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An antigenic protein gene of a phytoplasma associated with sweet potato witches' broom

Yen-Ling Yu,[1] Kai-Wun Yeh[2] and Chan-Pin Lin[1]

Author for correspondence: Chan-Pin Lin. Tel: +886 2 23661980. Fax: +886 2 23636490.
e-mail: cplin@ccms.ntu.edu.tw

Department of Plant Pathology and Entomology[1] and Department of Botany[2], National Taiwan University, Taipei, Taiwan 106, Republic of China

A gene encoding the major antigenic protein of phytoplasma associated with sweet potato witches' broom (SPWB) was cloned and analysed by screening the genomic library of SPWB phytoplasma with monoclonal antibodies for SPWB phytoplasma. The entire predicted structural gene encoded an antigenic protein composed of 172 amino acids with a computed molecular mass of 19·15 kDa and a pI value of 9·78. The −10 region of the promoter and the terminator region of the gene were identified and found to be similar to those of prokaryotes. The hydropathy profile of the deduced amino acid sequence consisted of two distinct regions, a strongly hydrophobic N-terminus and a highly hydrophilic C-terminus. This major antigenic protein was also present in phytoplasma associated with peanut witches' broom (PNWB) and the two showed homology based on the results of Western blot analysis, Southern hybridization, Northern hybridization, primer extension analysis and PCR. The homologous genes of the antigenic protein of SPWB phytoplasma and PNWB phytoplasma were not found in other phytoplasmas tested.

Keywords: phytoplasma, antigenic protein, monoclonal antibody

INTRODUCTION

Phytoplasmas, formerly mycoplasma-like organisms, are a group of cell-wall-free prokaryotes resistant to *in vitro* culturing (Lee & Davis, 1986). Understanding of the physiology, biochemistry and molecular biology of phytoplasmas is limited (Lim & Sears, 1989, 1991a, b; Lim *et al.*, 1992; Sinha, 1979; Sinha & Madhosingh, 1980), primarily because procedures available for purification of phytoplasmas from diseased plants are tedious and unsatisfactory (Clark *et al.*, 1989; Jiang & Chen, 1987) due to the inevitable contamination of plant components. However, recent studies have shown that phytoplasma DNA can be separated from plant DNA using CsCl equilibrium buoyant density centrifugation based on its extreme AT bias of codon usage (Kirkpatrick *et al.*, 1987; Sears *et al.*, 1989). Consequently, the molecular biology of phytoplasmas can now be studied. The 16S rRNA gene (Lim & Sears, 1989) and ribosomal protein genes *rpl2*, *rps19*, *rpl22* and *rps3* (Lim & Sears, 1991b, 1992) of phytoplasma have been identified and sequenced. The genome size of *Oenothera* phytoplasma was proved to be similar to those of animal mycoplasmas (Lim & Sears, 1991a). Phytoplasmas are now tentatively classified as members of the class *Mollicutes* (Lim & Sears, 1989, 1991b, 1992). The presence of certain characteristic oligonucleotide sequences in the 16S rRNA (Lim & Sears, 1989) and the UGA codon usage of phytoplasmas in the ribosomal protein genes (Lim & Sears, 1991b, 1992) suggest that phytoplasmas are evolutionarily closer to acholeplasmas than to mycoplasmas and spiroplasmas.

In an effort to elucidate the molecular biology of phytoplasmas, a gene encoding the major antigenic protein of the phytoplasma associated with sweet potato witches' broom (SPWB) was cloned and characterized using monoclonal antibodies for SPWB phytoplasma (Shen & Lin, 1993). The presence of the antigenic gene in the closely related peanut witches' broom (PNWB) phytoplasma (Chen & Lin, 1997; Hsyu & Lin, 1996a, b; Ko & Lin, 1994; Shen & Lin, 1993) was also demonstrated.

METHODS

Diseased plants and monoclonal antibodies. SPWB-phytoplasma-infected periwinkle [*Catharanthus roseus* (L.) G. Don] originally obtained by transmission through dodder (*Cuscuta australis* R. Broom) was provided by I. L. Yang

Abbreviations: PNWB, peanut witches' broom; SPWB, sweet potato witches' broom.

The GenBank accession number for the sequence reported in this paper is U15224.

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(Taiwan Agricultural Research Institute, Wufang, Taiwan) and was maintained and propagated in periwinkle by side grafting (Ko & Lin, 1994; Shen & Lin, 1993). Phytoplasmas associated with several other diseases were also maintained in periwinkle plants or collected from fields as previously described by Ko & Lin (1994) and Shen & Lin (1993). These diseased plants included periwinkle affected by PNWB, loofah witches' broom, paulownia witches' broom, elm yellows, aster yellows and *Ipomoea obscura* witches' broom; rice affected by rice yellow dwarf disease; and bamboo affected by bamboo little leaf disease. SPWB phytoplasma monoclonal antibodies MA40, MA35, MA21, MA16 and MA6 developed in our laboratory (Shen & Lin, 1993) were used in this study for Western blotting and immunological screening of a genomic library of SPWB phytoplasma. The antibodies cross-reacted with the closely related PNWB phytoplasma but not with other phytoplasmas (Shen & Lin, 1993).

Phytoplasma-enriched protein preparations, SDS-PAGE and Western blot analysis. Phytoplasma proteins were partially purified from periwinkle following the method described by Jiang & Chen (1987) with some modifications. Briefly, approximately 20 g diseased plant tissue was ground in 80 ml isolation medium (Shen & Lin, 1993) and the homogenate was subjected to two cycles of differential centrifugation (3000 *g* for 10 min and 14000 *g* for 40 min). The pellet, containing phytoplasma cells, was resuspended in 2 ml PBS (0·137 M NaCl, 10 mM Na_2HPO_4, 1·5 mM KH_2PO_4, 2·7 mM kCl, pH 7·4). Proteins thus prepared were analysed by SDS-PAGE (Laemmli, 1970). The separated proteins were then electrotransferred to a nitrocellulose filter (Schleicher & Schuell) and probed with SPWB phytoplasma monoclonal antibodies as described previously (Shen & Lin, 1993).

Cloning of the major antigenic protein gene of SPWB phytoplasma. Total DNA was isolated from healthy or phytoplasma-affected plant tissues according to the method described by Ko & Lin (1994). To separate phytoplasma DNA, a CsCl–bisbenzimide density-gradient centrifugation method was used (Ko & Lin, 1994). Approximately 250 ng *Eco*RI-digested SPWB phytoplasma DNA was ligated to 2 µg *Eco*RI-cleaved calf-intestine-phosphatase-dephosphorylated lambda ZapII cloning vector, and then packaged with Gigapack II Gold packaging extract (Stratagene).

Immunological screening of the genomic library of SPWB phytoplasma was performed using an equal volume ratio mixture of SPWB phytoplasma monoclonal antibodies MA6, MA16, MA21, MA35 and MA40. Overnight cultures of *Escherichia coli* XL-1 Blue grown in LB supplemented with 0·2 % maltose and 10 mM $MgSO_4$ were harvested and infected with the recombinant phage (5000 p.f.u. per plate of 150 mm in diameter) at 37 °C for 20 min. Infected cells were then plated on NZY plates (0·5 % yeast extract, 1 % NZ amine, 0·5 % NaCl, 0·2 % $MgSO_4$, 1·5 % Bacto agar, pH 7·5) and incubated at 37 °C overnight. The plates were overlaid with IPTG-saturated nitrocellulose filters and incubated at 37 °C for 4 h before lifting the first filter, and then 4·5 h for the replica filters. Filters were then immunologically detected as described above to identify positive clones.

In vivo excision of the recombinant pBluescript SK(−) phagemid containing the cloned gene was performed using the ExAssist/SOLR system (Stratagene).

Characterization of the antigenic protein gene. Induction of the *lacZ*-promoter-modulated antigenic protein was performed by adding IPTG to a final concentration of 5 mM to an *E. coli* culture of OD_{600} 0·6 (measured with a Hitachi U2000 spectrophotometer). After being induced for 2 h at 37 °C, *E. coli* was recovered by centrifugation (4000 *g* for 10 min). The pellet was washed twice with 0·02 M PBS (pH 7·4), resuspended in 2·5 ml 0·02 M PBS, and sonicated (W-385, Heat Systems-Ultrasonics). The protein samples were clarified by centrifugation (4000 *g* for 10 min). The supernatant was analysed by SDS-PAGE and immunoblotting as described previously using a pre-absorbed antibody prepared according to Sambrook *et al.* (1989).

Southern and Northern hybridization analyses. For Southern hybridization, total plant DNA from healthy periwinkle, periwinkle plants infected with SPWB, PNWB, loofah witches' broom, paulownia witches' broom, elm yellows, aster yellows or *I. obscura* witches' broom, rice affected by rice yellow dwarf disease, and bamboo affected by bamboo little leaf disease were digested with *Eco*RI, *Xba*I, *Hin*dIII and *Pst*I, respectively, and then separated in a 0·8 % agarose gel. The 2·3 kb insert of pSPAG1, containing the major antigenic protein gene, was labelled with digoxigenin-11-dUTP (Boehringer Mannheim). Hybridization was performed under low and high stringencies at 55 °C and 68 °C, respectively (Chen & Lin, 1997; Ko & Lin, 1994). For Northern hybridization, total RNA of healthy, SPWB-phytoplasma-infected and PNWB-phytoplasma-infected periwinkle plants was isolated according to the method of Yeh *et al.* (1991), and hybridization with the digoxigenin-labelled 2·3 kb insert was conducted at 55 °C.

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 DNA sequencing kit (US Biochemical). The pUC/M13 forward and reverse sequencing primers and a set of synthesized oligonucleotide primers were used. The entire nucleotide sequence of the 2·3 kb insert of pSPAG1 was read on each strand and analysed using the computer program IntelliGenetics Suite. A hydropathy profile of the deduced amino acid sequence was drawn with the DNA Strider computer program (Marck, 1988) following the method of Hopp & Woods (1981).

Primer extension. For primer extension analysis, primer IA (5′-ATGAACTATGTGTTAAAGCG-3′, complementary to nt 219–238), primer IC (5′-ATGCTACACAAACAGCTTGA-3′, complementary to nt 378–397) and primer III (5′-AATTTTGCCGTTTTTAGTTTGTAA-3′, complementary to nt 1617–1640) were adopted. Primer extension experiments were conducted using the Primer extension system with avian myeloblastosis virus (AMV) reverse transcriptase (Promega). Total RNAs prepared from healthy, SPWB-phytoplasma-infected and PNWB-phytoplasma-infected periwinkle plants were used as templates. The reaction products were analysed in a 6 % denaturing polyacrylamide sequencing gel in parallel with sequencing products of the cloned insert of pSPAG1 using the oligonucleotide 5′-GAGCAGTTTTTTAGAGAAGGATTA-3′ (nt 128–151) as a primer.

PCR. Two oligonucleotide primers, primer A (5′-ATGTTATAATTGAAGGCGAT-3′, nt 1504–1523) and primer B (5′-GTCTGTTTTAGCTTGACTTAATAA-3′, complementary to nt 2073–2096), were used for PCR. Total DNAs prepared from healthy periwinkle, periwinkle plants affected by SPWB, PNWB, loofah witches' broom, paulownia witches' broom, elm yellows, aster yellows and *I. obscura* witches' broom, rice affected by rice yellow dwarf disease, and bamboo affected by bamboo little leaf disease were used as DNA templates. PCR was performed as described previously (Ko & Lin, 1994). Thirty PCR cycles were conducted in a DNA Thermal Cycler 480 (Perkin Elmer) with the following parameters: denaturation for 25 s at 94 °C, annealing for 25 s at 45 °C and extension for 25 s at 72 °C. A 15 µl aliquot of PCR products was analysed in a 1·2 % agarose gel.

RESULTS AND DISCUSSION

Western blot analysis

The 18·4 kDa antigenic proteins of SPWB phytoplasma and PNWB phytoplasma were detected by immunoblot analysis to be the major signal when probed with MA40 (Fig. 1, lanes 2 and 3, respectively), MA35, MA21, MA16 and MA6 (data not shown). No signal was observed when the protein preparation from healthy periwinkle was probed with these antibodies (Fig. 1, lane 1). The results indicated that SPWB phytoplasma was antigenically related to PNWB phytoplasma, consistent with our previous data (Shen & Lin, 1993). The minor polypeptide bands of 84·7 and 38·4 kDa might carry epitopes with conformation similar to that of the major 18·4 kDa antigenic protein recognized by the antibodies.

Cloning of the major antigenic protein gene

After screening recombinant phages of the SPWB phytoplasma genomic library, several positive clones designated SPAG1, SPAG2, SPAG4, SPAG9 and SPAG54 were obtained. The molecular mass of the antigenic protein expressed in *E. coli* by these clones was the same as that synthesized *in situ* when probed with the SPWB phytoplasma monoclonal antibodies with (Fig. 2) or without (data not shown) IPTG induction. No peptide band was detected in the immunoblotting profile of the lysate of *E. coli* SOLR cells transformed by pBluescript (Fig. 2, lane 3). These results suggest that the protein originated from SPWB phytoplasma and reacted specifically with SPWB phytoplasma monoclonal antibodies. Furthermore, we could conclude that: (i) the selected clones contained the complete antigenic protein gene; (ii) the expression of the antigenic protein gene was independent of the *E. coli* promoter; and (iii) the genetic

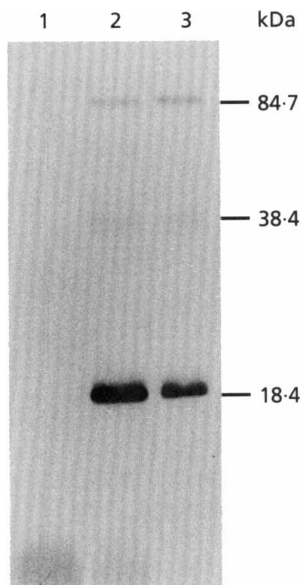

Fig. 1. Western blot analysis of SPWB phytoplasma and PNWB phytoplasma. Samples prepared from healthy and diseased periwinkle plants were analysed by 12% SDS-PAGE, electrophoretically transferred, and then probed with SPWB phytoplasma monoclonal antibody MA40. Lanes: 1, preparation from healthy periwinkle; 2, preparation from periwinkle affected by SPWB phytoplasma; 3, preparation from periwinkle affected by PNWB phytoplasma. Molecular masses (in kDa) of the immunostaining signals are shown on the right.

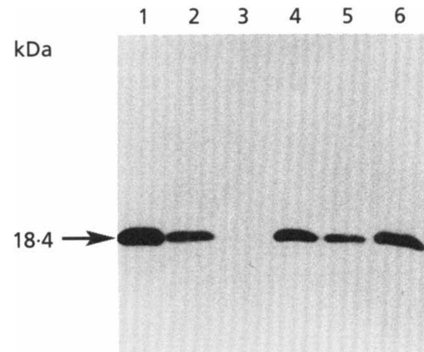

Fig. 2. Analysis of the expression of the cloned gene, encoding the 18·4 kDa antigenic protein of SPWB phytoplasma, in *E. coli* cells by Western blot analysis. Samples (from *E. coli* culture under inducing conditions) were analysed by 12% SDS-PAGE, electrophoretically transferred, and then probed with the pre-absorbed SPWB phytoplasma monoclonal antibody mixture. Lanes: 1, clone SPAG1; 2, clone SPAG2; 3, *E. coli* SOLR cells as a control; 4, clone SPAG4; 5, clone SPAG9; 6, clone SPAG54. The molecular mass (in kDa) of the immunostaining signal is shown on the left.

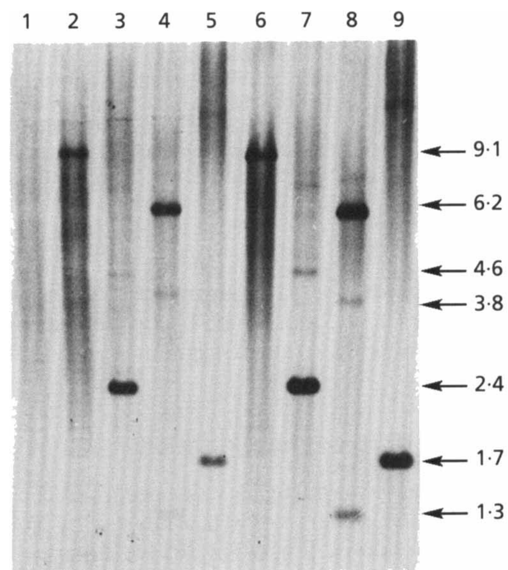

Fig. 3. Southern hybridization analysis of total DNA from healthy periwinkle digested with *Eco*RI (lane 1), and total DNA from diseased periwinkle affected by SPWB phytoplasma (lanes 2–5) and PNWB phytoplasma (lanes 6–9) digested with *Eco*RI (lanes 2 and 6), *Xba*I (lanes 3 and 7), *Hin*dIII (lanes 4 and 8) and *Pst*I (lanes 5 and 9). Hybridization was conducted at 68 °C using the digoxigenin-labelled 2·3 kb insert of pSPAG1 as a probe. Sizes (in kb) of the main hybridization signals are shown on the right.

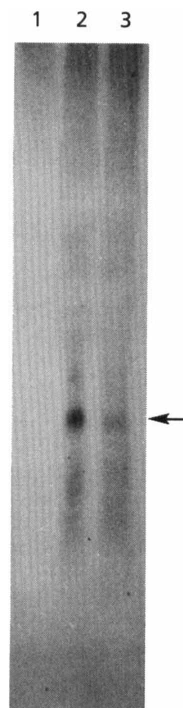

Fig. 4. Northern hybridization analysis of total RNA (50 µg per lane) from healthy (lane 1), SPWB-phytoplasma-infected (lane 2) and PNWB-phytoplasma-infected (lane 3) periwinkle plants. Hybridization was conducted at 55 °C using the digoxigenin-labelled 2·3 kb insert of pSPAG1 as a probe. The arrow at about 650 nt indicates the position of the transcripts for the antigenic protein gene.

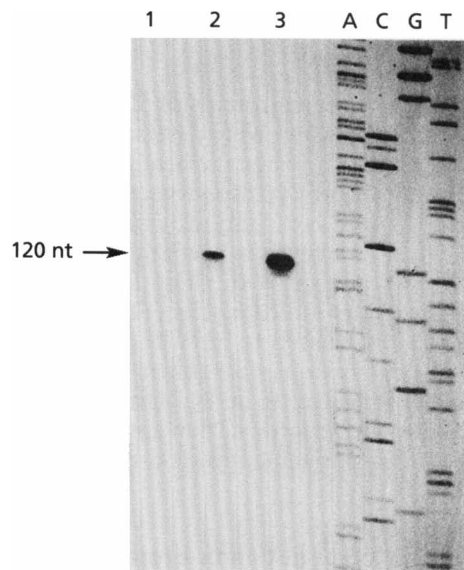

Fig. 5. Primer extension analysis of the transcription start site of the antigenic protein gene of SPWB phytoplasma. Primer III, 5′-end-labelled using [γ-^{32}P]ATP, was annealed with 40 µg total RNA from healthy periwinkle (lane 1), periwinkle infected with PNWB phytoplasma (lane 2) and periwinkle infected with SPWB phytoplasma (lane 3), then extended with AMV reverse transcriptase. Primer extension products were analysed in a 6% denaturing polyacrylamide gel in parallel with sequencing products of the cloned insert of pSPAG1 using oligonucleotide 5′-GAGCAGTTTTTTAGAGAAGGATTA-3′ (nt 128–151) as a primer. The size of the extended fragment (in nucleotides) is shown on the left.

codes are based on the same universal codon usage as genes of *E. coli*. Clone SPAG1 containing plasmid pSPAG1 with a 2·3 kb insert fragment was selected for further studies.

Southern and Northern hybridization

Similar hybridization patterns were obtained for SPWB and PNWB phytoplasmas when analysed by Southern (Fig. 3) and Northern (Fig. 4) hybridizations using the 2·3 kb insert of pSPAG1 as a probe. No hybridization signal was observed for other phytoplasmas in the Southern hybridization analysis under both low and high stringencies. This demonstrated that the antigenic protein genes of SPWB and PNWB phytoplasmas shared a high sequence homology and a transcript (about 650 nt) capable of encoding the 18·4 kDa product existed in both phytoplasmas but not in healthy plants (lanes 1 of Fig. 3 and Fig. 4, respectively).

Sequence analysis of the antigenic protein gene

The nucleotide sequence of the 2·3 kb insert DNA fragment of pSPAG1 has been assigned the GenBank accession number U15224. Based on the universal codon usage, putative ORFs, ORFs IA, IC and III, were identified. In primer extension analysis, a 120 nt cDNA product was extended only from primer III using total RNA templates prepared from PNWB-phytoplasma-infected and SPWB-phytoplasma-infected periwinkle plants (Fig. 5, lanes 2 and 3), but no product was obtained using template prepared from healthy periwinkle (Fig. 5, lane 1). It was concluded that the antigenic protein gene is located in ORF III and the transcription start site was 75 nt upstream of the initiation codon ATG of ORF III.

The coding region of the gene contains 519 nt (nt 1596–2114), starting from the ATG initiation codon and stopping at translation termination codon TAA. A putative Shine–Dalgarno sequence AGGAG (nt 1575–1579) (Shine & Dalgarno, 1974) and the consensus −10 sequence TATAAT (nt 1508–1513) recognized by RNA polymerase (Reznikoff *et al.*, 1985; Rosenberg & Court, 1979) were found, but not a −35 sequence. Inverted repeat sequences (32–53 nt downstream of the TAA stop codon, nt 2146–2167) followed by six T residues that could lead to a rho-independent transcription termination (Rosenberg & Court, 1979) were also identified. The transcript, therefore, should contain about 652 nt, a size in agreement with the result of Northern hybridization (Fig. 4).

The base composition of the gene is 45·1 mol% of A, 12·7 mol% of C, 14·6 mol% of G and 27·6 mol% of T. A low G+C content (27·3 mol%) of the gene, a preferential use of A- and T-rich codons, and the high

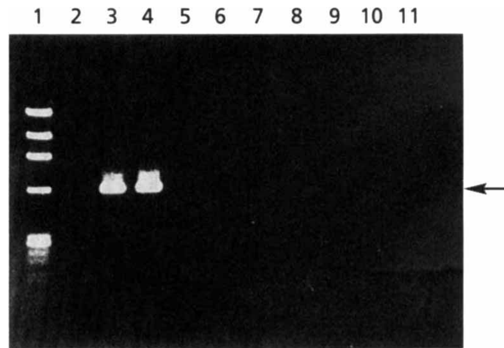

Fig. 6. Identification of the homologous antigenic protein gene in various phytoplasmas by PCR with the synthesized oligonucleotide primers A and B corresponding to the sequences at the 5′- and 3′-ends of ORF III, respectively. Lanes: 1, molecular mass standards; 2, template DNA prepared from healthy periwinkle; 3–11, template DNA prepared from periwinkle infected with SPWB phytoplasma, PNWB phytoplasma, loofah witches' broom, paulownia witches' broom, elm yellows, aster yellows and *I. obscura* witches' broom, rice infected with rice yellow dwarf disease, and bamboo infected with bamboo little leaf disease. The arrow on the right indicates the 590 bp PCR products.

frequency of the use of A or T residues at the 5′-end (1st base) (60·1 mol%) and 3′-end (3rd base) (86·1 mol%) of codons were observed, which are similar to the features of other phytoplasmas (Kirkpatrick *et al.*, 1987; Lim & Sears, 1991b; Sears *et al.*, 1989). Twenty-five AAA lysine codons appeared in the gene, which made it the most frequent codon. In the antigenic gene, two tryptophan residues are encoded by UGG. The result agrees with a previous study on the ribosomal protein genes for *Oenothera* phytoplasma (Lim & Sears, 1991b). In the class *Mollicutes*, mycoplasmas and spiroplasmas both utilize UGG and UGA triplets as tryptophan codons (Inamine *et al.*, 1990; Lim & Sears, 1992), but *Acholeplasma laidlawii* utilizes UGG only (Lim & Sears, 1992; Tanaka *et al.*, 1989). These results suggest that phytoplasmas might be evolutionarily closer to acholeplasmas than to mycoplasmas and spiroplasmas (Lim & Sears, 1992). However, further analysis of other genes or cloning of all tryptophanyl tRNA genes of phytoplasmas are required to support this hypothesis.

ORF III encodes a polypeptide of 172 amino acids with a molecular mass of 19·15 kDa, slightly greater than that determined by Western blot analysis (18·4 kDa), and a pI value of 9·78. Searches in the GenBank and EMBL databases for nucleotide sequences and in the PIR 39 and SWISS-PROT 28 databases for amino acid sequences indicated the uniqueness of the major antigenic protein of SPWB phytoplasma, with no acceptable resemblance to other known proteins.

Two distinct regions, a strongly hydrophobic N-terminus (amino acids 10–50) and a highly hydrophilic C-terminus (amino acids 50–172), were identified in the hydropathy profile of the deduced amino acid sequence. It is likely that the hydrophobic NH_2 moiety might be buried within the membrane and the hydrophilic COOH part exposed at the cell surface. Our previous studies using immunofluorescence staining and tissue-blotting staining of SPWB phytoplasma with the monoclonal antibodies for the major antigenic protein (Shen & Lin, 1993, 1994) also suggested that the antigenic protein might be located on the cell surface.

PCR

A specific PCR product (590 bp) was amplified using DNA templates prepared from SPWB-phytoplasma-infected and PNWB-phytoplasma-infected periwinkle plants (Fig. 6, lanes 3 and 4), but no PCR product was obtained using DNA templates prepared from healthy plants or from other phytoplasma-infected plants (Fig. 6, lanes 2 and 5–11).

Therefore, based on the results obtained from Southern hybridization, Northern hybridization, Western blotting and PCR analysis, we conclude that the cloned antigenic protein genes exist only in SPWB phytoplasma and PNWB phytoplasma, but not in other phytoplasmas tested. The results are in agreement with our previous studies on the phylogenetic relationships of phytoplasmas, indicating that SPWB and PNWB phytoplasmas were closely related based on the analysis of the sequences of the 16S–23S rDNA spacer and the RFLPs of 16S rDNA (Hsyu & Lin, 1996a, b).

Recently, several major antigenic proteins of various phytoplasmas with molecular mass ranging from 15·7 to 23 kDa were identified (Chang *et al.*, 1995; Clark *et al.*, 1989; Errampalli & Fletcher, 1993; Garnier *et al.*, 1991; Jiang *et al.*, 1988; Onuki *et al.*, 1992; Saeed *et al.*, 1992; Seddas *et al.*, 1993); however, the significance and the functions of these proteins are still unknown. How the major antigenic protein from SPWB phytoplasma compares to these proteins should be explored and answered. Furthermore, research to reveal the conformation and activity of the protein by expressing it in an appropriate host such as *E. coli* should also be conducted. The finding in this paper that the 18·4 kDa antigenic protein possesses a strongly hydrophobic N-terminus and a highly hydrophilic C-terminus suggests that it may be a transmembrane protein, hence shedding light on its function.

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