頂芽壞疽病毒 (*Groundnut bud necrosis virus*, GBNV)[69]、花生輪斑病毒 (*Groundnut ringspot virus*, GRSV)[62]、花生黃斑病毒 (*Groundnut yellow spot virus*, GYSV)[75]、

鳳仙花壞疽斑點病毒 (*Impatiens necrotic spot virus*, INSV)[2, 27, 51]、鳶尾花黃斑病毒 (*Iris yellow spot virus*，IYSV)[22]、蕎麥輪斑病毒 (*Polygonum ringspot virus*, PolRSV)[21]、番茄黃化斑點病毒 (*Tomato chlorotic spot virus*, TCSV)[24]、番茄斑點萎凋病毒 (*Tomato spotted wilt virus*, TSWV)[25]、西瓜頂芽壞疽病毒 (*Watermelon bud necrosis virus*, WBNV)[43]、西瓜銀斑病毒 (*Watermelon silver mottle virus*, WSMoV)[85, 86, 87]和矮南瓜致死黃化病毒 (*Zucchini lethal chlorosis virus*, ZLCV)[85, 86, 87]；以及18個非正式的病毒種：水仙百合壞疽條斑病毒 (Alstroemeria necrotic streak virus, ANSV)[39]、豆類壞疽嵌紋病毒 (Bean necrosis mosaic virus，BeNMV)[29, 30]、彩色海芋黃化斑點病毒 (Calla lily chlorotic spot virus, CCSV)[11, 55]、番椒黃化病毒 (Capsicum chlorosis virus, CaCV)[54]、菊花莖部壞疽病毒 (Chrysanthemum stem necrosis virus, CSNV)[3, 5]、花生黃化扇斑病毒 (Groundnut chlorotic fan-spot virus, GCFSV)[9, 10, 19]、孤挺花黃化輪斑病毒 (Hippeastrum chlorotic ringspot virus, HCRV)[32]、洋桔梗壞疽輪斑病毒 (Lisianthus necrotic ringspot virus, LNRV)[78]、甜瓜嚴重嵌紋病毒 (Melon severe mosaic virus, MeSMV)[20]、甜瓜黃斑病毒 (Melon yellow spot virus, MYSV)[13, 14, 46]、桑葉脈條斑病毒 (Mulberry vein banding virus, MuVBV)[57]、辣椒壞疽斑點病毒 (Pepper necrotic spot virus, PNSV)[80]、甜椒黃化斑點病毒 (Pepper chlorotic spot virus, PCSV)[16]、大豆葉脈壞疽病毒 (Soybean vein necrosis-associated virus, SVNaV)[94]、番茄黃輪病毒 (Tomato yellow ring virus, TYRV)[40]、番茄壞疽輪斑病毒 (Tomato necrotic ringspot virus, TNRV)[76]、番茄壞疽斑點相關病毒 (Tomato necrotic spot associated virus, TNSaV)[90]及番茄輪斑病毒 (Tomato zonate spot virus, TZSV)[31]等。在親緣演化與血清學關係方面，*Tospovirus*屬可歸類為五個主要的血清群 (serogroups)，分別以TSWV、WSMoV、IYSV、GYSV及SVNaV為代表種命名及一個獨立的血清型 (serotype) LNRV，各血清群內的病毒，彼此會有血清反應，相反地，各群彼此間不會有血清反應的發生[2, 14, 15, 23, 52, 55]。從病毒的危害程度及區域分佈來看，TSWV血清群及WSMoV血清群最為重要，分別危害歐美及亞洲重要的農業經濟區域[4, 36, 44, 64]。台灣由於地處熱帶、亞熱帶地區，氣候適宜，非常適合tospoviruses及其媒介薊馬的生長，包含TSWV在內，目前台灣已發現有7種tospoviruses之危害[9, 10, 11, 14, 16, 19, 56, 66, 85]，尤其以WSMoV和MYSV危害瓜類作物栽培最為嚴重[67, 90]，CaCV危害蝴蝶蘭產業[12, 17, 92, 93]，而GCFSV、CCSV及PCSV則為首次發現的新病毒[9, 10, 11, 16, 20]。TSWV在台灣首次記錄出現於1995年，在新竹縣竹東地區栽培的彩色海芋上發現[86]，之後並未有病害出現，一直到了2009年在南投縣仁愛山區的甜椒作物上大發生，造成重大的損失[94]。

Tospoviruses之田間診斷主要以NP的多元及單株抗體進行血清學分析[1, 15, 16, 43, 78, 88]，或以病毒RNA為標的所衍生的偵測技術[70, 71]。過去利用血清學的方式，如間接式酵素連結免疫吸附分析法 (indirect enzyme-linked immunosorbent assay, indirect ELISA)，由於操作簡單、成本低廉，非常適用於田間大量樣本之檢測[38, 83]，但因為同一血清群的病毒彼此之間NP的序列同源性高，要區分病毒種類，在技術層次上有一定的難度，甚至有無法區分的情形出現[15]。因此過去在病毒種的診斷上，都利用病毒N基因序列設計專一性的引子對，結合反轉錄-聚合酶連鎖反應 (reverse transcription-polymerase chain reaction, RT-PCR) [30, 61, 62]或real time RT-PCR進行病毒檢測[6, 72]，甚至結合抗體及RT-PCR技術的Immunocapture-RT-PCR進行病毒種的診斷[34]。利用病毒基因體L RNA上的高保留性序列 (conserved region) 設計簡併引子對，則可用於所有Tospovirus屬病毒之診斷[18]。

由於*Tospovirus*屬病毒的種類繁多，為了有效率地執行病毒的防檢疫工作，本研究嘗試利用TSWV血清群內各病毒種N基因序列上的高保留區間設計多組簡併引子 (degenerate primers)，透過交叉配對的方式，產生多對引子對組 (primer pairs)，藉由各引子對與病毒之間的RT-PCR反應結果，探討本技術於TSWV血清群病毒種間之鑑定及田間應用的可行性。

材料與方法

病毒N基因選殖質體之純化

由亞洲大學生物科技學系陳宗祺教授提供，TSWV血清群正對照品，包括ANSV、CSNV、GRSV、INSV、MeSMV、TSWV及TCSV N基因之選殖質體，分別命名為pTOPO-ANSV-N、pTOPO-CSNV-N、pTOPO-GRSV-N、pTOPO-INSV-N、pTOPO-MeSMV-N、pTOPO-TSWV-N及pTOPO-TCSV-N，以微量質體純化試劑組(Plasmid Miniprep Purification Kit) (GeneMark, GMbiolab Co., Ltd., Taichung, Taiwan) 進行質體DNA之純化，實驗步驟參考產品使用說明，將純化後的質體DNA置於-20℃保存備用。

病毒來源與罹病植物總量RNA之萃取

TSWV之彩色海芋分離株 (TSWV-Z) 分離自台灣南投仁愛地區[93]。INSV來自美國的鳳仙花 (INSV-M)，由Dr. J. Moyer所提供[51, 53]。病毒以機械方式接種於單斑寄主奎藜 (*Chenopodium quinoa* Willd.) 與系統性寄主菸草 (*Nicotiana benthamiana* Domin) 上，置於恆溫隔離溫室中 (26-28℃) 生長，再以Total RNA Miniprep Purification Kit (GeneMark) 進行植物總量RNA之提純，實驗步驟參考產品使用說明，抽取後的總量RNA保存於-80℃備用。

引子之設計

參考NCBI基因庫已登錄之ANSV、CSNV、GRSV、INSV、MeSMV、TCSV、TSWV及ZLCV之S RNA序列 (表一)，分別將其N基因序列，以DNASTAR Lasergene之核酸分析MegAligt Jotun Hein Method (DNASTAR, Inc.) 進行TSWV血清群各病毒種間N基因保留區與變異區的分析，以利簡併性及專一性引子的設計。

PCR反應

以pTOPO-ANSV-N、pTOPO-CSNV-N、pTOPO-GRSV-N、pTOPO-INSV-N、pTOPO-MeSMV-N、pTOPO-TSWV-N及pTOPO-TCSV-N質體DNA為模板進行引子對於TSWV血清群病毒種間專一性之測試。每一PCR反應的配方含有2.5 μl 10× Taq buffer [50 mM Tris-HCl (pH 8.0)、1 mM EDTA、1 mM DTT及50%(v/v) glycerol] (Protech, Taipei, Taiwan)、1 μl 10 mM dNTPs (Protech)、0.1 μl 100 μM上游引子、0.1 μl 100 μM下游引子、0.1 μl Pro Plus Taq DNA polymerase (5 U/μl) (Protech)、1 μl病毒N基因質體DNA (100 ng/μl)、加水至總反應體積為25 μl。以Applied Biosystems GeneAMP PCR System 9700 (Thermo Fisher Scientific Inc., MA, USA) 進行以下PCR反應程式：95℃進行變性反應5分鐘，再以95℃反應30秒、50℃煉合40秒及72℃反應30秒，進行

表一、用於本研究分析之*Tospovirus*屬病毒核鞘基因序列編號一覽表。

TABLE 1. Accession codes of the nucleocapsid (N) genes of tospoviruses used for analysis in this study

Species*	Abbreviation	Chinese name	Accession code
Alstroemeria necrotic streak virus	ANSV	水仙百合壞疽條斑病毒	GQ478668
Chrysanthemum stem necrosis virus	CSNV	菊花莖部壞疽病毒	AF067068
Groundnut ringspot virus	GRSV	花生輪斑病毒	AF251271
Impatiens necrotic spot virus	INSV	鳳仙花壞疽斑點病毒	AB109100
Melon severe mosaic virus	MeSMV	甜瓜嚴重嵌紋病毒	EU275149
Tomato chlorotic spot virus	TCSV	番茄黃化斑點病毒	AF521102
Tomato spotted wilt virus	TSWV	番茄斑點萎凋病毒	AB010997
Zucchini lethal chlorosis virus	ZLCV	矮南瓜致死黃化病毒	AF067069

* Italic typing represents official species and standard typing represents tentative species.

35個溫度循環，最終再以72℃反應6分鐘，以4℃儲存增幅的產物。取10 µl PCR產物，以2%凝膠瓊脂 (Agarose I, Amresco)，於0.5倍TAE緩衝液中(GeneMark)進行PCR產物電泳分析，膠體經溴化乙錠 (ethidium bromide) (Protech) 染色後，置於紫外燈箱檢視，並拍照紀錄分析結果。

RT-PCR

以萃取自感染TSWV及INSV的菸草 (*N. benthamiana*) 總量RNA為模板進行RT-PCR測試。每一反應含有1 µl總量RNA (200 ng/µl)、0.2 µl M-MuLV反轉錄酶 (5 U/µl) (Protech)、2.5 µl 10× Taq buffer [50 mM Tris-HCl (pH 8.0)、1 mM EDTA、1 mM DTT及50%(v/v) glycerol] (Protech)、1 µl 10 mM dNTPs (Protech)、0.1 µl 100 µM上游引子、0.1 µl 100 µM下游引子、0.1 µl Pro Plus Taq DNA polymerase (50 U/µl) (Protech)、加水至總反應體積為25 µl，以Applied Biosystems GeneAMP PCR System 9700於45℃進行反轉錄作用30分鐘，95℃反應5分鐘終止反應，再以95℃反應30秒；50℃反應40秒及72℃反應30秒，進行35個溫度循環，最後以72℃反應6分鐘。取10 µl PCR產物，以2%凝膠瓊脂，於0.5倍TAE緩衝液中 (GeneMark)，進行電泳分析。

結　果

引子設計及引子對的組成

分析TSWV血清群各病毒種間N基因之保留區與變異區，結果顯示同一個血清群的病毒種，N基因上具有連續性高度的同源性保留區間及病毒種差異化的變異區。經比對後以同源性較高的保留區內第1-300個核苷酸的位置設計上游簡併引子FT1、FT2、FT3、FT4及FT5；在第450-755個核苷酸的區間設計下游簡併引子RT1、RT2、RT3、RT4及RT5，各引子的序列及對應位置如表二所示。將上游與下游引子分別以交叉配對的方式進行重組，可以產生25對引子對組，各引子對之編號及預期產物大小如表三所示。

表二、利用TSWV血清群病毒N基因保留區間設計之引子序列及其對應位置。

TABLE 2. The nucleotide sequences and corresponding positions of primers designed from the N gene conserved regions of the TSWV-serogroup tospoviruses.

Primer name	Position in N gene of TSWV	Sequence (5'→3')
FT1	1-20	ATGTCTAAGGTHAAGCTYAC
FT2	13-35	AAGCTTACNAAGGARAACATTGT
FT3	172-197	AAGAATCGCCAGDGYATAATGAARGT
FT4	211-233	GATTTTACTTTTGGNAMARTCAC
FT5	270-290	AGCTAATGAYATGACTTTCAG
RT1	451-476	GAAGCAATVAGAGGDADACTACCTCC
RT2	607-627	ATCCATDBCAAATCCTTTGCT
RT3	703-722	TCARTGTAATGYTCCATWGC
RT4	731-755	ACTCCRAACATBTCATAGAAYTTGAT
RT5	731-755	ACTCCAAACATGCTGTAAATVGATM

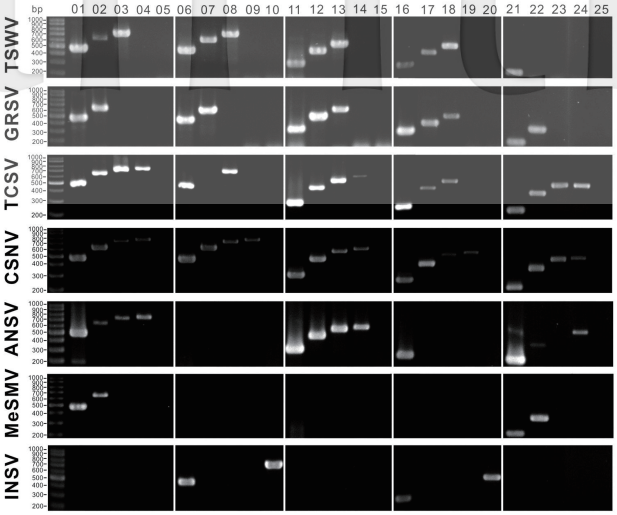
表三、引子交叉配對組合及預估擴增產物的大小。

TABLE 3. Cross matching of primer pairs and the expected molecular sizes of correspondingamplicons.

Primer	RT1	RT2	RT3	RT4	RT5
FT1	01: FT1/RT1	02: FT1/RT2	03: FT1/RT3	04: FT1/RT4	05: FT1/RT5
	475 bp	626 bp	721 bp	754 bp	754 bp
FT2	06: FT2/RT1	07: FT2/RT2	08: FT2/RT3	09: FT2/RT4	10: FT2/RT5
	463 bp	614 bp	709 bp	742 bp	742 bp
FT3	11: FT3/RT1	12: FT3/RT2	13: FT3/RT3	14: FT3/RT4	15: FT3/RT5
	304 bp	455 bp	550 bp	583 bp	583 bp
FT4	16: FT4/RT1	17: FT4/RT2	18: FT4/RT3	19: FT4/RT4	20: FT4/RT5
	265 bp	416 bp	511 bp	544 bp	544 bp
FT5	21: FT5/RT1	22: FT5/RT2	23: FT5/RT3	24: FT5/RT4	25: FT5/RT5
	206 bp	357 bp	452 bp	485 bp	485 bp

引子對測試及電泳圖譜分析

將上述25對引子對組分別以ANSV、CSNV、GRSV、INSV、MeSMV、TCSV及TSWV等七種病毒N基因選殖質體DNA進行PCR專一性測試，結果顯示，編號: 05、10、15、20及25對所有病毒N基因選殖質體DNA完全沒有任何PCR反應，其餘的引子對組則依不同的病毒種類會有增幅產物出現有無反應的現象，擴增出之PCR產物的分子量大小範圍約在200~800 bp之間 (圖一)。這些有無反應的組成，在各病毒種的測試上，顯現出其特異的電泳圖譜，透過檢索表的整合製作發現，該引子對組所呈現的有無反應，可以成功地鑑定出TSWV血清群內的病毒種，包括TSWV、GRSV、TCSV、CSNV、INSV、ANSV及MeSMV等 (表四)，各引子對增幅的產物分子量大小詳如表三所示。



圖一、引子對用於聚合酶連鎖反應 (PCR) 檢測TSWV血清群病毒之專一性測試。以帶有TSWV、GRSV、TCSV、CSNV、ANSV、MeSMV及INSV N基因之質體做為模板進行試驗。PCR產物以2%膠體電泳分析。引子對之編號詳見表三。

Fig. 1. The specificity test of primer pairs for detecting TSWV-serogroup tospoviruses in polymerase chain reaction (PCR). The plasmids carrying the N gene sequences of TSWV, GRSV, TCSV, CSNV, ANSV, MeSMV and INSV were used as the templates for assay. PCR products were analyzed by 2% agarose gel electrophoresis. Lanes represent the codes of primer pairs as shown in Table 3.

表四、引子對用於檢測TSWV血清群不同tospoviruses之專一性測試。

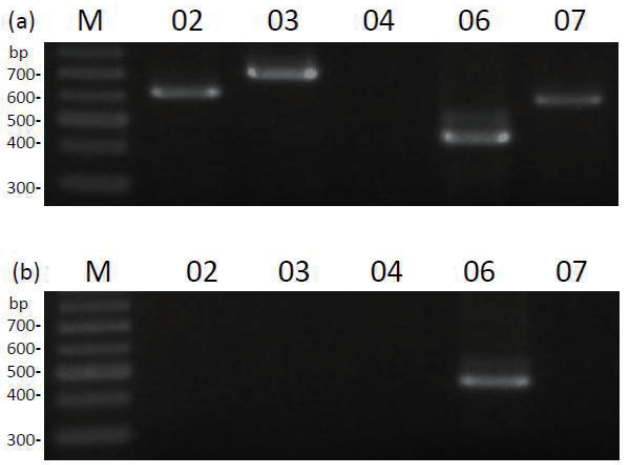
TABLE 4. The specificity test of individual primer pairs for detecting different tospoviruses of TSWV serogroup.

Virus	Primer pair* 01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
TSWV	+	+	+	−	−	+	+	+	−	−	+	+	+	−	−	+	+	+	−	−	+	−	−	−	−
GRSV	+	+	−	−	−	+	+	−	−	−	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−
TCSV	+	+	+	+	−	+	−	+	−	−	+	+	+	−	−	+	+	+	−	−	+	+	+	+	−
CSNV	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−
ANSV	+	+	+	+	−	−	−	−	−	−	+	+	+	−	+	−	−	−	−	−	+	+	−	+	−
MeSMV	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
INSV	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−

* The codes of individual primer pairs are shown in Table 3.

引子對組應用於病毒鑑定之分析

挑選編號02：FT1/RT2、03: FT1/RT3、04: FT1/RT4、06: FT2/RT1及07: FT2/RT3 等5組引子對，與感染病毒的菸草總量RNA進行RT-PCR測試，結果顯示，感染TSWV的菸草樣本除了編號04引子對沒有反應外，其他引子對包括編號02、03、06及07可分別擴增出626 bp、721 bp、463 bp及614 bp等產物 (圖二、a)；而感染INSV的菸草樣本則可由引子對組編號06，增幅出463 bp的產物 (圖二、b)外，其餘引子對包括編號02、03、04及07皆不會有反應。此結果與上述N基因選殖質體所呈現的病毒特異性電泳圖譜一致，說明此方法可成功地區分此二病毒。



圖二、引子對組應用於反轉錄聚合酶連鎖反應 (RT-PCR) 進行番茄斑點萎凋病毒 (TSWV) (a)及鳳仙花壞疽斑點病毒 (INSV) (b) 之鑑定。

Fig. 2. Identification of Tomato spotted wilt virus (TSWV) (a) and Impatiens necrotic spot virus (INSV) (b) using the primer pair sets in reverse transcription-polymerase chain reaction (RT-PCR). Lanes represent the codes of selected primer pairs as shown in Table 3.

討　論

從N基因的核酸序列及胺基酸序列比較分析上可以發現，TSWV血清群的病毒，其NP identity都高於55 %以上，具有許多連續性的保留序列(conserved sequence)區間，因此在演化上具有很高的親緣關係，如果想要利用抗血清做為病毒種的鑑定工具，在技術層次上的確具有相當的難度[15]。而利用病毒N基因的序列做為病毒種類鑑定的依據，一直是Tospovirus屬病毒分類上重要的依據，本研究利用本病毒血清群內7種病毒的N基因序列的保留序列區間，設計多組共通型引子，透過彼此交叉配對產生多個引子對組，以病毒N基因質體DNA進行測試，可以成功地自各病毒N基因嵌入序列，擴增出特定的電泳圖譜，用以區別TSWV、GRSV、TCSV、ANSV、CSNV、MeSMV及INSV等病毒。這些引子對對於其他非TSWV血清群的病毒，包括WSMoV、IYSV、GYSV及SVNaV等血清群，皆不具任何反應 (結果未出示)，顯示本研究所設計之引子對組僅對於TSWV血清群病毒具有高度的專一性反應。

由圖一結果可知，各引子對，對於不同病毒偵測之效果及靈敏度互有差異，從結果分析來看，編號02：FT1/RT2、03: FT1/RT3、04: FT1/RT4、06: FT2/RT1及07: FT2/RT3 等5個引子對，可以將進行測試的七種病毒N基因的質體DNA區分出來，因此將5對引子分別以感染TSWV及INSV的植物總量RNA進行鑑定分析，結果顯示可成功地擴增出與質體DNA測試所得到的結果相同(圖一及圖二)，顯示此引子對組未來可實際應用於田間進行此種二種病毒的鑑定工作，而其餘未選用之引子對組仍具病毒鑑定之潛力。由於台灣尚未發現其他TSWV血清群的病毒，此檢測方法的建立將有助於病毒防檢疫的工作。本研究所

設計的多組引子對可因應檢測對象的不同，適時調整配對方式和引子對組數的彈性，以提高本檢測方法之實用性和擴充性。

引子對設計方面，過去引子的設計皆採N基因的變異區設計專一性引子對，這種一對一病毒檢測的設計概念操作簡單，專一性高，對於已知病毒的防疫檢疫效果佳[59]，過去是應用檢測與鑑定 *Tospovirus* 屬病毒種最常用的方法之一。然而，對於新病毒種或一旦設計的引子對在病毒的對應序列配對處出現變異時，就會造成未檢出或偽陰性的可能。另外，在大量篩檢病材時，如果出現複合性感染，該方法的應用上不管在材料及人力的輸出上，都相當的費時及吃力。因此，利用複合性專一性引子 (multiplex specific primers) 於單一檢測的條件下，同時進行多種病毒的鑑定技術因運而生。本技術看似可行，但面臨克服的技術障礙也不少，例如引子對數量的限定、各引子對在同一反應內彼此相互競爭、干擾、反應液dNTPs的濃度、酵素競爭及作用效率、鎂離子濃度等，皆有導致檢測靈敏度下降的風險，如何將上述因子適量化，將是此技術成功的重要關鍵[8, 51]。本研究在鑑定病毒種的設計概念上與上述不同，主要是利用N基因序列連續的保留區間，設計共通型引子，利用多組的共通引子對來進行同血清群病毒種的鑑定，一來在血清群間可以成功的區隔；二來藉由各引子對反應有無呈現的特異性電泳圖譜，進行病毒種的鑑定，以彌補專一性單一引子對及複合性專一引子對檢測可能因為配對處發生變異造成未檢出而導致偽陰性出現的風險。在同群中新病毒種的防疫上，多組共通引子對的應用，相較單一及複合性引子對而言，更加的積極安全，透過病毒種呈現的特異電泳圖譜的建立及比較差異化分析，可以很容易讓防疫者注意到有新病毒入侵的可能。一旦發現有病毒種特異性電泳圖譜未載入資料庫者，可以透過定序的方式，讓新病毒快速被鑑定出來。

病毒種鑑定方面，利用病毒基因體S RNA上N基因或L RNA序列保留區間 (conserved region) 所設計的共通型簡併引子對 (degenerate primer pairs)，可結合不同限制酵素 (restriction enzyme) 的剪切作用，將引子對所擴增的產物切割成不同大小片段，呈現特異的DNA電泳圖譜，透過種間彼此的差異化，來進行病毒種的鑑定[18, 30, 61]。此方法與本研究策略類似，但在技術操作上，本研究技術可省去限制酶的作用，相較之下更為簡便，亦可避免過多操作流程造成失誤而影響鑑定的結果。

靈敏度方面，本研究的技術方法，相較於複合性專一引子對法，雖然在量化檢測和快速篩檢上不若其方便快速，但在靈敏度上，則保留如同單一檢測的優勢，不受複合性引子對存在的干擾影響，未來可結合複合性專一引子對檢測技術進行量化篩檢的檢疫工作，將未篩檢出的樣本，利用本技術進行防疫確效，兩技術的結合，將有助於本國檢疫防疫網綿密的架構，以避免新種病毒入侵對我國農業造成無可彌補的危害。

本研究的設計概念，採用同源性較高的保留區間，設計共通型引子，透過交叉配對法，組成同源性高的引子對群，以PCR有無反應的電泳結果，呈現同血清群間各病毒種特異的電泳圖譜，透過差異化分析，建立「病毒N基因共通型簡併引子對組特異電泳圖譜資料庫」，進而成功地將各病毒種鑑定出來。這樣的方式以分別感染TSWV及INSV的植物材料進行RT-PCR驗證，測試的結果也相符合，表示此設計理念，應用在同血清群病毒種的鑑定是可行的，未來將可推廣應用至其他病毒屬病毒種之鑑定。

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ABSTRACT

Liu, L.-Y., Chen, T.-C. and Chen, T.-H. 2016. Development of primer sets for identification of the member species of Tomato spotted wilt virus (TSWV) serogroup. J. Plant Med. 58(1): 17-24.

Tospovirus is one of important plant virus genera that has a wide host rang infecting more than 1090 species of 82 plant families and causes symptoms of spots, chlorosis, mottle, stunt, wilt and necrosis. According to the serological relationship of nucleocapsid protein (NP), tospoviruses are divided into serogroups. Most of tospoviruses are classified in two major serogroups named from *Watermelon silver mottle virus* (WSMoV) and *Tomato spotted wilt virus* (TSWV). The WSMoV-serogroup tospoviruses are prevailing in Asia while the TSWV-serogroup tospoviruses are mainly distributed in Europe and America. The members of TSWV serogroup, such as Alstroemeria necrotic streak virus (ANSV), Chrysanthemum stem necrosis virus (CSNV), *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), Melon severe mosaic virus (MeSMV), Pepper necrotic spot virus (PNSV), *Tomato chlorotic spot virus* (TCSV), *Tomato spotted wilt virus* (TSWV) and *Zucchini lethal chlorosis virus* (ZLCV), are important quarantine viruses in Taiwan. In this study, the nucleotide sequences of N genes of TSWV-serogroup tospoviruses were analyzed to design forward primers FT1, FT2, FT3, FT4 and FT5 and reverse primers RT1, RT2, RT3, RT4 and RT5. The forward and reverse primers were cross-mated to form 25 primer pairs that were used to test the accessibility for amplifying signals from the cloned plasmids carrying the N genes of ANSV, CSNV, GRSV, INSV, MeSMV, TCSV and TSWV in polymerase chain reaction (PCR). Except the primer pairs 05: FT1/RT5, 10: FT2/RT5, 15: FT3/RT5, 20: FT4/RT5 and 25: FT5/RT5, most primer pairs could be used to amplify certain DNA fragments, ranging from 200 bp to 800 bp, from different N gene clones. Moreover, tospovirus species could be identified from the polymorphisms of amplicons. The primer sets 02: FT1/RT2, 03: FT1/RT3, 04: FT1/RT4, 06: FT2/RT1 and 07: FT2/RT3 were successfully used to identify TSWV and INSV from total RNAs of virus-infected *Nicotiana benthamiana* leaf tissues by reverse transcription-PCR. Our results showed that the primer sets have a great potential for identification of TSWV-serogroup tospoviruses.

Keywords: *Tomato spotted wilt virus* (TSWV), serogroup, virus identification