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Molecular characterisation of two plasmids from paulownia witches'-broom phytoplasma and detection of a plasmid-encoded protein in infected plants

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Abstract Two plasmids were cloned from paulownia witches'-broom phytoplasma-Nanyang strain (PaWBNy). Southern blotting using pPaWBNy-1ORF1 probe confirmed the existence of the two plasmids. The 4485 bp plasmid, designated pPaWBNy-1, had a nucleotide content of 24 mol% G+C and contained six putative open reading frames (ORFs). The 3837 bp plasmid, designated pPaWBNy-2, had a nucleotide content of 25.9 mol% G+C and contained five putative ORFs which showed similarity with ORFs in pPaWBNy-1. The two plasmids contained a series of tandem repeats and encoded a replication-associated protein (RepA) and a single-stranded DNA-binding protein (SSB), which were necessary for the replication of plasmids. Seven putative proteins encoded by two plasmids were predicted to contain one or more hydrophobic transmembrane domains, respectively, and presumably to be localised to the membrane. ORF4 from pPaWBNy-1 was partially cloned and the recombinant protein with His-tag expressed in *Escherichia coli*. The fusion protein was used for immunisation and the polyclonal antiserum to ORF4 protein detected the native expression of ORF4 protein in Western blot analysis from infected but not healthy plants.

Keywords 16S rRNA · Replication-associated protein · Tandem repeat · Transmembrane protein

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Introduction

Paulownia (*Paulownia* sp.) is one of the most economically important trees in China, and the disease of witches'-broom spreads throughout paulownia-cultured regions and causes devastating damage to paulownia growth and wood production (Tian and Raychaudhuri 1996). Paulownia witches'-broom (PaWB) phytoplasma, which is the causative agent of paulownia witches'-broom disease, is transmitted by leafhopper (*Cicadella viridius*) as well as two kinds of stink bugs (*Halyomorpha mista* and *H. picus*) (Doi et al. 1967; Shiozawa et al. 1979; Zheng et al. 1990). Phytoplasmas are intracellular parasites which belong to the class *Mollicutes*, a diverse group of gram-positive low G+C bacteria lacking a cell wall. Phytoplasmas (*Candidatus* Phytoplasma) transmitted by phloem-feeding insects cause diseases in hundreds of plants. Due to their inability to be cultured in vitro, they were poorly characterised until the application of molecular biological techniques. Subsequently, phyto-

plasmas achieved *Candidatus* status and were classified based on 16S rRNA gene sequences (IRPCM Phytoplasma Spiroplasma Working Team Phytoplasma taxonomy group 2004). PaWB phytoplasma belongs to the *Candidatus* Phytoplasma asteris group (16SrI-D), the largest of the phytoplasma groups (Lee et al. 1998, 2004; Li et al. 2005).

Plasmids associated with some spiroplasmas, mycoplasmas and acholeplasmas were first found in maize bushy stunt phytoplasma (Davis et al. 1988). Up to now, 21 plasmids from 10 phytoplasma strains have been sequenced and published, which indicates that plasmids of various phytoplasmas differ significantly in both number and size (Nakashima and Hayashi 1997; Nishigawa et al. 2003; Liefting et al. 2004, 2006; Bai et al. 2006; Tran-Nguyen and Gibb 2006). Genes *repA*, *dnaG* and *ssb* involved in self-replication and *cop* involved in copy-number control of plasmids have been identified by sequence similarity, and RepA protein was proven to be expressed *in planta* (Nishigawa et al. 2001, 2003). Remarkably, most proteins encoded by the four plasmids from AYWB were secreted or membrane proteins (Bai et al. 2006). It has been reported that membrane protein was very important for the interaction between mycoplasmas and their hosts (Ye et al. 1997). Similarly, plasmids presumably have played key roles in phytoplasma evolution, and have contributed to the diversity of phytoplasma species, through horizontal exchange of genes encoding virulence factors, niche adaptation factors, and factors conferring competitive advantages on the phytoplasma cell (Davis et al. 1988).

To characterise the plasmids in PaWB and to understand their functions, we cloned and sequenced two plasmids from PaWB strain Nanyang (PaWBNy). Southern blot using pPaWBNy-1ORF1 (*repA* gene) probe confirmed the two plasmids in PaWB. Seven proteins encoded by the two plasmids were predicted to be membrane proteins and a protein encoded by pPaWBNy-1ORF4 was detected in phytoplasma-infected plants by Western blot.

Materials and methods

Phytoplasma and phytoplasma-infected plants

PaWBNy strain was collected from Nanyang in Henan province, China. Paulownia branches infected with phytoplasmas were graft-transmitted to healthy plantlets and maintained *in vitro* on MS medium (Tian et al. 1999).

Extraction of total DNA and phytoplasmal DNA

Total DNA from healthy and infected paulownia plantlets was extracted using the CTAB method (Doyle and Doyle 1987). Phytoplasmas were enriched using differential speed centrifugation according to procedures described previously (Kirkpatrick et al. 1987). The resulting pellet was then used for extraction of phytoplasmal DNA using the CTAB method.

PCR amplification and sequencing of plasmids

Phytoplasmal DNA extracted from infected paulownia plantlets and DNA of healthy controls was used as templates for PCR amplification. Two primers, p1F1fwd and p1F1rev (Table 1) were designed according to the sequence of plasmids from onion yellows phytoplasma (OY) (accession no. AB076262) to amplify the F1 fragment. Purified PCR products were cloned into the pMD18-T vector (Takara, Japan) and sequenced. Outward primer pairs, p1F2fwd and p1F2rev (Table 1) were designed according to the sequenced F1 fragment and the second fragment (F2) was inversely amplified. From four sequenced pPaWBNy-1ORF1, one sequence with 77.38% identity to the other three was presumed to be a partial sequence of another plasmid in the PaWB phytoplasma. Therefore, two pairs of forward and outward primers (p2F1fwd- p2F1rev and p2F2fwd- p2F2rev) (Table 1) were designed in the sites of inconsistent nucleotides to obtain a complete sequence of the second plasmid. PCR was performed in 25 μl reactions containing 10–50 ng template DNA, 150 μM dNTPs, 0.4 μM of each primer and 1 U ExTaq DNA polymerase with the recommended PCR buffer containing $MgCl_2$ (Takara). Amplification involved an initial denaturation cycle at 95°C for 5 min, then 35 cycles of 95°C for 30 sec, 54°C for 30 sec and 72°C for 120 sec, followed by a final 10 min extension at 72°C.

Southern blot

One microgram of purified PCR products of ORF1 was used as the templates to prepare probes labelled

Table 1 Primers used in this study

Name	Primer sequences (5′–3′)[a]	Resulting products
p1F1fwd	TAAAGGAGTAGGTTGGA	F1 fragment of pPaWBNy-1
p1F1rev	CCTGTTTGAGAGTCAAATG	
p1F2fwd	GGATCCTAACCAAACCAAGAC	F2 fragment of pPaWBNy-1
p1F2rev	GTCGACTATTGACTCTCCAAC	
p2F1fwd	TGGTCTGAGAATAACGGTCATAAG	F1 fragment of pPaWBNy-2
p2F1rev	TGAATCGAAAGAATAGTCTGGTTTG	
p2F2fwd	CAA ACCAGACTATTCTTTCGATTCA	F2 fragment of pPaWBNy-2
p2F2rev	CTTATGACCGTTATTCTCAGACCA	
p1ORF1fwd	ATGAAAAAAACTAAATTTAAAATAA	ORF1 of pPaWBNy-1
p1ORF1rev	ATATAATTTATCAGGGACATA	
p1ORF4fwd	atGGATCCAAACAATCCCCACAATCGTTT	ORF4 excluding 5′ 99 nucleotides of pPaWBNy-1
p1ORF4rev	atGTCGACAAAATATCTTACTTTTTCAAG	

[a] Nucleotides underlined were restriction sites carrying protection nucleotides (lower-case letters).

with DIG-UTP using the Klenow fragment. Total DNA extracted from PaWBNy-infected paulownia was restricted by *Sca*I and *Eco*RI. About 3 μg of total DNA and restricted total DNA for Southern hybridisation were separated by 0.8% agarose gel, respectively. Electrophoresis conditions were at 2 V/cm for 6 h at room temperature in 1×TAE buffer. DNA from the gel was transferred to a nylon membrane (Hybond-N+, Amersham Bioscience) and then the membrane was dried at 120°C for 30 min. The blots were pre-hybridised for 3 h at 50°C in the recommended Dig Easy Hyb Granules (Roche) and hybridised to ORF1 probes in hybridisation solution for 12 h at 50°C. Post-hybridisation washes were performed twice with 2×SSC (0.3 M sodium chloride, 0.03 M sodium citrate), 0.1% sodium dodecyl sulphate (SDS) for 5 min at room temperature and twice with 0.1×SSC, 0.1% SDS at 68°C for 30 min. The detection of probes was carried out using a DIG detection kit according to the manufacturer's instructions (Roche Diagnostic, Germany).

Sequence alignment and protein prediction

The sequenced overlapping DNA fragments were assembled using the DNAMAN 6.0 software package. ORFs were predicted by the ORF finder of the NCBI server with the standard genetic code (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Searches on the GenBank database for homologous sequences were performed using the blastx programme (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Potential transmembrane domains were predicted using TMPred programmes (www.ch.embnet.org/software/TMPRED_form.html) by comparing the deduced amino acid sequence with characterised membrane proteins to identify potential transmembrane domains. To predict the subcellular localisation and the topological structure of proteins, the TargetP programme (http://www.cbs.dtu.dk/services/targetP/) and TopPred2 (http://bio web.pasteur.fr/seqanal/interfaces/toppred.html) were performed, respectively. Phylogenetic analyses were performed with the software Molecular Evolutionary Genetics Analysis, version 3.1 (MEGA 3.1).

Preparation of ploclonal antiserum specific to the ORF4 protein

With primers p1ORF4fwd and p1ORF4rev (Table 1), the partial pPaWBNy-1ORF4 (771 bp) was amplified excluding the 99 nucleotides at the 5′ end. The PCR product was digested with *Sal*I and *Bam*HI, and inserted into the pET-28a(+) vector. Fusion protein with 6-His tag was expressed in *Escherichia coli* Rossetta (DE3) (Novagen) after IPTG (isopropyl-2-D-thiogalactopyranoside) induction according to Sambrook and Russell (2001), and purified with a His-Bind resin column following the manufacturer's guidelines (Promega). The antiserum was prepared in German white rabbits by injection of 0.2 mg each time of purified protein emulsified with an equal volume of Freund's incomplete adjuvant, intramuscularly in the back and subcutaneously four times at 7-day intervals. The rabbits were bled two weeks after the last

injection. The antiserum was fractionated and 0.1% NaN_3 was added before being stored at −20°C.

Western blot analysis

Total proteins were extracted from healthy and PaWBNy-infected paulownia plantlets, respectively. About 0.3 g of plant tissue was ground in liquid nitrogen and suspended in 0.2 ml of protein cracking buffer (40 mmol l^{-1} Tris–HCl, pH 6.8, 5% SDS, 8 M Urea, 0.1 mM EDTA, 10 mM 2-mercaptoethanol and 5 mM PMSF). After centrifugation at 12,000 rpm for 5 min at 4°C, the supernatant was added by equal volume to 2×loading buffer, boiled for 5 min and then subjected to electrophoresis on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Another aliquot of plant tissue ground in liquid nitrogen was suspended in 0.2 ml of soluble protein extraction buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM 2-mercaptoethanol) (Sambrook and Russell 2001). After centrifugation at 12,000 rpm for 5 min at 4°C, the supernatant containing soluble proteins was added by equal volume to 2×loading buffer, and the pellet containing insoluble proteins was resuspended in 0.2 ml of protein cracking buffer. The supernatant and pellet were processed with the same method as described above for SDS-PAGE. For Western blot analysis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes with a Trans-Blot apparatus (Bio-Rad). The membrane was blocked with 50 g l^{-1} non-fat milk powder in TBS buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) for 1 h at room temperature, and incubated for 1 h at room temperature with anti-ORF4 protein serum (1:1000 dilution) in TBST buffer (TBS with 0.5 ml l^{-1} Tween-20) on a shaker. The membrane was washed three times in TBST buffer (10 min) and incubated with protein A-alkaline phosphatase conjugate (Sigma) (1:2500) for 1 h. The membrane was washed again and the bands of interest were visualised by reaction with the substrate using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium tablets (BCIP/NBT) (Sigma).

Results

Sequence analysis of two plasmids from PaWBNy phytoplasma

Two fragments, 1560 bp and 3025 bp, were cloned by PCR and inverse PCR using PaWBNy phytoplasmal DNA as templates, respectively. The two overlapped sequences were assembled as a circular DNA molecule and designated as pPaWBNy-1 which was 4485 bp in length with 24.8 mol% G+C content. The ORF search indicated that pPaWBNy-1 contained six predicted ORFs encoding products larger than 100 amino acids, all of which were orientated in the same direction (Fig. 1a, Table 2). The first nucleotide of ORF1 was designated as position 1 in the plasmid. Two unique *Sca*I and *Xba*I restriction sites were found on the sequence of pPaWBNy-1 and two sets of tandem repeats were identified. One was 20 bp (TTTCAATGTGAGT TATTTTT) which repeated twice between ORF1 and ORF2 and another was 13 bp (TATTTTTTAGGAT) which repeated twice within ORF2.

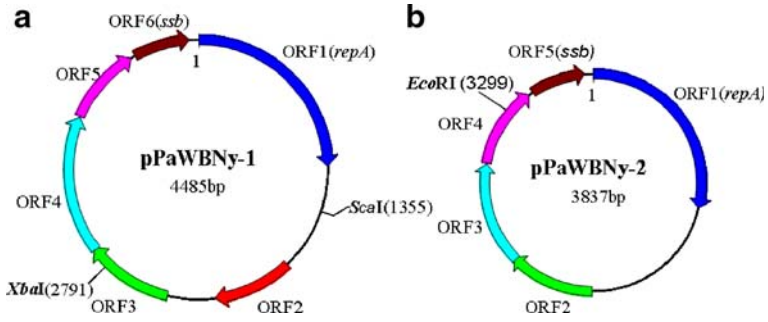

Fig. 1 Maps of the two plasmids of pPaWBNy-1(a) and pPaWBNy-2(b) from PaWBNy. The first nucleotide of ORF1 (*repA*) is designated as position 1.ORFs are presented as *block arrows*, and *arrows* of paralogous ORFs on two plasmids have the same colour. Unique restriction sites (*Sca*I, *Xba*I, *Eco*RI) are indicated. *repA* encodes plasmid replication-associated protein; *ssb* encodes the single-stranded DNA-binding protein

Table 2 Predicted ORFs in pPaWBNy-1

Predicted ORFs	Position (nucleotide)	Amino acid number	Homologous protein (gene designation) (GenBank accession no.)	Phytoplasma showing best match	%Identity	*E*-value
ORF1	1–1104	367	RepA (YP_456877)	pAYWB-III of AYWB	61	3e-105
ORF2	1704–2150	148	HP (YP_456877, AYWB_404)	Chromosomal gene of AYWB	89	1e-25
ORF3	2414–2905	163	HP OYPNIM_03 (YP_214981)	Plasmid of OYNIM	73	2e-26
ORF4	2892–3662	256	Conserved HP AYWB_148 (YP_456344)	Chromosomal gene of AYWB	45	1e-15
ORF5	3665–4099	144	HP PAM458 (NP_950710)	Chromosomal gene of OY-M	85	2e-37
ORF6	4124–4438	104	SSB (BAA36390)	Plasmid of OY-W	94	2e-48

Abbreviations: *HP* hypothetical protein, *AYWB* Aster yellows witches' broom, *OYNIM* onion yellows non-insect-transmissible strain, *OY-M* onion yellows mild strain, *OY-W* onion yellows wild-type strain.

Similarly, a circular 3837 bp plasmid was assembled by two overlapping fragments of 454 bp and 3432 bp and designated as pPaWBNy-2 which had 25.9 mol% G+C content, and contained five ORFs encoding products larger than 100 amino acids, all of which were orientated in the same direction (Fig. 1b, Table 3). A unique *Eco*RI restriction site was found on the sequence of pPaWBNy-2 and three sets of tandem repeats were identified. The first tandem repeat was 20 bp (TCAATGTGTGTACTTTTTTA) which repeated twice between ORF1 and ORF2, the second one was 12 bp (CAAACTGAAAAA) which repeated twice in ORF4, and the third one was 24 bp (AATTATCACAAGAAGAAATAGATA) which repeated three times in ORF3.

To reveal the relationship between the plasmids pPaWBNy-1 and -2, complete nucleotide sequence alignment was performed. The results showed that pPaWBNy-1 had 70.74% identity to pPaWBNy-2. Gene sequence alignment showed that the ORFs, except for ORF2 on pPaWBNy-1, had their counterparts on pPaWBNy-2 (Fig. 1a,b; Table 3). Both sequences have been deposited into GenBank with accession nos. EF426472 and EF426473 for pPaWBNy-1 and pPaWBNy-2, respectively.

Detection of plasmids from PaWB phytoplasma

Southern blot analysis using the pPaWBNy-1ORF1 probe was performed to confirm that plasmids existed in the phytoplasma cell as circle DNA molecules, and that the full-length version of the plasmids had been cloned. Four bands were detected in total DNA extracted from PaWBNy-infected paulownia plantlets (Fig. 2, lane 1), whereas no band was observed with total DNAs extracted from healthy plantlets (Fig. 2, lane 3). Two bands of approximately 4.8 kb and 3.9 kb in restricted total DNA by *Sca*I/*Eco*RI extracted from PaWBNy-infected paulownia were detected and presumed to be linear forms of the two cloned plasmids (Fig. 2, lane 2).

Characterisation of proteins encoded by pPaWBNy-1 and pPaWBNy-2

To characterise proteins encoded by the two plasmids of PaWBNy, the BLAST programme was performed to search for homologous proteins in GenBank. The

Table 3 Identity of ORFs between pPaWBNy-1 and pPaWBNy-2

Predicted ORFs of pPaWBNy-2	Position (nucleotide)	Amino acid number	Alignment to ORFs of pPaWBNy-1	%Identity (nucleotide)	%Identity (amino acid)
ORF1	1–1104	367	ORF1	77.45	67.57
ORF2	1926–2417	163	ORF3	93.70	91.41
ORF3	2404–2988	194	ORF4	64.10	41.80
ORF4	2991–3452	153	ORF5	91.03	86.11
ORF5	3476–3790	104	ORF6	93.33	92.31

results (Table 2) showed that the deduced amino acids of the protein encoded by pPaWBNy-1ORF1 shared 61% homology with RepA of the pAYWB-III from aster yellows witches'-broom (AYWB). The motif

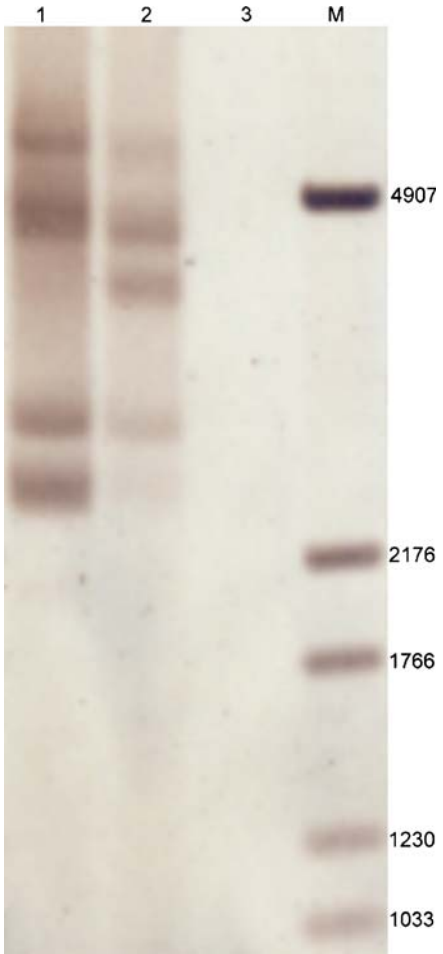

Fig. 2 Southern blot analysis of plasmids from PaWBNy with the pPaWBNy-1ORF1 probe. Lane *1*, Total DNA (3 μg) extracted from PaWBNy-infected plantlets was loaded. Lane *2*, Restricted total DNA by *Sca* I/*Eco*RI extracted from PaWBNy-infected plantlets; Lane *3*, Total DNA (3 μg) extracted from healthy paulownia plantlets; M, pBR328 *Eco*RI/*Hin*dIII marker

HSHVLF in the ORF1-encoded protein was identified to be a conserved sequence consisting of HisHydr-HisHydrHydrHydr (Hydr—bulky hydrophobic residue), which was involved in the initiation and termination of rolling circle DNA replication. The P-loop (GASRLGKT) as the nucleotide-binding domain was also identified in ORF1. The deduced amino acid sequence of the putative protein encoded by ORF6 was 94% identical to the single-stranded DNA-binding protein (SSB) of pOYW from the onion yellows wild strain (OY-W). The four putative proteins encoded by ORF2–5 were homologous to the hypothetical proteins encoded by OY-W or AYWB phytoplasmas.

Transmembrane domain analysis by the TMPred programme revealed that putative proteins deduced from pPaWBNy-1ORF1 and ORF6 had no significantly predicted hydrophobic transmembrane regions. Three predicted hydrophobic regions corresponding to transmembrane helices were identified for pPaWBNy-1ORF2. Two predicted hydrophobic segments were identified within the pPaWBNy-1 ORF3 protein. One predicted hydrophobic segment was identified at the N-terminus of the pPaWBNy-1ORF4 protein and two predicted hydrophobic segments were identified at the N- and C-terminus of the pPaWBNy-1 ORF5 protein. The subcellular localisation of pPaWBNy-1 encoding proteins determined by the TargetP programme demonstrated that pPaWBNy-1ORF2–5 proteins, which contained signal peptides to the membrane, were further confirmed as being likely localised at the membrane, while pPaWBNy-1 ORF1 and ORF6 contained no signal peptide for specific localisation. Therefore, four proteins except for ORF1 (RepA) and ORF6 (SSB) encoded on pPaWBNy-1 were predicted to be membrane proteins. Similarly, three proteins encoded by pPaWBNy-2 ORF2-4 were predicted to be localised at the membrane (data not shown).

Comparative analysis of phytoplasmal plasmids

Phylogenetic analysis based on complete sequences of plasmids from five phytoplasma groups showed that there were five subclades which were distinguished in general among different phytoplasma groups (Fig. 3). pPaWBNy-1 and pPaWBNy-2 from PaWBNy were more closely related to pAYWB-I and pAYWB-III from AYWB (16SrI-B) than those from other phytoplasmas. However, two other plasmids from AYWB were classified into another subclade along with pJHW from Japanese honeywort witches'-broom and pCPa from *Candidatus* P. australiense (Table 4).

pPaWBNy-1ORF4 was expressed in infected paulownia

To confirm whether the ORF4 protein was expressed in PaWBNy-infected paulownia, recombinant pPaWBNy-1 ORF4 protein with the N-terminal amino acids 1 to 33 deletion was expressed in *E. coli* and then purified for raising antiserum specific to the ORF4 protein. Western blot using total proteins from PaWBNy-infected paulownia showed that a protein of about 33 kD was detected in paulownia infected with PaWBNy, but not in healthy paulownia, which indicated that pPaWBNy-1ORF4 was indeed expressed in PaWB-infected paulownia (Fig. 4). Moreover, the ORF4 protein was detected in insoluble proteins but not in soluble proteins.

Discussion

To our knowledge, this is the first report that two plasmids in PaWB phytoplasma were identified and sequenced. The molecular characterisation of the plasmids was performed and their encoded proteins predicted, and the expression of a protein encoded by pPaWBNy-1ORF4 was detected in PaWB-infected paulownia. The results provide clues for us to explore the function of phytoplasmal plasmids.

Seven of the eleven ORFs in the two plasmids were predicted to be putatively secreted or membrane-localised proteins by the TopPred programme. A large proportion of membrane proteins identified in genomes and plasmids of phytoplasmas might contribute to phytoplasmal survival and host interactions (Berg et al. 1999; Bai et al. 2006; Mounsey et al.

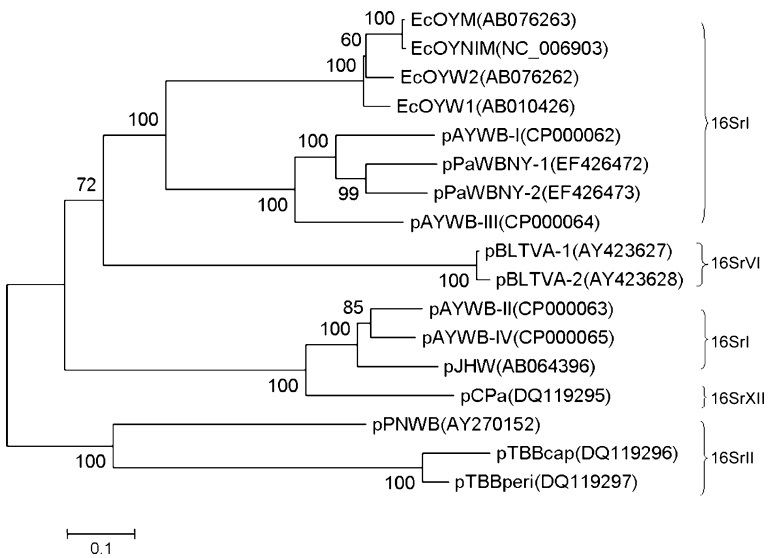

Fig. 3 Bootstrapped phylogenetic analysis for phytoplasmal plasmids based on the full-length sequences of all given plasmids. The first nucleotide of *rep* on all plasmids was used as a start site during phylogenetic analysis. Abbreviations: EcOYW1 and EcOYW2, plasmids from onion yellows wild strain; EcOYM, from onion yellows mild strain; EcOYNIM, from onion yellows non-insect-transmissible strain; pAYWB-I, -II, -III and -IV, plasmids from aster yellows witches'-broom phytoplasma; pBLTVA-1 and -2, from beet leafhopper-transmitted virescence agent-1; pJHW, from Japanese honeywort witches'-broom; pCPa, from *Candidatus* P. australiense; pPNWB, from peanut witches'-broom phytoplasma; pTBBperi and pTBBcap, from tomato big bud;. GenBank accession numbers are given in parentheses

Table 4 Number and size of plasmids in different phytoplasmas

Phytoplasma strain	Disease caused and/or natural host	Classification	Number of plasmids	Size of plasmids (bp)[a]	Reference
JHW	Japanese honewort witches' broom	16SrI	1	4278	Lee et al. 2004
AYWB	Aster yellows witches'-broom	16SrI-A	4	5104, 4316, 4009, 3827	Bai et al. 2006
OYW	Onion yellows wild	16SrI-B	3	7005, 5056, 3933	Nishigawa et al. 2003
OYM	Onion yellows mild	16SrI-B	2	5025, 3932	
OYNIM	Onion yellows Non-insect-transmissible	16SrI-B	2	5045, 3062	
PNWB	Peanut witches'-broom	16SrII-A	1	4226	
TBB	Tomato big bud	16SrII-E	2	4092, 3319	Tran-Nguyen and Gibb 2006
Candidatus P. australiense	*Phormium* yellow leaf	16SrII-E	1	3607	Liefting et al. 2006
Candidatus P. australiense	Strawberry lethal yellows	16SrII-E	1	3635	
BLTVA	Beet Leafhopper-transmitted virescence agent	16SrVI-A	2	10785, 2587	Liefting et al. 2004
SCWL	Sugarcane white leaf	16SrXI-B	1	2645	Nakashima and Hayashi 1997
Candidatus P. australiense	*Gomphocarpus physocarpus*	16SrXII	1	3773	Liefting et al. 2006
PaWB	Paulownia witches'-broom	16SrI-D	>2	4485, 3837	

[a] See legend of Fig. 3 for GenBank accession numbers

2006; Suzuki et al. 2006). To further characterise plasmid proteins, we expressed pPaWBNy-1 ORF4 protein with the deletion of N-terminal amino acids 1 to 33 in *E. coli* and prepared the specific antiserum. The TopPred programme predicted that the N-terminal amino acids 11 to 31 of pPaWBNy-1ORF4 protein traversed through the membrane, and the C-terminal region was localised outside the membrane. A putative cleavage site between positions 30 and 31 of the N-terminal region was identified; the probability of cleavage was 0.682. If the signal peptide was cleaved after being translocated into the membrane, the mature peptide encoded by ORF4 would be secreted into the host plant cell to interact with proteins from the host. If not, its N-terminus would be anchored to the membrane. The Western blot result indicated that the high-level pPaWBNy-1 ORF4 protein was expressed in PaWBNy-infected tissue as an insoluble protein. Further experiments are needed to determine whether the ORF4 protein was localised to phytoplasma membrane, and to identify interacting proteins of the host plant in order to elucidate its biological functions.

Fig. 4 Expression of pPaWBNy-1ORF4 protein in a phytoplasma-infected plant. Lane *1*, Total proteins from Healthy Paulownia; lane *2*, Total proteins from phytoplasma-infected Paulownia; lane *3*, soluble proteins; lane *4*, insoluble proteins

Comparative analysis of all sequenced plasmids from various phytoplasmas indicated the diversity of phytoplasmal plasmids (Fig. 3). Although there existed variation between plasmids from different phytoplasmas, the phylogenetic relationship of plasmids was consistent with the classification based on the 16S rDNA sequence of phytoplasmas. The exceptions of pAYWB-II pAYWB-IV and pJHW suggested that plasmids in phytoplasmas presumably have different origins. A number of repeat nucleotide sequences and tandem repeats were identified in pPaWBNys as well as in pBLTVAs, and gene *repA* occurring twice in pBLTVA-1 was identified in all sequenced plasmids from phytoplasmas (Liefting et al. 2004). There is evidence that the diversity of plasmids partially results from the intermolecular recombination occurring between plasmids in phytoplasmas (Nishigawa et al. 2002). The remarkable variation of plasmids in number and size found in different phytoplasma groups or strains might be related to pathogenicity, vector transmission, or host adaptation, according to the roles of plasmids from

other bacteria (Schneider et al. 1992; Chopra and Roberts 2001; Oshima et al. 2001; Tian et al. 2001; Vivian et al. 2001; Novichkov et al. 2004; Christensen et al. 2005; Wegrzyn 2005).

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