Plant Pathology Bulletin 15:211-218, 2006

Cloning and Sequence Analyses of *recA* Gene of Phytoplasma Associated with Peanut Witches' Broom

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Accepted for publication: August, 30, 2006

ABSTRACT

Chu, Y. R., Chen, W. Y., and Lin, C. P. 2006. Cloning and sequence analyses of *recA* gene of phytoplasma associated with peanut witches' broom. Plant Pathol. Bull. 15: 211-218.

The *recA* gene, which plays an important role in DNA repair of eubacteria, was cloned and analyzed from phytoplasma associated with peanut witches' broom (PnWB) in this study. In order to clone the *recA* gene, a pair of degenerate PCR primers, recA-1f and recA-1r, was designed according to the sequences of *recA* gene of *Escherichia coli*, *Bacillus subtilis*, *Acholeplasma laidlawii*, *Mycoplasma genitalium*, *Mycoplasma pulmonis*, and *Mycoplasma mycoides* subsp. *mycoides*. Total DNA prepared from the diseased periwinkle plant infected with PnWB phytoplasma was then used as the DNA template for booster PCR to amplify the partial sequences of *recA* gene of PnWB phytoplasma with primer pair recA-1f/ recA-1r. An amplified 323 bp-PCR product harboring an *Eco*RI site was cloned in vector pCR2.1 and the recombinant plasmid pTA1 was obtained. The cloned insert of pTA1 and the 76 bp fragment of the *Eco*RI restricted 323 bp-PCR product was then used as a nucleic acid probe for screening the λ ZAP II *Eco*RI genomic library of PnWB phytoplasma. Two *in vivo* excised recombinants, pREC1 (with a 2.3 kb insert) and pREC9 (with a 2.5 kb insert) were obtained and sequenced. The sequences of the 323 bp-PCR product overlap with the sequences of the cloned inserts of pREC1 and pREC9 that flanking to each other at the *Eco*RI cutting sequences of the 323 bp-PCR product. The cloned insert of pREC1 contained the upstream sequences and the 5' region of *recA* gene, and the cloned insert of pREC9 contained the 3' region and the downstream sequences of the gene. A complete open reading frame (ORF) was thus constructed and identified based on the sequences of the 323 bp-PCR product, and the DNA inserts of pREC1 and pREC9. The Shine-Dalgano sequence complementary to the 3'-end sequence of 16S rRNA of phytoplasmas was found 12-16 nucleotides upstream of AUG start codon of the ORF. The gene organization, the nucleotide sequence, and the deduced amino acid sequence in the conserved regions of the ORF are similar to those of the other *recA* genes of eubacteria. Therefore, this gene was identified as a putative *recA* gene.

Key words: Peanut witches' broom phytoplasma, *recA* gene

INTRODUCTION

Peanut (*Arachis hypogaea* L.) witches' broom (PnWB) first discovered in a geographically isolated area, the Penghu Islands, in 1975 in Taiwan [36] is a plant disease associated with plant pathogenic phytoplasma. Phytoplasmas, used to be known as mycoplasmalike organisms (MLOs), are now classified as members of the class *Mollicutes* [14, 17, 18, 19]. They are a group of prokaryotes without cell wall and remain unculturable *in vitro* [7]. Therefore, understandings in physiology, biochemistry and molecular biology about phytoplasmas are limited. For the detection and identification of phytoplasmas, polyclonal and monoclonal antibodies, DNA probes, and PCR primers have been developed for various phytoplasmas [1, 2, 15, 20, 21, 29]. Besides the detection purpose, extrachromosomal DNA, insertion sequence [34], and various genes [3, 4] of peanut witches' broom phytoplasma have also been cloned and analyzed in our lab.

RecA protein, the product of *recA* gene, is a key protein involved in DNA recombination and DNA repair. In *Escherichia coli*, the *recA* gene coding for RecA protein of 352 amino acids has been extensively characterized and proved to be essential for numerous cell functions, such as homologous recombination, DNA repair, and induction of the SOS response after DNA damage [5, 6, 22, 23, 24, 30].

Though the *recA* gene in Gram-negative bacteria has been extensively studied, little is known about this key enzyme in other Gram-positive bacteria other than *Bacillus subtilis* [33]. Especially, only few *recA* genes have been cloned from the mycoplasmas [8, 9, 10, 11, 13]. In this study, we report the first cloning and characterization of a putative *recA* gene of PnWB phytoplasma.

MATERIALS AND METHODS

Plant material

Peanut plants naturally infected with PnWB phytoplasma were collected from fields on the Penghu Islands. PnWB phytoplasma-affected periwinkle plants (*Catharanthus roseus* (L.) G. Don) provided by Dr. I. L. Yang (Taiwan Agricultural Research Institute, Wufeng) were originally transmitted from diseased peanut plants by dodder (*Cuscuta saustualis* R. Brown) and now maintained in periwinkle plants by top and side grafting. Periwinkle plants affected by sweet potato witches' broom (SPWB, another phytoplasma-associated disease) were also maintained by periodic grafting to healthy periwinkle plants in the green house as described previously [29].

Isolation of total DNA from periwinkle plants

The procedure reported by Kollar et al. [16] was used with minor modifications to isolate total DNA from healthy and diseased plants [15, 35]. Ten grams of PnWB phytoplasma-infected periwinkle or healthy periwinkle were ground to fine powder in liquid nitrogen with a mortar and pestle. The frozen powder was then suspended in 30 ml DNA extraction buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 500 mM NaCl; 0.01% proteinase K; 1% sodium lauroyl sarcosine; 60 mM 2-mercaptoethanol) and incubated at 50℃ for 1 hour [35] to extract nucleic acids. Crude nucleic acids were further clarified by repeated precipitations with ethanol and extractions with phenol and chloroform as described previously [25, 35]. Nucleic acid preparation was finally resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or sterile distilled water.

Separation of PnWB phytoplasma DNA from host plant DNA

Cesium chloride (CsCl)-bisbenzimide density gradient centrifugation was used to separate phytoplasma DNA from host plant DNA as described previously [15]. After centrifugation, PnWB phytoplasma DNA was visualized under ultraviolet (UV) light as a unique band uppermost in gradients of DNA from infected plants and was then collected with a syringe. This DNA fraction was recentrifuged two more times as previously described [15]. After a final centrifugation, the phytoplasma DNA band was extracted five times with equal volumes of NaCl-saturated isopropanol. The DNA was precipitated for 2 hours at -20℃ after the addition of three volumes of sterile distilled water and eight volumes of cold ethanol. Upon collection by centrifugation at 13,000 g for 20 min, the DNA pellet was rinsed with 70% ethanol, dried, resuspended in TE buffer (pH 8.0), and quantified [25].

Construction of the genomic library of PnWB phytoplasma

The procedures used for molecular cloning of PnWB phytoplasma DNA and screening of recombinants were similar to those described previously [37]. CsCl-purified PnWB phytoplasma DNA was digested with EcoRI restriction endonuclease (Boehringer Mannheim GmbH, Mannheim, Germany), ligated with *Eco*RI-digested and calf intestine phosphatase-phosphorylated lambda Zap II cloning vector (Stratagene Cloning Systems, La Jolla, CA) [31], and subsequently packaged with Gigapack II Gold packaging extract (Stratagene Cloning Systems) according to the manufacturer's instructions.

Polymerase chain reaction

Complete or partial sequences of *recA* gene of *Escherichia coli* [26], *Bacillus subtilis* [33], *Acholeplasma laidlawii* [10], *Mycoplasma genitalium* [11], *Mycoplasma pulmonis* [13], and *Mycoplasma mycoides* subsp. *mycoides* [13] were collected from the database of GenBank. The CLUSTAL [12] program was applied to align the sequences of the six *recA* genes and to analyze the highly conserved regions of this gene. Based on the conserved sequences, a pair of degenerate PCR primers: recA-1f: 5'-ARATHGARAARMWRTTYGG-3', and recA-1r: 5'-GYTCNCCNBWRTCNGG-3' (R=A+G, Y=C+T, H=A+T+G, M=A+C, W=A+T, B=T+C+G, N=A+T+C+G) was designed.

Total DNA prepared from healthy periwinkle, periwinkle plants affected with PnWB phytoplasma or SPWB phytoplasma, and CsCl-purified PnWB-phytoplasma DNA were used as DNA templates for booster PCR. The PCR was performed in a 50 μl of reaction mixture containing 50 ng of template DNA, 10 μM of each primer, 250 μM of dNTP mixture, 5 μl of *Taq* DNA polymerase 10x Mg^{2+}-free buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.4), 2.25 mM $MgCl_2$, and 2 units of *Taq* DNA polymerase (Gibco BRL Life Technologies, Inc., Grand Island, NY). Forty PCR cycles were conducted in a PCR machine (DNA Thermal Cycler 2400, Perkin Elmer Corporation, Norwalk, CT) with the following parameters: denaturation for 50 sec at 94℃, annealing for 50 sec at 50 ℃, and extension for 50 sec at 72℃. After the first forty cycles, another 2 units of *Taq* DNA polymerase was added to perform another forty PCR cycles with the same parameters.

Cloning of PCR-amplified fragments

To further clone and analyze the PCR-amplified fragment, the target fragments from the PCR products were extracted. After separating the PCR products in a 2% agarose gel electrophoresis, the target DNA fragments were sliced out, purified by QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), and subsequently cloned in vector pCR2.1 using PCR II TA Cloning Kit (Invitrogene Corporation, San Diego, CA). The molar ratio of the target DNA fragments to the plasmid is 1 to 3. The cloning procedures were conducted by following the vendor's instructions. White colony transformants that grew on a Luria-Bertani (LB) medium containing ampicillin (100 μg/ml), X-gal (5-bromo-4-chloro-3-indoyl-β-galactoside, 0.004%), and IPTG (isopropyl-β-D-thiogalacto-pyranoside, 0.5 mM) were subcultured. To tentatively identify recombinants containing cloned PCR fragments, plasmid DNA was extracted from white colonies by alkaline lysis method and analyzed by 1.5% agarose gel electrophoresis after cutting with appropriate restriction enzymes as described later [37].

Screening of the PnWB phytoplasma genomic library

Overnight cultures of *Escherichia coli* XL1-blue grown in LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$ were pelleted, suspended with 1/3 volume of 10 mM $MgSO_4$, and then infected with recombinant phages (5,000 pfu per plate of 150 mm in diameter) at 37℃ for 20 min. Infected cells mixed with 55 ℃ prewarmed NZY top agar (0.5% yeast extract, 1% NZ amine, 0.5% NaCl, 0.2% $MgSO_4$, 0.7% agarose, pH 7.5) were then plated on 37℃ prewarmed NZY plate (NZY top agar except replacing 0.7% agarose with 1.5% bactoagar). After being incubated at 37℃ for 8-10 hours, the plates were overlaid with nylon membranes (MegnaGraph, nylone membrane, pore size 0.45 μm, 132 mm in diameter, Micron Separations, Inc., Westboro, MA) for 5 min at 37℃ before lifting the filter followed by a 30 min incubation for the consequent replica membranes. Membranes were washed sequentially with denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 5 min, neutralization solution (0.5 M Tris-HCl, pH 7.5, 3 M NaCl) for 5 min, and then rinse buffer (2x SSC) for 15 min. Membranes were then air-dried and cross-linked using an UV-crosslinker (Spectronics Corporation, Westburg, NY). The procedures used for hybridization and high stringency washes were described by Ko and Lin [15]. The 323 bp PCR-amplified DNA fragment cloned in vector pCR2.1 (described later) was labeled with digoxigenin-dUTP according to the manufacturer's protocols (Boehringer Mannheim GmbH) and used as a probe to hybridize with the phage DNA. The signal detection was performed by using digoxigenin nucleic acid detection kit (Boehringer Mannheim GmbH) following the supplier's instructions. The blots were visualized by incubating the membranes in the dark in a solution containing the colorimetric substrate, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, X-phosphate). Sizes of the cloned inserts in each selected recombinant were determined by agarose gel electrophoresis (0.8%) after *Eco*RI digestion.

Southern hybridization

For each blot, 3 μg of total DNA from healthy periwinkle plant and PnWB phytoplasma-infected periwinkle plants was digested with *Eco*RI, electrophoresed in 0.8% agarose gels, alkaline-denatured (0.5 M NaOH in 1.5 M NaCl for 45 min), neutralized (1 M Tris, pH 7.4, 1.5 M NaCl for 15 min), and transferred to the nylon membranes following the procedures described

by Southern [32]. The membranes were cross-linked, prehybridized, hybridized with digoxigenin-labeled DNA probes of the 323 bp-PCR product or the 76 bp *Eco*RI-digested partial fragment of the 323 bp-PCR product (described later), and washed under moderate stringency as previously described [15].

DNA sequencing

Recombinant plasmids, pTA1, pREC1 and pREC9 (described later), containing a PnWB phytoplasma DNA fragment were purified with Wizard minipreps DNA purification system (Promega Corporation, Madison, WI) for DNA sequencing. The inserts of recombinant plasmids were determined by the dideoxy chain termination method [27] using Sequenase DNA sequencing kit (US Biochemical, Cleveland, OH). Sequence reactions were primed with pUC/M13 forward, pUC/M13 reverse, and synthesized oligonucleotide primers when necessary in order to continue sequencing. The sequence was confirmed by analysis of the complementary strand. Sequencing gel electrophoresis was conducted in a 6% denaturing polyacrylamide gel as described by Sambrook et al. [25].

RESULTS AND DISCUSSION

PCR-amplified product

Based on the sequences of *recA* genes of *Escherichia coli* [26], *Bacillus subtilis* [33], *Acholeplasma laidlawii* [10], *Mycoplasma genitalium* [11], *Mycoplasma pulmonis*, and *Mycoplasma mycoides* subsp. *mycoides* [13], two conserved regions were identified and used for designing the degenerate PCR primer pair recA-1f/ recA-1r. According to the amino acid sequence, and the special characteristics of an extreme AT bias in the DNA of phytoplasmas [28], A or T was usually used in the degenerate primers.

After conducting the booster PCR, a distinct DNA product of 323 bp in size was obtained by using primer pair recA-1f/ recA-1r with total DNA prepared from PnWB phytoplasma-infected periwinkle and SPWB phytoplasma-infected periwinkle (lanes 2 and 3, Fig. 1). The PCR products of the same size were also obtained by using CsCl-bisbenzimide purified PnWB phytoplasma DNA as a template (data not shown). No specific PCR product was obtained in all other PCR reactions using the DNA templates prepared from healthy plants (Fig. 1).

Cloning of PCR-amplified fragment

After cloning the 323 bp-PCR product in plasmid vector pCR2.1, three transformants pTA1, pTA2, pTA3 were selected for further studies. When the recombinant plasmids were digested with *Eco*RI, besides the vector DNA band of about 3.9 kb in size, there were 2 more bands in the size of 247 bp and 76 bp observed. This result indicated that there is an *Eco*RI recognition sequence in the 323 bp-PCR product, and the 323 bp-PCR product can yield 247 bp and 76 bp fragments after *Eco*RI digestion (Fig. 2). To confirm the presence of the 323 bp fragment in the recombinant plasmids, restriction enzymes *Bam*HI and *Xba*I were then used for the double digestion of these recombinant plasmids, and a band in the size of about 420 bp was observed in the agarose gel electrophoresis analysis. In vector pCR2.1, the recognition sites of *Bam*HI and *Xba*I are about 40 bp and 50 bp away from the integrated sequence, respectively. The result indicated that the recombinant plasmids pTA1, pTA2, pTA3 did carry the cloned inserts.

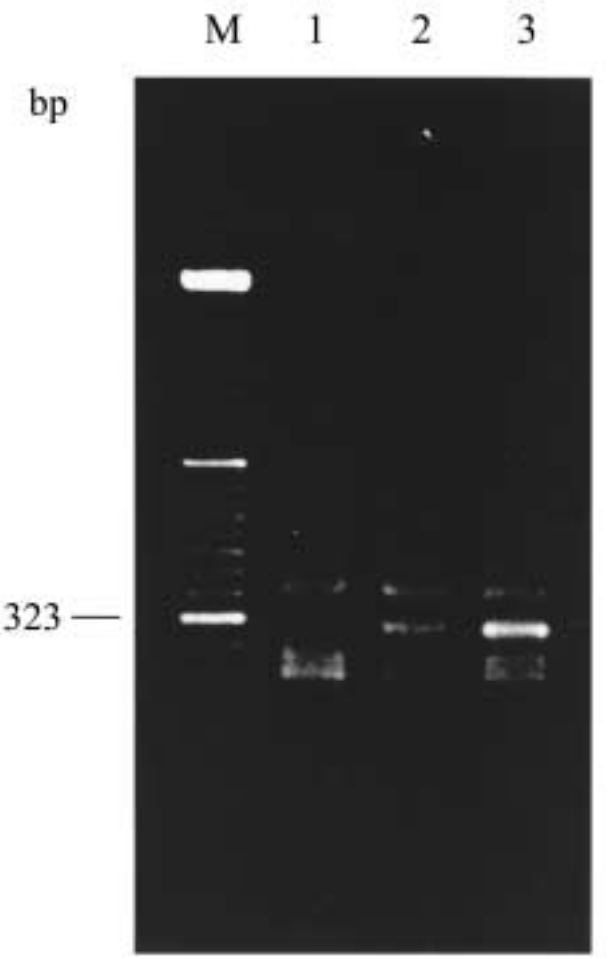

Fig. 1. DNA products amplified by booster PCR with total DNA extracted from: lane 1, healthy periwinkle; lane 2, periwinkle infected with PnWB phytoplasma; lane 3, periwinkle infected with SPWB phytoplasma. M, 50 bp ladder as molecular weight standards. The size (in bp) of PCR product is shown on the left.

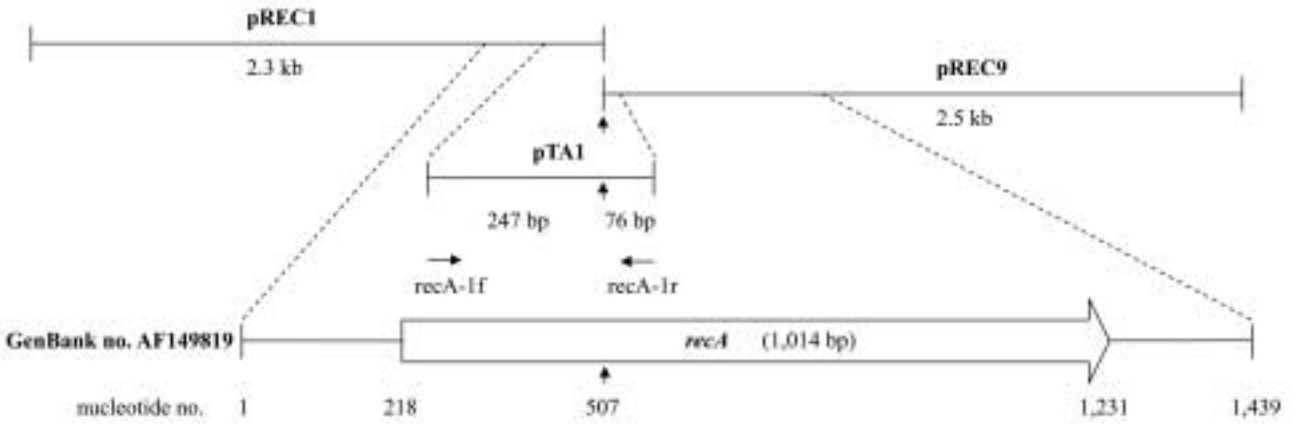

Fig. 2. Diagram showing the relative positions and length of *recA* gene, 323 bp PCR-product (the DNA insert of pTA1), the DNA insert of pREC1, and the DNA insert of pREC9, and the annealing sites of degenerate primer pair recA-1f/ recA-1r. Upward arrows refer to the *Eco*RI cutting sites, large arrow refers to the direction of transcription, and small arrows refer to the 5' to 3' direction of oligonucleotide primers.

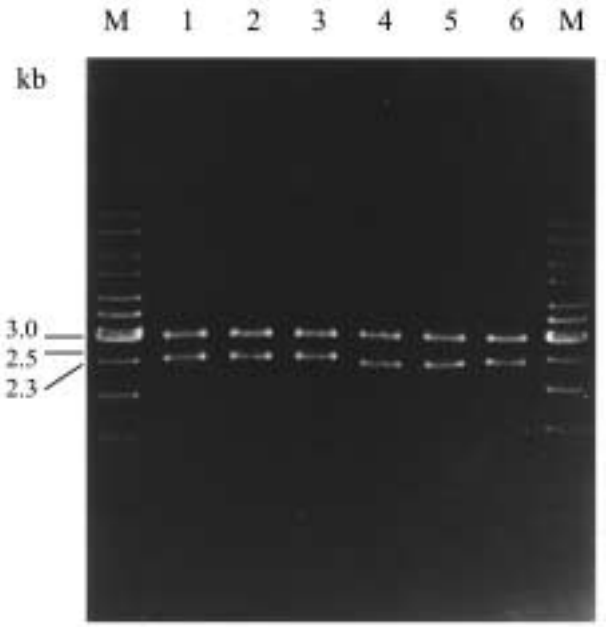

Fig. 3. Analysis of recombinant plasmids pREC9 (lane 1), pREC10 (lane 2), pREC11 (lane 3), pREC1 (lane 4), pREC2 (lane 5), and pREC3 (lane 6) in a 1% agarose gel electrophoresis after digested with the restriction enzyme *Eco*RI. M, 1 kb ladder as molecular weight standards. The sizes of plasmid vector (3.0 kb) and inserts (2.3 or 2.5 kb) are shown on the left.

After sequencing the recombinant plasmids pTA1, pTA2, and pTA3, the DNA sequences of the 323 bp PCR product among them revealed to be identical to each other, and the *Eco*RI recognition sequences in these fragments were all identified (Fig. 2). Recombinant plasmid pTA1 was then chosen for further studies.

Screening of the PnWB phytoplasma genomic library

The PnWB phytoplasma genomic library was screened by using the PCR-amplified 323 bp PCR-fragment cloned in recombinant plasmid pTA1 as the probe. After several times of screening, eight clones of lambda Zap II recombinants were selected and *in vivo* excised to yield recombinant plasmids pREC1, pREC2, pREC3, pREC4, pREC5, pREC6, pREC7, and pREC8 that carried the PnWB phytoplasma DNA in pBluescript plasmid vector. After digested with restriction enzyme *Eco*RI, all eight recombinant plasmid carried an 2.3 kb insert DNA fragment (Fig. 2) besides the vector DNA of 3.0 kb in size (lanes 4, 5, 6, Fig. 3).

The universal primers T7 and T3 were used to sequence these eight recombinant plasmids. The DNA sequences of all these eight plasmids were identical to each other, and one end of the sequences completely overlaps with the sequence of the 247 bp *Eco*RI-digested fragment (Fig. 2) of the 323 bp-PCR fragment. Further sequence analyses revealed that the inserts of these eight recombinant plasmids carried the upstream region and the 5'-end of the *recA* gene (Fig. 2). Recombinant plasmid pREC1 was then chosen for further studies.

Preparation of 76 bp DNA probe and screening of the PnWB phytoplasma genomic library

In order to obtain the sequences of the 3'-end of the *recA* gene that flanking to the insert DNA sequence of pREC1 at the *Eco*R1 site and overlapping with the sequence of the 76 bp fragment (Fig. 2) that located on the other side of the *Eco*RI cutting site of the 323 bp-PCR

fragment, the 76 bp fragment was purified from agarose gel after electrophoresis and labeled with digoxigenin-dUTP as a probe for further screening of the PnWB phytoplasma genomic library. Six recombinant plasmids pREC9, pREC10, pREC11, pREC12, pREC13, and pREC14 were thus obtained. All of these six recombinant plasmids carried a 2.5 kb insert after digested with *Eco*RI and analyzed in agarose gel electrophoresis (lanes 1, 2, 3, Fig. 3). The DNA sequences of these six recombinant plasmids were identical to each other, and one end of the sequences completely overlaps with the sequence of the 76 bp *Eco*RI-digested fragment (Fig. 2) of the 323 bp-PCR fragment. Further sequence analyses revealed that the inserts of these recombinant plasmids carried the 3'-end and the downstream region of the *recA* gene that flanking to the insert DNA sequence of pREC1 at the *Eco*RI site (Fig. 2). Recombinant plasmid pREC9 was then chosen for further studies.

DNA sequence of PnWB phytoplasmal *recA* gene

A 1,439 bp nucleotide sequence which contains the complete sequence of PnWB phytoplasmal *recA* gene harboring an *Eco*RI recognition sequence (Fig. 2) were assembled from the partial sequences of the insert DNAs of pREC1 and pREC9 that flanking to each other at the *Eco*R1 site and from the complete sequence of the insert DNA of pTA1 that overlaping it's 247 bp and 76 bp sequences with the sequences of insert DNAs of pREC1 and pREC9, respectively. The sequences were determined at a minimum of 2x sequencing coverage for each base position and submitted to GenBank. The accession number for the 1,439 bp nucleotide sequence is AF149819. This putative *recA* gene of PnWB phytoplasma is 1,014 bp in size. The RecA protein of PnWB phytoplasma deduced from the DNA sequence is of 337 a.a. residues and 37 KD in the molecular weight. Sequences of the ribosome binding site (RBS) (5'-AGAGG-3') are 12 to 16 nucleotides upstream of the start codon (nt 218-220). The A-T ratio in the open reading frame is 70.13%. The DNA sequence homology of this putative phytoplasmal *recA* gene is 57.7% to that of *Mycoplasma genitalium*, and amino acid sequence comparison shows homology of 44%.

Southern hybridization

The total DNA of the healthy and PnWB-infected periwinkles were digested by *Eco*RI, electrophoresed, and then transferred to the nylon membrane. The DIG-labeled insert DNA fragment of pTA1 and the 76 bp fragment of the *Eco*RI-digested 323 bp-PCR product described above were used as probes. Two hybridization bands (2.5 kb and 2.3 kb) were detected when using the probe of pTA1 insert (Fig. 4A), and only one band (2.5 kb) was detected by the probe of 76 bp fragment (Fig. 4B). No specific hybridization signal was detected in the DNA preparation of healthy plant. According to the results of the Southern hybridization analysis, it suggests that only one copy of *recA* gene exists in PnWB phytoplasma.

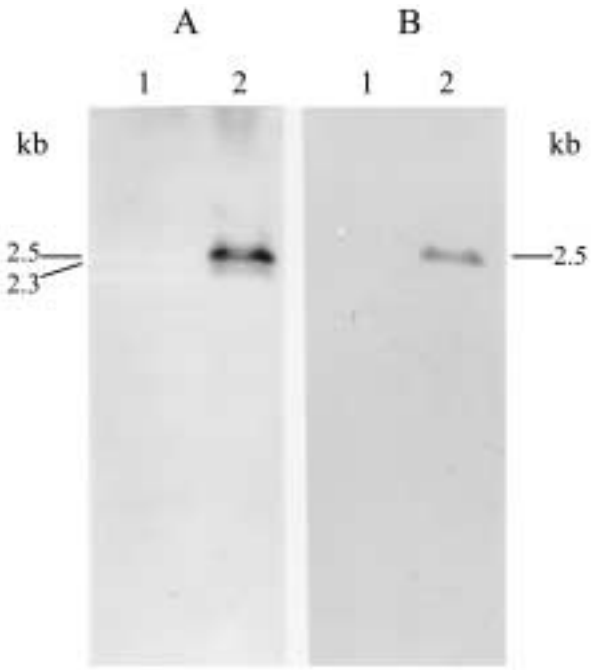

Fig. 4. Southern blot analysis of total DNA (3 μg per lane) prepared from healthy periwinkle (lanes 1) and diseased perwinkle infected with PnWB phytoplasma (lanes 2) digested with restriction enzyme *Eco*RI. Hybridization analyses were conducted at 55℃ using the DIG-labeled insert DNA fragment of pTA1 (A), and DIG-labeled 76 bp fragment of the 323 bp-PCR fragment (B) described in the text as probes. The sizes (in kb) of the hybridization signals are shown on the margins.

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摘 要

朱俞蓉[1]、陳武揚[1]、林長平[1,2]. 2006. 花生簇葉病菌質體 *recA* 基因之選殖與分析. 植病會刊 15: 211-218. （[1]臺北市 國立臺灣大學植物病理與微生物學系；[2]聯絡作者，電子郵件：cplin@ntu.edu.tw；傳眞：+886-2-23661980）

本實驗將 *Escherichia coli*, *Bacillus subtilis*, *Acholeplasma laidlawii*, *Mycoplasma genitalium*, *Mycoplasma pulmonis* 及 *Mycoplasma mycoides* subsp. *mycoides* 等細菌之 *recA* 基因進行序列比對，根據其保守性較高之區域設計出 PCR 引子對 recA-1f/ recA-1r，以花生簇葉病病原菌質體 DNA 爲模板進行 booster PCR 反應，得到一 323 bp 之 PCR 產物，此 PCR 產物之序列中含有一 *Eco*RI 切位，將此 PCR 片段或將其 *Eco*RI 酵解後所獲之 76 bp 片段以 DIG 進行標識以作爲核酸探針，並用於篩選花生簇葉病病原菌質體之 λZAPII *Eco*RI 基因庫，得到選殖株重組質體 pREC1 (含 2.3 kb 之嵌入片段) 及 pREC9 (含 2.5 kb 之嵌入片段)。對此 323 bp 之 PCR 產物、pREC1 及 pREC9 之嵌入片段進行核苷酸序列分析，將其重疊之序列聯結後可得到一個完整的 ORF (open reading frame)，且在轉譯起始密碼 AUG 上游有互補於植物菌質體 16S rRNA 之 3' 端核酸序列之 Shine-Dalgarno 序列之存在，其可能爲核糖體結合位置 (ribosomal binding site)。此 ORF 之核苷酸序列經推衍爲胺基酸序列後，發現其基因大小及基因結構均與其他的細菌之 *recA* 基因相似，故由此推斷其爲植物菌質體之 *recA* 可能基因。其基因在密碼利用性上並無以 UGA 作爲 tryptophan 轉譯密碼之現象，且該基因序列有與其他 Mollicutes 綱細菌在基因體特性上相同之 AT-rich 現象。而在南方氏雜配反應的結果則可發現在花生簇葉病植物菌質體之基因體中應僅具有單一套組 (single copy) 之 *recA* 基因。

關鍵詞：花生簇葉病菌質體、*recA* 基因