花生簇葉病菌質體 RNA 聚合酵素 Sigma factor 基因之選殖與分析
Cloning and analysis of sigma factor gene of phytoplasma associated with peanut witches' broom

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主持人：林長平　台大植病系

一、中文摘要

在台灣許多重要病害諸如花生簇葉病，甘藷簇葉病，水稻黃萎病等均為植物菌質體 (phytoplasmas) 所引起，其原被稱為似菌質體 (mycoplasmalike organisms, MLOs)。長久以來，因植物菌質體均無法成功地被培養出來，且其細胞的純化仍有許多困難，因此有關其基本生物及生理、生化特性之研究仍屬有限，而有關植物菌質體之分子生物學之探討更是付之闕如。本研究室近年來除積極從事於利用核酸探針及單元抗體進行植物病原植物菌質體之偵測工作外，亦已著手探討植物菌質體之 DNA polymerase, elongation factor, ribosomal RNA 等基因之研究，在本計畫中將利用 PCR 增幅方式，從事 RNA 聚合酵素 Sigma factor 基因片段之選殖，以獲得花生簇葉病菌質體 Sigma factor 基因篩選用之探針，從建立於 lambda 噬菌體之甘藷簇葉病菌質體基因庫篩選出攜帶 RNA 聚合酵素 Sigma factor 基因之選殖株，其後利用生體內剪接作用製備出質體選殖株，再從事南方氏漬染，北方漬染，核甘酸定序、分析及引子伸展反應等一系列之測驗，以完成對花生簇葉病菌質體 Sigma factor 基因之探討，本計畫將於二年完成，本年度為第二年執行，於 1998/ 8 月迄今已完成花生簇葉病菌質體 DNA 之純化、基因庫之構築、利用 PCR 增幅方式研製花生簇葉病菌質體 Sigma factor 篩選用核酸探針，並完成該探針之選殖、核酸解序及比對、探針標識等工作。

關鍵詞：植物菌質體，RNA **聚合酵素** Sigma factor **基因**

Abstract

In Taiwan, many important plant diseases such as peanut witches' broom (PNWB), sweetpotato witches' broom, rice yellow dwarf were caused by phytoplasmas, former mycoplasmalike organisms (MLOs). Up to now, the phytopathogenic phytoplasmas still resist to be cultured in any available media. It's

also very difficult to purify phytoplasmas from affected plants without the contamination of plant antigens. The biological and biochemical data of phytoplasmas are also next to nothing. In our lab, many diagnostic monoclonal antibodies and nucleic acid probe have been developed and applied for disease detection recently. We are also currently working on the cloning and characterization of the DNA polymerase, elongation factor, and ribosomal RNA genes of phytoplasmas. In this study, the gene responsible for the Sigma factor of RNA polymerase of peanut witches' broom phytoplasma will be cloned and analyzed. PCR-amplified Sigma factor gene fragment will be applied as a probe in the screening of the genomic library of peanut witches' broom. The proposed study will be accomplished in two years. In the first year (1998/8-present), the genomic library of PNWB-phytoplasma was constructed in lambda vector. DNA fragment of sigma factor gene was amplified by PCR using primers designed based on the conserved sequences of various organisms. The PCR-amplified fragment was cloned and amplified as a probe for the screening of the library.

Keywords: phytoplasma, sigma factor gene

二、緣由與目的

植物病原菌質體(phytoplasma)，是一種相當重要之植物病原菌，原稱為似菌質體(mycoplasma like organism, MLO)。可引起植物的病徵包括枝條增生(proliferation)，花器葉化(phyllody)，葉片變小，質株矮化(stunting)，黃化(yellowing)，簇葉(witches' broom)。由於其所造成的病徵與病毒所引起病害之病徵相似(Black, 1943; Steere, 1967)，可造成系統性感染，且病原無法以人工方式培養(Lee and Davis, 1986)。由於培養植物菌質體的技術無法突破，因此無法以分類細菌的生理、生化試驗而決定植物菌質體之分類地位，但因植物菌質體細胞形態與菌質體(mycoplasmas)相似，且受植物菌質體感染的植物在以四環黴素(tetracyclines) 治療後，能使植物菌質體細胞在篩管中消失而恢復健康的特性(McCoy et al., 1989)，因此長久以來植物菌質體一直被認為應屬於 Mollicutes 綱的細菌。由於目前仍無法人工培養植物菌質體 (Denes and Sinha, 1992； Lee and Davis, 1986)，且其細胞的純化有許多困難仍待克服 (Jiang and Chen, 1987；Jiang et al., 1988)，因此有關其基本生物及生理、生化特性之研究仍有待加強。

在 eubacteria 中，位於 Mollicutes 綱的細菌一般稱為菌質體，為最小的原核生物，其共同的特徵為缺乏細胞壁，僅以細胞膜 (plasma membrane) 構成細胞對外界的屏障。因菌質體是具有最小基因體 (genome) 的生物，以如此小的基因體卻能維持細胞的生命力則是令學者們熱衷於研究菌質體細胞生物特性的原因

(Razin, 1985； Razin, 1989)。依基因體 DNA (genomic DNA) 的大小，也可將菌質體分為兩群，一群為基因體 DNA 大小為 500 MDa 的 *Mycoplasma* 及 *Ureaplasma*，一群為基因體 DNA 大小為 1,000 MDa 的 *Spioplasma* 、*Acholeplasma*、*Anaeroplasma* 及 *Asteroleplasma* 1,000 MDa 的基因體 DNA 在原核生物已是少見，而 500 MDa 的基因體 DNA 更是只有在 *Mycoplasma* 及 *Ureaplasma* 才可見，如此小的基因體所能解譯 (coding) 的蛋白質約 700 個，僅為一個生物生存所必要的蛋白質數目的二倍，其基因體 DNA 組成的另一項重要特徵是其基因體 DNA 組成具偏低的 G+C 值 (G+C content)，菌質體基因體 DNA 組成的 G+C 值約在 24% 至 40%之間，而對一個生物而言， 26% 的 G+C 值是一個生物解釋具有正常胺基酸組成之蛋白質所必須具有的最小 G+C 值 (Razin, 1985)，菌質體能以如此 AT rich 的基因體 DNA 組成生存，其所代表的意義是菌質體偏好利用 A 或 T rich 的密碼 (codons) 的特殊生物特性。

不論是在真核生物或是原核生物，所有的生理生化反應都是由 DNA 上的訊息，經由轉錄(transcription)及轉譯(translation)操控進行。因此轉錄可說是生命必須的基礎生化反應。原核生物的轉錄催化酵素，也就是 RNA 聚合 (RNA polymerase)，是由α、β、β'及σ等四種 subunit 所組成。轉錄反應進行時，由 2 個α subunits 及一個β subunit，一個β'subunit 組成 core enzyme，core enzyme 必須和σ factor 結合後成為 holoenzyme 後始能進行轉錄反應，轉錄反應進行時，sigma factor 負有啟動子辨識(promotor recognition)的責任，其必須辨識出基因之啟動子區域，並且結合(binding)在-10 至-35 區域，再與中心酵素結合，轉錄反應才能開始進行(Burgess *et al.,* 1969; Chamberlin, 1974; Doi and Wang, 1986; McClure, 1985; Renznikoff, 1985; Travers and Burgess, 1969; von Hipppel *et al.,* 1984; Helmann and Chamberlin, 1987)。

由許多細菌之 sigma factor 之胺基酸序列比對(alignment)的結果，大致上可以將整個 sigma factor 基因由 N 端至 C 端區分成 1-4 等四個區域(Landick *et al.,* 1984; Gribskov and Burgess, 1986; Stragier *et al.,* 1985)，其中區域 1 及 2 之蛋白質二級結構由胺基酸序列分析可得其大致上為β平板(β sheet)，而區域 3 及 4 則為α螺旋(α helix) (Helmann and Chamberlin, 1987; Stragier *et al.,* 1985)，區域 1 及 3 之保守性較低，胺基酸組成多為酸性胺基酸，區域 2 及 4 之胺基酸序列則有較高之保守性，胺基酸組成則多為鹼性胺基酸，又由胺基酸序列分析可推測此蛋白質之 N 端部份與 C 端部份分屬兩個不同之作用區位(domain)，兩個不同區位間之連結區域則有可供蛋白質分解酵素(protease)作用的位置，其他位置則無蛋白質分解酵素作用位置被發現(Garnier *et al.,* 1978; Zvelebil *et al.,* 1987)

於本計畫中，有鑑於 RNA 聚合酵素之 sigma factor 在轉錄層次的基因調控上(regulation of gene expression at transcriptional level)扮演非常重要的角色。而由其基因轉錄系統及基因表現調控著手，亦不失為瞭解植物菌質體特性之有效方法，故 sigma factor 之研究對菌質體生理生化特性的探討上，當屬極為重要。而 sigma factor 因前述之特性，故其高保守性區域即可為引子設計之根據，而可以藉由 PCR

反應增幅出 sigma factor 基因片段以為基因篩選用探針，而對菌質體之 sigma factor 基因進行選殖。故本實驗即依此研究背景，針對花生簇葉病植物菌質體之 sigma factor 基因進行選殖，並探討其特性，以求對植物菌質體之遺傳及生物特性能有進一步的瞭解。

三、結果與討論

花生簇葉病植物菌質體基因庫之建立

將 PNWB 植物菌質體 DNA 以 *Eco*RI 核　酸內限制　進行完全酵解(complete digestion)後，以 250 ng 之花生簇葉病菌質體 DNA 與 2 μg 之 lambda ZapII 載體 DNA 進行黏結反應；根據菌質體之基因體 DNA 組成為 G+C 值較低之特性(Kirkpatrick *et al.,* 1987； Sears *et al.,* 1989)，設定 30 %為花生簇葉病植物菌質體 DNA 之 G+C 值，則 DNA 以 *Eco*RI (recognition sequence：GAATTC) 進行酵解所獲得之 DNA 片段的平均大小約為 3 kb (1/ $(0.15)^2$ $(0.35)^4$= 3000)，而 lambda ZapII 載體 DNA 之大小為 40.8 kb (Short *et al.,* 1988)，則經過計算後，在黏結反應中，花生簇葉病植物菌質體 DNA 與載體 DNA 之分子數比值應為 5：3。完成黏結反應後，再進行包被反應，則獲得花生簇葉病菌質體之基因庫。本實驗建立之花生簇葉病植物菌質體基因庫的包被效率(packaging efficiency)為 3.67×10^6 pfu/ μg of vector DNA，獲得轉型株(即為白色溶菌斑)之機率為 88 %。

PCR 反應引子之設計

由 GCG 基因資料庫可獲得 *E. coli*、*B. subtilis*、*Lactococcus lactis*、*Staphylococcus aureus*、*Clostridium acetobutylicum*、*Listeria monocytogenes* 等六種物種之 *sigA*，*rpoD* 之基因之核　酸及胺基酸序列，及由 Science 期刊的 The *Mycoplasma genitalium* Genome Database (http://www.tigr.org/tdb/mdb/mgdb/mgdb.Html)，連接其中的資料庫可取得 *Mycoplasma genitalium* 之 sigma factor 之核　酸及胺基酸序列，經 CLUSTAL 軟體比對後可找到五處具高保守性區域，經參照前人實驗分析所確定之特殊作用區域(Helmann and Chamberlin, 1987; Stragier *et al.,* 1985)，發現在 rpoD box 及-35 辨識結合區之胺基酸序列之保守性最高，經找出這些區域之核　酸序列再加比對並後，即設計出一對 degenerate primer:SF-f1 及 SF-r1，其序列分別如下：

SF-f1：5’-GATTTRATYCADGARGGWAA-3’(20mer)

SF-r1：5’-GCTTTKKYTTCDATTTG-3’(17mer)

PCR 反應及 PCR 反應產物之選殖

以 SF-f1, SF-r1 為引子, 並以感染 PNWB 之植物全 DNA 為模板, 進行之 PCR 反應結果，以電泳分析 PCR 產物，顯示在 600 bp 有一明顯之亮帶，除此則無其他亮帶可以被偵測到；而再以健康日日春 DNA 為模版之對照組中則無任何明顯可見之亮帶，表示增幅出來之 DNA 片段為植物菌質體所專有，且此一 PCR 產物與由基因序列推測之預期完全符合。將此 PCR 反應產物純化後取少許出來再進行一次 PCR 反應, 並將反應之引子黏合溫度提昇至 45 ℃以提高反應之嚴苛度。結果仍然可以得到此一 600 bp 之明顯亮帶，如此即可確定此 PCR 反應產物並非為非專一性之產物。

接著對此 600 bp 之 PCR 反應產物進行選殖，以利於以後之定序分析及製備核酸探針之製備，經轉型反應後得到之轉型株共有 67 個，取其中 6 個轉型株，分別命名為 TA1, TA2, TA3, TA4, TA5 及 TA6，並繼續進行以下之轉型株特性分析，確定所選殖之片段即為預期之 sigma facor 基因片段。

轉型株之特性分析

分別對上述 6 個轉型株抽取其重組質體 DNA，對其嵌入片段作特性分析。以 SF-f1,SF-r1 為引子，重組質體為模版進行 PCR 反應，對 TA1 到 TA6 等 6 個選殖株之重組質體(pTA1-pTA6)為模版之 PCR 反應仍均可得到 600 bp 之 PCR 反應產物，可見轉型株之重組質體均已含原先 PCR 反應產物(圖四)。再以內限制 *Eco*RI 對 pTA1 到 pTA6 進行酵解以分析嵌入片段之大小，對此反應結果進行水平電泳分析，在 pTA1 到 pTA6 均可在 3.9 kb 大小處見到質體 DNA 之亮帶, 另有一約 600 bp 大小之亮帶即為嵌入片段(圖五)。由 PCR 反應及內限制 作用結果可確定 pTA1 到 pTA6 均為選殖成功之轉型株。接著再針對 pTA1,pTA2 及 pTA3 進行核酸序列分析, 以確定此嵌入片段為 sigma facor 基因片段。由核 酸序列分析結果與 Science 期刊的 The *Mycoplasma genitalium* Genome Database (http://www.tigr.org/tdb/mdb/mgdb/mgdb.Html)，連接其中的資料庫並取得 *Mycoplasma genitalium* 之 sigma factor 之核 酸及胺基酸序列並與之比對, 在 *rpoD* box 及-35 辨識及結合區域之胺基酸序列，p1,p2 及 p3 均與其他 *rpoD* 基因之胺基酸序列相同，故可推測此片段確為 sigma facor 基因片段，並選擇 pTA1 選殖株之重組質體之嵌入片段作為篩選基因庫之核酸探針。

四、計畫成果自評

本計畫於 1998/ 8 月執行迄今已完成花生簇葉病菌質體 DNA 之純化、基因庫之構築, 利用 PCR 增幅方式研製花生簇葉病菌質體 Sigma factor 篩選用核酸探針，並完成該探針之選殖、核酸解序及比對、探針標識等工作。第二年將利用第一年度選殖出之 Sigma factor 基因探針，對構築完成之基因庫進行篩選、並進行

噬菌體生體內剪接質體化、質體選殖株嵌入片段之鑑定、南方氏雜配、北方雜配、嵌入片段之核甘酸定序與分析、引子伸展反應等試驗以確定基因之位置、轉錄及轉譯之起迄位置，於本年度將可順利完成此一計畫。

本計畫可充分利用貴會補助之重要儀器設備，發揮其研究效益，而對基礎科學之研究上，則可因研究之成果而能對植物菌質體提供重要之分子生物學上之資訊，尤以在植物菌質體基因之研究上有所貢獻，而且或能因對其基因之瞭解，進而對植物菌質體與寄主植物或媒介昆蟲細胞間之辨識關係能有進一步之認知，如此對整體防治策略之擬定當有極大助益。

參與之工作人員能熟悉植物病原菌質體之抗原及核酸分離技術，亦能熟悉基因選殖及各種核酸分析之技術（如 hybridization, sequencing, PCR ...**等)，此外亦能參與實驗之設計，瓶頸之克服，並學習將基礎研究之成果延用於實際植物病理研究之相關工作上。**

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