Available online at www.sciencedirect.com
ScienceDirect
Plasmid 56 (2006) 153–166
PLASMID
www.elsevier.com/locate/yplas

# Extrachromosomal DNA isolated from tomato big bud and *Candidatus* Phytoplasma australiense phytoplasma strains

L.T.T. Tran-Nguyen *, K.S. Gibb

*Charles Darwin University, Darwin, NT 0909, Australia*

Received 2 March 2006, revised 8 May 2006
Available online 1 August 2006
Communicated by Saleem Khan

**Abstract**

The nucleotide sequences of two extrachromosomal elements from tomato big bud (TBB) and one extrachromosomal element from *Candidatus* Phytoplasma australiense (*Ca.* P. australiense) phytoplasmas were determined. Both TBB plasmids (3319 and 4092 bp) contained an open reading frame (~570 bp) with homology to the rolling circle replication initiator protein (Rep). This gene was shorter than the *rep* genes identified from other phytoplasma plasmids, geminiviruses and bacterial plasmids. Both TBB extrachromosomal DNAs (eDNAs) encoded a putative DNA primase (*dnaG*) gene, a chromosomal gene required for DNA replication and which contains the conserved topoisomerase/primase domain. We speculate that the replication mechanism for the TBB phytoplasma eDNA involves the *dnaG* gene instead of the *rep* gene. The *Ca.* P. australiense eDNA (3773 bp) was shown to be circular and contained four open reading frames. The *rep* gene was encoded on ORF 1 and had homology to both plasmid (pLS1) and geminivirus-like domains.


*Keywords:* Phytoplasma; Extrachromosomal DNA; Tomato big bud; *Candidatus* Phytoplasma australiense; Rep and DnaG proteins

## 1. Introduction

Extrachromosomal DNA (eDNA) elements play important roles in the pathogenicity, host specificity and virulence of many plant pathogenic bacteria (Panopoulos and Peet, 1985; Vivian et al., 2001). They are mobile genetic elements that can replicate autonomously. Plasmids as a form of eDNA can incorporate genetic material from their host by recombination or transposition, and transfer it to a new host either by conjugation, mobilisation, transformation, or transduction (del Solar et al., 1996). A large majority of small, multicopy plasmids of Gram-positive bacteria replicate using the rolling circle replication (RCR) mechanism (Khan, 2000). This mechanism involves the generation of a site-specific nick by the plasmid-encoded replication initiator protein (Rep) that has a nucleotydil-transferase activity. The nascent strand is extended at the 3′ hydroxyl end by a DNA polymerase (Khan, 1997). RCR mechanism is also used

* Corresponding author. Fax: +61 8 8946 6847.
*E-mail address:* lucy.tran-nguyen@cdu.edu.au (L.T.T. Tran-Nguyen).

0147-619X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.plasmid.2006.05.009

by families of prokaryotic and eukaryotic single stranded (ss) DNA viruses such as geminiviruses (Stenger et al., 1991).

Phytoplasmas associated with numerous plant diseases in northern Australia have been extensively studied and documented (Collmer, 1998; Davis et al., 1997; Davis et al., 2003; De La Rue et al., 1999; Gibb et al., 1995; Padovan and Gibb, 2001; Schneider et al., 1999b; Tran-Nguyen et al., 2000). Two phytoplasmas, tomato big bud (TBB) and *Candidatus* Phytoplasma australiense (*Ca.* P. australiense) are widespread in Australia. The TBB phytoplasma has a wide host range including papaya, sesame, eggplant, and passionfruit (Davis et al., 1997; Padovan and Gibb, 2001; Schneider et al., 1999b). The *Ca.* P. australiense phytoplasma has a more limited host range of crop and non-crop host species (Davis et al., 1997; Davis et al., 2003; Padovan et al., 2000; Schneider et al., 1999b; Streten et al., 2005).

To date, eDNAs have been isolated from phytoplasmas associated with maize bushy stunt (Davis et al., 1988); phyllody of *Oenothera hookeri* (Sears et al., 1989), western aster yellows (AY) (Kuske and Kirkpatrick, 1990), chrysanthemum yellows, tomato big bud, periwinkle little leaf, American eastern AY (Bertaccini et al., 1990), witches' broom disease of pigeon pea (Harrison et al., 1991), walnut witches broom (Chen et al., 1992), numerous phytoplasmas from herbaceous plants, and woody dicots (Schneider et al., 1992); sugarcane white leaf (Nakashima and Hayashi, 1995); peanut witches' broom, and aster yellows (unpublished GenBank Entry Accession No. AY270152 and NC_003353); onion yellows (OY) (Kuboyama et al., 1998; Nishigawa et al., 2003; Nishigawa et al., 2002a; Nishigawa et al., 2002b; Nishigawa et al., 2001; Oshima et al., 2001a), beet leafhopper-transmitted virescence agent (BLTVA) (Liefting et al., 2004; Shaw, 1991); aster yellows witches' broom (AYWB) (Bai et al., 2006); TBB (Australian strain), and sweet potato little leaf strain V4 (Schneider et al., 1999a).

Although phytoplasma eDNAs have been detected, nothing is known about the role of these eDNAs in phytoplasma pathogenicity, if indeed they do have such a role. Only the seven phytoplasma eDNAs from OY, two from BLTVA and four from AYWB have been fully sequenced and published. This study aimed to expand our knowledge of eDNAs from Australian phytoplasmas.

## 2. Materials and methods

### 2.1. Sources of plants and DNA

Tomato big bud and *Ca.* P. australiense phytoplasma DNA were isolated from periwinkle (*Catharanthus roseus*) plants maintained at Charles Darwin University (CDU). The TBB phytoplasma strain was originally transmitted from tomato (*Lycopersicon esculentum*) collected near Adelaide, South Australia. The *Ca.* P. australiense phytoplasma strain was originally transmitted from *Gomphocarpus physocarpus*, Nambour, QLD. These TBB and *Ca.* P. australiense phytoplasma strains were transmitted to periwinkle by grafting and maintained in periwinkles by serial grafting. Field samples of the TBB phytoplasma were obtained from capsicum samples with little leaf (Childers, South QLD) and a chicory plant with little leaf and phyllody (Bundaberg, central QLD). A field sample of the *Ca.* P. australiense phytoplasma was obtained from strawberry (*Fragaria x ananassa*) with green petal symptoms (Deception Bay, southeast QLD). Other sources of TBB phytoplasma DNA were from a DNA archive maintained at CDU. Random clone TBB88 containing TBB from periwinkle was obtained as described in Schneider et al. (1999a).

### 2.2. DNA extractions

To identify the best method for isolating phytoplasma eDNA, several extraction methods were used. The methods included a small-scale extraction from fresh material (Doyle and Doyle, 1990) and total DNA extraction using the Dellaporta method (Dellaporta et al., 1983). Genomic DNA was extracted from 100 mg of lyophilised plant material using the DNeasy Kit (Qiagen, Australia) following the manufacturer's instructions. DNA prepared for Southern hybridisation was extracted using 2 g of lyophilised plant material according to Doyle and Doyle (1990) and resuspended in 200 μL TE buffer pH 8.0. To separate chromosomal host and phytoplasma DNA in a large scale total DNA extraction, 30 g of lyophilised strawberry plant material was extracted as described by (Porebski et al., 1997), resuspended in 5 mL of TE buffer and partially purified using a cesium chloride (CsCl) bisbenzimide gradient (Kollar and Seemüller, 1989; Kollar et al., 1990). Briefly, the DNA suspension was centrifuged for 70 h at 33,000 rpm. Hoechst stain preferentially binds the AT-rich phytoplasma DNA, thereby lowering the DNA buoyant density, and facilitating separation of phytoplasma and plant host DNA. The phytoplasma DNA band was located by comparing with a healthy plant DNA control. The phytoplasma band was extracted using a small Teflon tube attached to a micropipette tip, stained with Hoechst solution again and centrifuged at 33,000 rpm for a further 70 h, in total three spins. The final partially purified DNA solution was destained using isopropanol and precipitated

with 3 M sodium acetate pH 4.6 and 100% ethanol. DNA pellets were resuspended in 100 μL SDW. Two to five micrograms of partially purified CsCl DNA were loaded on a 1% agarose gel for separation at 80 V for 1 h. Pulsed field gel electrophoresis (PFGE) was performed by the contour-clamped homogeneous-electric field (CHEF) technique using the CHEF-DR III system (Bio-Rad) with 1% Seakem agarose (FMC). PFGE was used to provide an accurate determination of eDNA band sizes. Electrophoresis conditions were 1.5–5 s at 6 V/cm for 15 h at 14 °C in 0.5× TBE buffer.

Plasmid DNA from random clone TBB88 (pTBB88) was prepared from 4 mL LB media supplemented with 50 μg/mL ampicillin using the Miniprep Kit (Qiagen, Australia) following the manufacturer's instructions. DNA was eluted from the columns using 50 μL SDW. All DNA samples were stored at −20 °C until required.

### 2.3. PCR amplification of rep and dnaG genes

Preliminary sequence analysis of pTBB88 (Schneider et al., 1999a) contained a segment of the *rep* gene. PCR primers TBBRepF (5′-CAT GAT TGC AAA GAT GCT G-3′) and TBBRepR (5′-GTG GAC GAT CAA ATT GGC-3′) were designed to amplify a 650 bp fragment of the TBB extrachromosomal phytoplasma *rep* gene. PCR assays were performed in 50 μL reactions using 0.1 mM dNTP, 0.25 μM of each primer, 1× polymerase buffer, 1.25 U *Taq* DNA polymerase (Geneworks, Australia) and 50–100 ng of DNA. The optimal PCR conditions used were 30 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 1.5 min.

To determine whether phytoplasma eDNA was circular, eDNA was subjected to inverse PCR using primers based on the *rep* gene. For TBB eDNA, the inverse primers were TBBRepinvr144 (5′-GTT TTG TCA TGG AAT TAG TG-3′) and TBBRepF322 (5′-AGA TGG AAT TTC GAA GCA A-3′). PCR assays were 25 μL reactions using 0.2 mM dNTP, 1 μM of each primer, 1× PCR buffer and 1× Advantage Polymerase Mix (BD Biosciences, Australia). PCR conditions were 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 3 min. For *Ca.* P. australiense eDNA, the inverse primers were PaRepinvr40 (5′-GAG ATT TGT TTT GCT AAA GT-3′) and PaRepF185 (5′-CAG CTT GAT CAG TGA A-3′). PCR conditions were 25 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension for 3 min at 68 °C.

PCR primers fPrim2 (5′-CAG CTC TCG ATT ATT TGT T-3′) and rPrim2 (5′-CCA TAT TTG CGT AAT AAT TC-3′) designed from the alignment of phytoplasma *dnaG* sequences from chromosomal peanut witches' broom (PNWB) (GenBank Accession No. AY270153) and phytoplasma eDNAs, were used to amplify a 650 bp segment of the *dnaG* gene. PCR conditions used were 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1.5 min.

### 2.4. eDNA sequence analysis

Samples used for sequencing were plasmid DNA of clone TBB88, inverse PCR products of TBBperi (periwinkle), TBBcap (capsicum), and CPa (strawberry), hereafter referred to as pTBBperi, pTBBcap, and pCPa respectively, The nucleotide sequences of the pTBB88 insert, pTBBperi, pTBBcap, and pCPa eDNAs were determined on both strands by primer walking using the ABI Big Dye Terminator Kit (Australian Genome Research Facility, (AGRF), Brisbane, QLD) and analysed at the AGRF. Primers were designed using the Primer3 program (http://www.genome.wi.mit.edu/genome_software.other/prime3.html) based on melting temperature and their location on the sequence. Sequencing primers were synthesised by Sigma Customs Oligos (Sigma Genosys, Australia). Sequences were assembled using AssemblyLIGN (Eastman Kodak, USA). Open reading frames were predicted using MacVector (Eastman Kodak), ORF finder (NCBI, http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and FlipORF (BioManager, Entigen Corporation, http://www.entigen.com) using the bacterial genetic code with the minimum setting of 80 amino acids for ORFs. Homologous sequences were identified from the GenBank database using the NCBI BlastX network (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). Tandem repeats finder server (http://tandem.bu.edu/trf/trf.html) was used to identify repeated regions in the sequences (Benson, 1999). Conserved domains were identified using software available from http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (Marchler-Bauer and Bryant, 2004). Multiple sequence alignments of related amino acids were performed using ClustalW (accurate) (Thompson et al., 1994) and displayed using PrettyBox (GCG package 1998). Phylogeny based upon *dnaG* was estimated using the general bootstrapping tool 'Seqboot' in which 100 replicate data sets were generated. The bootstrapped set of phylogenetic trees was generated by parsimony using the program 'DNAPars'. A consensus tree was generated using the majority-rule consensus tree method (Felsenstein, 1989) and viewed using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). MacVector (Eastman Kodak) was used to construct plasmid diagrams.

### 2.5. Southern blot analysis

Two to five micrograms of DNA was loaded onto a 1% agarose gel and transferred to a nylon membrane as described by (Sambrook and Russell, 2001). Aliquots from the same samples were also loaded onto a 1% Seakem agarose gel (FMC) and subjected to pulsed-field gel electrophoresis using the contour-clamped homogeneous-electric field (CHEF) technique using the CHEF-DR III system (Bio-Rad). Electrophoresis conditions were 1.5–5 s at 6 V/cm for

15 h at 14 °C in 0.5× TBE buffer. DNA from the gel was transferred to a nylon membrane and southern hybridisations were performed as described by Davis et al., 1997, using digoxigenin labelled DNA probes. Post hybridisation washes were performed twice with 2× SSC, 0.1% sodium dodecylsulfate (SDS) for 5 min at room temperature and twice with 0.1× SSC, 0.1% SDS at 50–55 °C for 30 min. The blots were generally hybridised and washed at 50 °C first, exposed to film and then re-evaluated after the wash temperature was increased to 55 °C. Digoxigenin labelled probes of clone pTBB88 and *rep* gene were made using the DIG PCR labelling kit (Roche Diagnostic, Australia) according to the manufacturer's instructions. PCR condition used for labelling the *rep* genes were similar to those described above. Universal forward (5′-GTA AAA CGA CGG CCA GT-3′) and reverse (5′-AAC AGC TAT GAC CAT G-3′) M13 primers were used to label the pTBB88 insert. PCR conditions used were 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The TBB phytoplasma 16S rRNA gene was labelled using fP1 (Deng and Hiruki, 1991) and rP7 (Smart et al., 1996), PCR conditions were 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min.

### 2.6. Two-dimensional gel electrophoresis

To identify different structural conformations of *Ca.* P. australiense eDNA, two-dimensional gel electrophoresis was conducted as described by Hintermann et al. (1981) with slight modifications. One and a half to two micrograms of CsCl purified *Ca.* P. australiense DNA was loaded onto a 0.85% agarose gel and subjected to electrophoresis at 60 V for 6 h. The gel was stained in 50 μg/mL ethidium bromide (EtBr) for one hour and destained for 30 mins. The lane containing phytoplasma bands was excised, exposed to UV light for 2 min and inserted into a new 0.85% agarose gel at 90° to the original orientation. Gel electrophoresis was conducted at 60 V for 6 h, followed by staining and destaining as described above.

## 3. Results

### 3.1. eDNA detection

Extrachromosomal DNA, if present, was obtained for each of the DNA extraction methods tested. Cesium chloride buoyant density centrifugation was found to be optimal for obtaining high titres of eDNA for *Ca.* P. australiense, since eDNA bands could be seen on normal 1% agarose gels. While TBB eDNA could not be observed on normal 1% agarose gels, it was detected using PCR amplification; southern hybridisation using eDNA probes or by cloning and screening CsCl purified DNA.

### 3.2. Candidatus Phytoplasma australiense eDNA

#### 3.2.1. PCR amplification of pCPa

A 600 bp fragment of the *rep* gene was first identified by sequence analyses of clones from a *Ca.* P. australiense phytoplasma library (data not shown). Inverse PCR based upon the *rep* gene was used to amplify a 3.6 kb product from one strawberry sample, thereby confirming that the eDNA from *Ca.* P. australiense phytoplasma was approximately 3.7 kb, and circular.

#### 3.2.2. Sequence analysis of Candidatus Phytoplasma australiense eDNA

The complete 3773 bp *Ca.* P. australiense eDNA sequence (GenBank Accession No. DQ119295), was sequenced and had a G + C content of 28.38 mol% and four open reading frames (ORF) (Fig. 1, Table 1). A conserved domain for the replication (Rep) protein was identified at the 5′ end of ORF 1, and a RNA helicase at the 3′ end (data not shown). A multiple amino acid residue alignment for the replication protein from pCPa, from other phytoplasma eDNAs and pCI411 (plasmid from *Leuconostic lactis* 533) indicated that four of five conserved motifs (RII-V) (del Solar et al., 1993) typical of bacterial plasmids were located on ORF 1 (Fig. 2). The Walker A and Walker B (Saraste et al., 1990; Walker et al., 1982) motifs, known as nucleotide-binding motifs and typically found in geminiviruses (Koonin and IIyina, 1992; Oshima et al., 2001a), were located on the C terminus of ORF1 (Fig. 2). Tandem repeat DNA is two or more contiguous copies of a pattern of nucleotides (Benson, 1999). One repeat of 15 bp (GATAATAAA TCAACT) was identified and found to repeat three times within ORF3 (data not shown).

#### 3.2.3. Two-dimensional electrophoresis

*Candidatus* Phytoplasma australiense CsCl purified DNA was subjected to horizontal gel electrophoresis and bands of approximately 4.1 and 6 kb was observed (Fig. 3a). The sample was subjected to 2D electrophoresis to determine the nature of these bands. This method is used to distinguish different forms of plasmid DNA, open circular (OC), closed circular (CC) and linear (L) forms. When the closed circular form is nicked with UV light in the presence of ethidium bromide, it will convert to the relaxed open circular form, designated OC*. After the second dimension electrophoresis, the original OC, the CC and the newly converted OC* form a triangle (Hintermann et al., 1981). The first dimensional electrophoresis of pCPa that gave bands at 4.1 and 6 kb (Fig. 3a), when subjected to

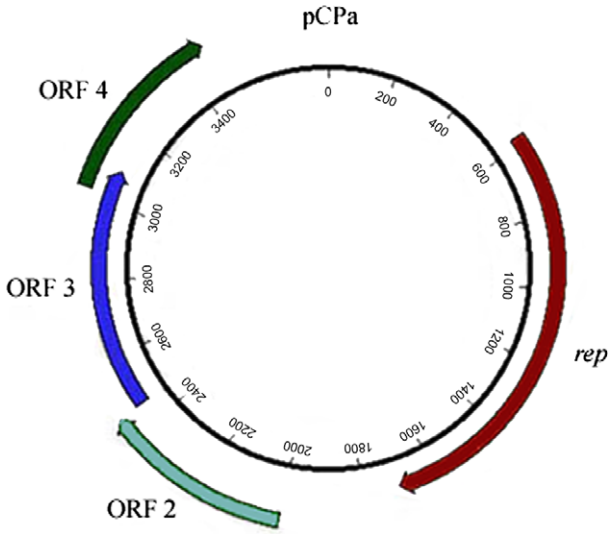


Fig. 1. Plasmid diagram of *Ca.* Phytoplasama australiense.

Table 1
Predicted ORFs in phytoplasma eDNAs

| Phytoplasma eDNA | Plant host | Predicted ORFs | Size (bp) | Protein[a] | Organism | % Similarity (amino acid) |
|---|---|---|---|---|---|---|
| pTBBperi (3319 bp) | Periwinkle | ORF 1 | 996 | DNA primase | pPNWB | 84 |
| | | ORF 2 | 567 | Replication protein | pPNWB | 97 |
| | | ORF 3 | 366 | No significant homology | — | — |
| | | ORF 4 | 528 | Threonine synthase | pPNWB | 96 |
| pTBBcap (4092 bp) | Capsicum | ORF 1 | 1656 | DNA primase | pPNWB | 80 |
| | | ORF 2 | 570 | Replication protein | pPNWB | 94 |
| | | ORF 3 | 528 | Threonine synthase | pPNWB | 96 |
| pCPa (3773 bp) | Strawberry | ORF 1 | 1128 | Replication protein | OY-NIM | 61 |
| | | | | ATP-dependent protease, Clp, ATPase subunit | OY-M | 62 |
| | | ORF 2 | 453 | No significant homology | — | — |
| | | ORF 3 | 642 | Hypothetical protein | OY-M | 29 |
| | | ORF 4 | 441 | Hypothetical protein | OY-M | 35 |

*Abbreviations:* pPNWB, peanut witches' broom; OY-NIM, onion yellows non-insect transmissible mutant; and OY-M, onion yellows mutant strain.

[a] Based on closest match found in database.

2D electrophoresis, produced a triangle formed by the OC form at 6 kb, the CC form at 4.1 kb and the newly converted OC* form at 6 kb (Fig. 3b).

### 3.3. Tomato big bud eDNA

#### 3.3.1. PCR amplification of tomato big bud eDNAs (pTBBperi and pTBBcap)

PCR *rep* primers based upon the TBB Rep protein were used to screen TBB positive plants in which the DNA had been extracted using different methods (Table 2). All samples except for *Solanum* sp. and *Gomphocarpus* sp. were positive. Inverse PCR based upon the Rep protein was conducted on samples from periwinkle and capsicum and these gave a 3.2 and 4.5 kb product respectively. The remaining TBB samples listed in Table 2 were also subjected to inverse PCR but a product was not observed. The same TBB samples were subjected to PCR using primers designed to amplify the chromosomal *dnaG* gene, giving a 650 bp product.

#### 3.3.2. Sequence analysis of pTBBperi

The sequence of random clone pTBB88 (3 kb) and the full-length circular 3.3 kb eDNA from TBB periwinkle, designated pTBBperi, were compared and


Fig. 2. Multiple sequence alignment of amino acid residues showing the conserved motifs for the putative replication protein. Abbreviations are pOYNIM, plasmid from onion yellows on-insect transmissible mutant strain; pOYM, plasmid from onion yellows mutant strain, pOYW, plasmid from onion yellows wild-type strain; pJHW, plasmid from Japanese honewort; OYMATPase, onion yellows mutant strain ATPase-dependent protease Clp subunit; pCPa, plasmid from *Candidatus* P. australiense, and pCI411 from *Leuconostoc lactis*, plasmid from pLS1 family. Conserved motifs RII-V are characteristics of pLS1 plasmids. # and * are conserved histamine and tyrosine residues, respectively. Walker A and Walker B motifs are characteristic of geminiviruses replication proteins.

found to be 100% homologous over 3 kb. The exact length of pTBBperi was 3319 bp with a G + C content of 26.8 mol%. The pTBBperi sequence (GenBank Accession No. DQ119297) was analysed for ORFs encoding a minimum of 80 amino acids, and four ORFs (Fig. 4a, Table 1) were identified. ORF 1 was 996 bp and encoded the putative *dnaG* gene. ORF1 was also subjected to searches against a conserved domain database (CDD). DnaG protein and TOPRIM (topoisomerase/primase) conserved domains were identified (data not shown). ORF 2 was 567 bp and encoded a putative replication (Rep) protein. Compared with other *rep* genes from other phytoplasma eDNAs (Fig. 5), the pTBBperi *rep* gene was considerably shorter (Table 3). ORF 3 did not have significant homology to known proteins available from the database and ORF 4 encoded the putative threonine synthase protein. Two sets of tandem repeats were identified for pTBBperi. One repeat was the nucleotide (A) repeated 28 times between positions 2273 and 2300, the second was an 11 bp segment (TTGGCTCCAAG) repeated three times within ORF3.

### 3.3.3. Sequence analysis of pTBBcap

To determine if the short *rep* gene in pTBBperi was unique for TBB eDNAs, a field collected TBB sample from capsicum containing eDNA, designated pTBBcap, was sequenced. Using inverse PCR, a 4.5 kb product was amplified from the TBB capsicum field sample. A 4092 bp segment of the 4.6 kb pTBBcap (GenBank Accession No. DQ119296) was sequenced, and had a G + C content of 30.8 mol% and three ORFs (Fig. 4b, Table 1). ORF 1 encoded the putative *dnaG* gene, and using conserved domain searches for pTBBcap ORF 1, conserved domains for *dnaG* and the zinc finger were identified (data not shown). ORF2 encoded a short *rep* gene of 570 bp (Fig. 5) and ORF3 encoded the putative threonine synthase. No tandem repeats were identified in pTBBcap.

A spatial distribution of ORFs of the two TBB eDNAs, peanut witches' broom phytoplasma

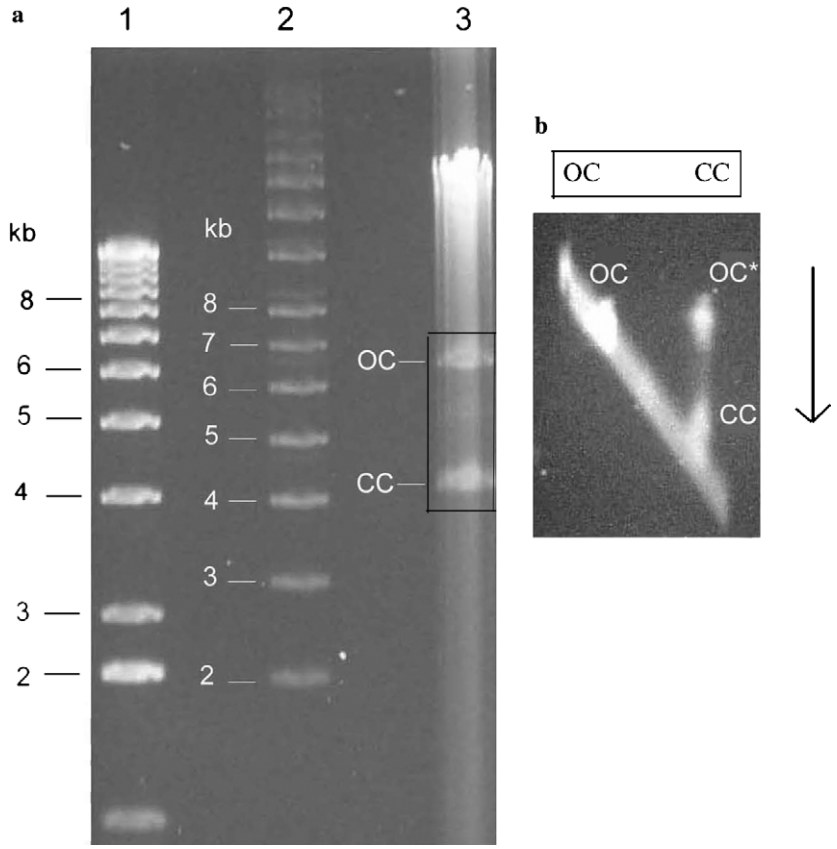


Fig. 3. Conformation of pCPa eDNA (a) 1D and (b) 2D gel electrophoresis. Lane 1 is 1 kb plus DNA marker; lane 2 is supercoiled marker; and lane 3 is *Ca.* P. australiense DNA. CC is the closed circular form, OC is the open circular form and OC* is the nicked closed circular form. After 1D electrophoresis, lane 3 indicated on the figure was extracted and placed at the top of the new gel at 90° to the original orientation, as described in the text. Arrow indicates the direction of 2D electrophoresis.

Table 2
Screening for eDNA by PCR

| Plant host | Location | DNA extraction method | Phytoplasma strain[a] | TBB *rep* PCR | TBB DNA primase PCR | Inverse rep PCR |
|---|---|---|---|---|---|---|
| *Catharanthus roseus* | NT | CsCl gradient | TBB[c] | + | + | + (3.2 kb) |
| *Catharanthus roseus* | NT | DNeasy | TBB | + | + | + (3.2 kb) |
| *Catharanthus roseus* | NT | Dellaporta | TBB | + | + | + (3.2 kb) |
| *Lycopersicon esculentum* L. | QLD | Doyle and Doyle | TBB | + | + | – |
| *Cichorium intybus* | QLD | DNeasy | TBB | + | + | – |
| *Capsicum annuum* | QLD | DNeasy | TBB | + | + | + (4.5 kb) |
| *Apium graveolens* | QLD | CsCl gradient | TBB | + | + | – |
| *Solanum melongena* L. | NT | Doyle and Doyle | TBB | – | + | – |
| *Solanum melongena* L. | NT | Doyle and Doyle | TBB | – | + | – |
| *Gomphocarpus physocarpus* | QLD | Doyle and Doyle | *Ca.* P.a.[b] | – | nt | nt |
| *Gomphocarpus physocarpus* | QLD | Doyle and Doyle | *Ca.* P.a. | – | nt | nt |
| *Fragaria x ananassa* | QLD | CsCl gradient | *Ca.* P.a. | nt | nt | + (3.6 kb) |

nt, not tested.
[a] Based upon 16S rRNA data.
[b] *Candidatus* Phytoplasma australiense phytoplasma.
[c] Tomato big bud phytoplasma.

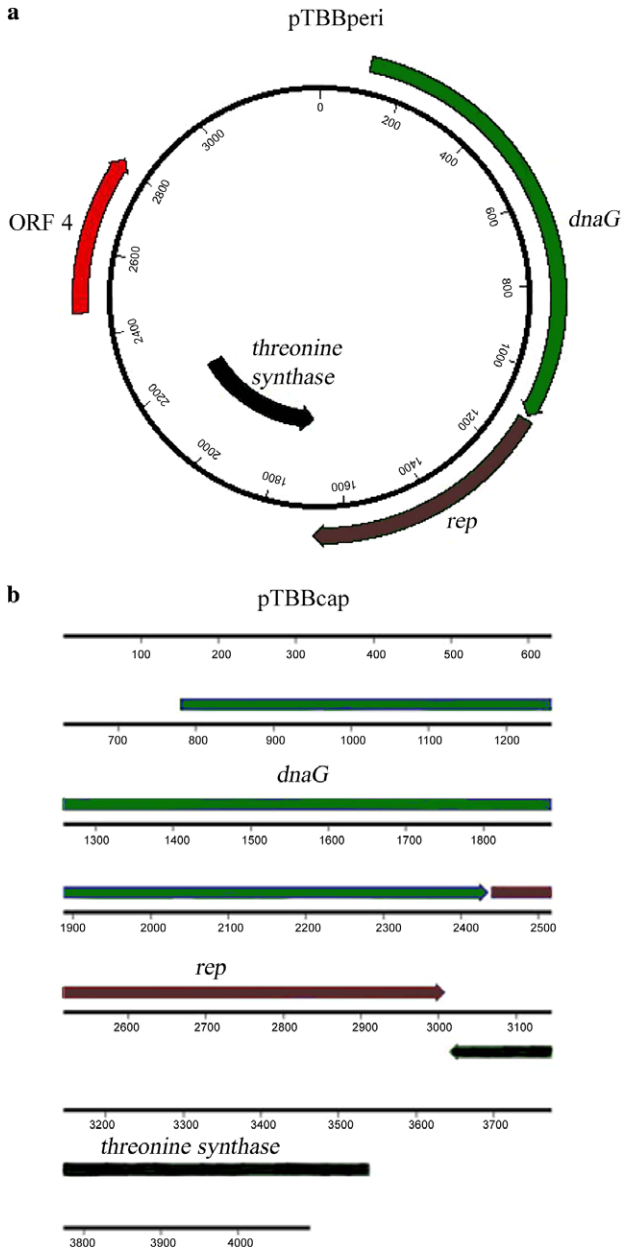


Fig. 4. Plasmid diagram of TBB eDNAs (a) pTBBperi and pTBBcap.

eDNA (pPNWB; GenBank Accession No. AY270152) and beet leafhopper-transmitted virescence agent (pBLTVA-1; GenBank Accession No. AY423627) eDNA (Fig. 5) not only demonstrated that the *rep* gene in the two TBB eDNAs was shorter than the *rep* gene for the other two phytoplasma eDNAs, but it also showed that the *dnaG* gene is common to all four phytoplasmas. A bootstrapped phylogenetic tree for the *dnaG* gene (Fig. 6) demonstrated the close relationship between TBB and peanut witches' broom phytoplasma eDNA.

### 3.3.4. Southern blot hybridisation

No TBB eDNA was observed on 1% agarose horizontal gels, so southern hybridisation was used to determine how many eDNAs were present in TBB phytoplasma samples. Extrachromosomal clone pTBB88 was labelled and hybridised to eDNAs in two field samples (capsicum and chicory). Two bands

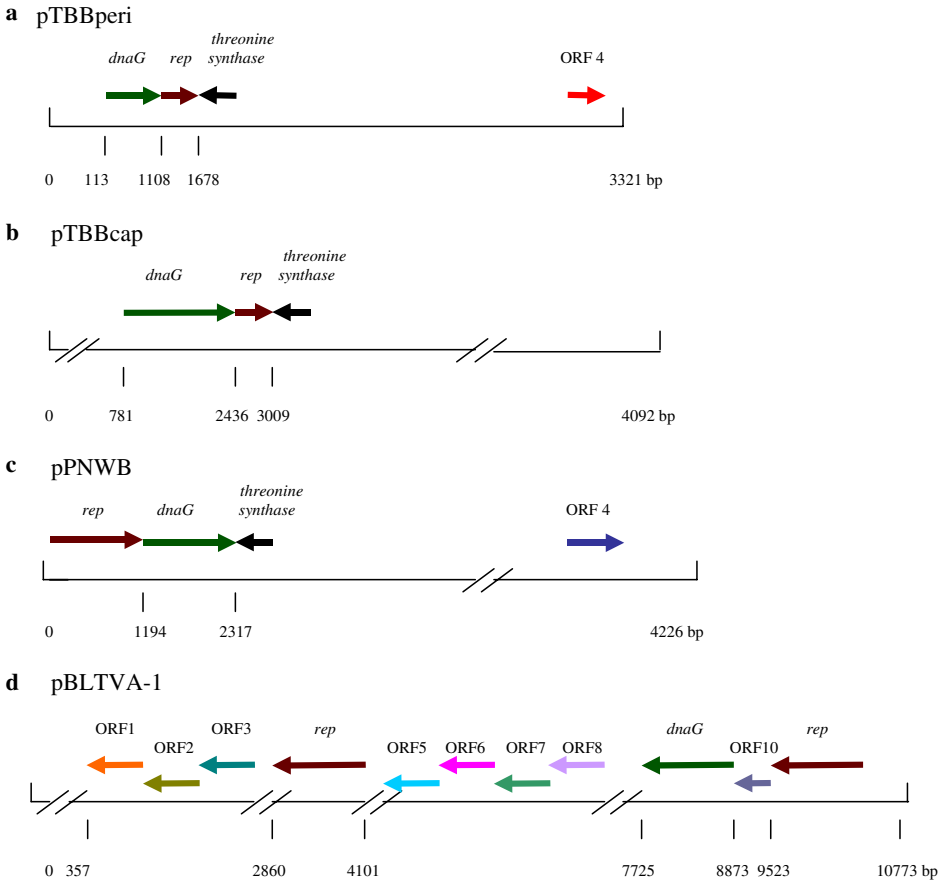


Fig. 5. Spatial distribution of ORFs in phytoplasma eDNAs. Abbreviations are pTBBcap, plasmid from tomato big bud isolated from capsicum; pPNWB, plasmid from Peanut witches' broom; pTBBperi, plasmid from tomato big bud from periwinkle; and pBLTVA-1, plasmid from beet leafhopper-transmitted virescence-1.

Table 3
Phytoplasma eDNA replication proteins

| Phytoplasma eDNA | Genbank Accession No. | Replication protein (bp) |
|---|---|---|
| pCPa | DQ119295 | 1128 |
| pTBBperi | DQ119297 | 567 |
| pTBBcap | DQ119296 | 570 |
| pJHW | AB064396 | 1125 |
| pOY-NIM | AB061724 | 1134 |
| pOYW | AB056858 | 1134 |
| pBLTVA1-1 | AY423627 | 1241 |
| pBLTVA1-2 | AY423627 | 1251 |
| pBLTVA 2 | AY423628 | 1251 |
| pPNWB | AY270152 | 1194 |
| pAYWB-I | CP000062 | 1119 |
| pAYWB-II | CP000063 | 1149 |
| pAYWB-III | CP000064 | 1107 |
| pAYWB-IV | CP000065 | 1128 |

approximately 5.5 and 12 kb were observed in both samples. Due to gel artefacts such as uneven heat dissipation from the gel or ionic strength of the two samples (Rodriguez and Tait, 1983), there was a slight difference in migration rate of the plasmids in lanes 1 and 2 (Fig. 7). The pTBB88 eDNA probe was highly specific and did not hybridise to healthy DNA or *Ca.* P. australiense phytoplasma DNA (Fig. 7; lanes 3–5). Several bands were observed for TBB CsCl purified DNA (Fig. 7; lane 6) and the pTBB88 DNA positive control (Fig. 7; lane 7) indicating the multiple forms of the plasmid. The probe also hybridised to high molecular weight TBB CsCl purified DNA (>48.5 kb) suggesting similar sequences of the plasmid in the TBB chromosome. To confirm the two bands in the field samples were plasmid in origin, a probe based upon the *rep* gene was used and hybridised to the lower two bands (data not shown).

## 4. Discussion

Two TBB phytoplasma eDNAs were studied in detail and the nucleotide sequences determined.

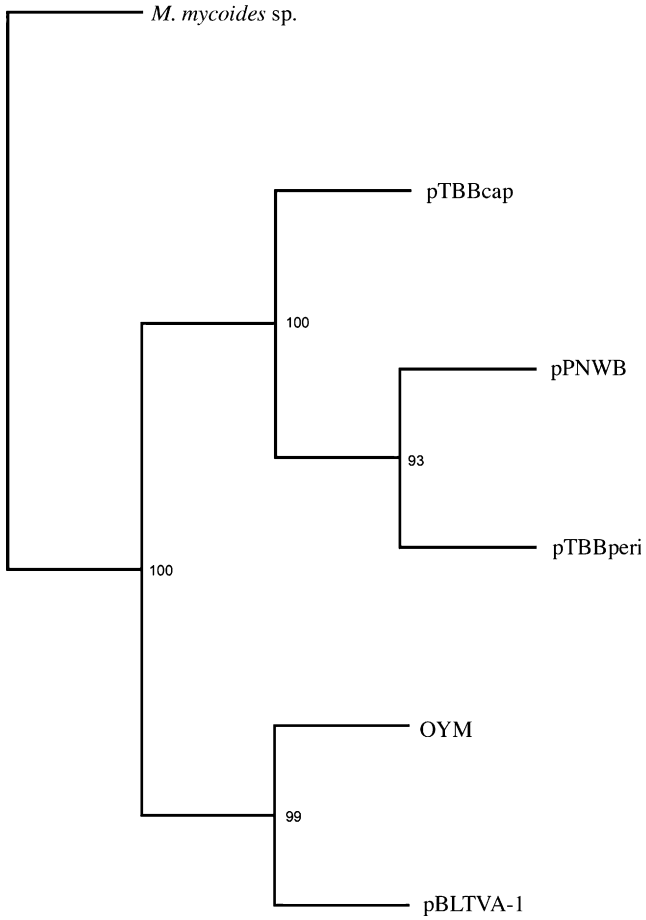


Fig. 6. Bootstrapped phylogenetic tree for DNA primase. Abbreviations are pTBBcap, plasmid from tomato big bud isolated from capsicum (GenBank Accession No. DQ119296); pPNWB- plasmid from peanut witches' broom (GenBank Accession No. AY270152); pTBBperi, plasmid from tomato big bud from periwinkle (GenBank Accession No. DQ119297); OYM, onion yellows mutant strain (GenBank Accession No. AP006628, PAM569); pBLTVA-1, plasmid from beet leafhopper transmitted virescence-1 (GenBank Accession No. AY423627), and *M. mycoides* sp. is *Mycoplasma mycoides subsp. mycoides* (GenBank Accession No. CAE77093). *M. mycoides* was selected as the outgroup for tree construction.

pTBBperi isolated from grafted TBB periwinkles and pTBBcap derived from a field collected capsicum plant contained circular eDNA of 3.3 and 4.8 kb respectively. A 3.7 kb plasmid was identified in one strawberry sample that was positive for the *Ca.* P. australiense phytoplasma. This is the first report of an eDNA for this phytoplasma. Due to the differing titres of phytoplasmas in their host, four different DNA extraction methods were compared and, of these, the CsCl buoyant gradient extraction method was best suited for eDNA detection.

*Candidatus* Phytoplasma australiense contained both closed circular and open circular forms of eDNA and this was demonstrated by the migration pattern observed in 2D electrophoresis. The size of the plasmid was estimated to be 4.1 kb by gel electrophoresis but the size estimated by inverse PCR was 3.6 kb which is a more accurate measure of actual size than migration through a gel. Any unknown bands observed after 2D electrophoresis that do not fit the triangle pattern usually indicate contaminating chromosomal DNA or linear forms of eDNA (Hintermann et al., 1981). We observed the smearing associated with chromosomal DNA but the linear form of the plasmid was not observed.

The pCPa *rep* gene contained the conserved domain for bacterial plasmid *rep* genes (del Solar et al., 1993; Khan, 1997; Oshima et al., 2001a) as well as the conserved Walker A and Walker B motifs characteristic of geminiviruses (Marchler-Bauer and Bryant, 2004). A geminivirus-like rolling circle

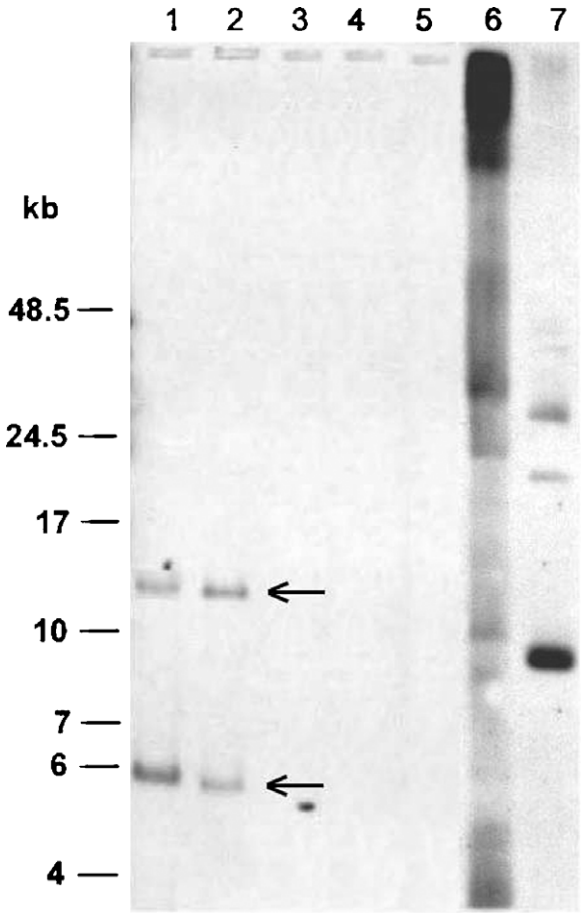


Fig. 7. Southern hybridisation of DNA using pTBB88 probe. Lane 1 is TBB (chicory); lane 2 is TBB (capsicum); lane 3 is *Ca.* P. australiense (strawberry); lane 4 is healthy periwinkle; lane 5 is CsCl purified *Ca.* P. australiense; lane 6 is CsCI purified TBBperi; and lane 7 is pTBB88. Arrows indicate presence of eDNAs.

replication mechanism has been previously reported for *Vaccinium* witches' broom (Liefting et al., 2004; Namba et al., 2005; Nishigawa et al., 2002a; Nishigawa et al., 2001; Rekab et al., 1999). The unusual finding of the pLS1 and the Walker motifs on the pCPa *rep* gene was also observed in plasmids isolated from the OY phytoplasma (pOYW) (Oshima et al., 2001a). Oshima et al. discussed two possibilities for this phenomenon. First, an ancestral pOYW plasmid may have acquired its eukaryotic DNA from host phytoplasma DNA, entered the eukaryotic cytoplasm and evolved into an ancestral eukaryotic ssDNA virus. Second, an ancestral pOYW obtained the helicase domain by recombination with a virus. When the phytoplasma *rep* genes were aligned with the OY phytoplasma ATPase subunit of the ATP-dependent protease Clp, the four conserved motifs characteristic of the bacterial plasmid pLS1 and the two Walker motifs were present. This is consistent with an ATPase role for nucleotide-binding proteins (Higgins et al., 1988; Koonin, 1993) and supports the replication role of the phytoplasma *rep* genes.

The TBBperi strain used in the study had been maintained in the shadehouse for over 10 years by grafting. Schneider et al. (1999a) detected eDNAs from TBBperi ranging in size from ~4.1 to 12.1 kb. The difference in sizes may reflect the different physical forms of eDNAs. In this study, the 12.1 kb eDNA was also observed in field samples from chicory and capsicum. In this present study, the TBB eDNA from periwinkles maintained in the shadehouses was estimated to be 3.3 kb and not 4.1 kb. It is possible that genetic material may have been lost during serial grafting. This has also been reported in studies of the onion yellows phytoplasma strain, where serial grafting of the wild-type OY gave rise to the OY-NIM strain which could not replicate in the insect host (Oshima et al., 2001b). pOY-NIM lacks two ORFs that were found in pOYW and pOYM, these two ORFs encoded for single-stranded DNA-binding protein (SSB) and a protein with no significant similarity to known proteins. Another possibility for the difference in TBBperi eDNA sizes from Schneider et al. (1999a) and this study, is that hybridisation patterns of eDNA are subject to change due to the multiple physical forms of eDNA resulting from different DNA extractions methods, storage conditions and the age of the DNA sample (Schneider et al., 1999a).

We were unable to obtain the complete sequence of pTBBcap due to technical difficulties encountered in sequencing the remaining 700 bp even after several attempts. It is possible that long stretches of single nucleotides, AT rich regions and tandem repeats were present that are known to confound sequencing of DNA fragments (Weitzmann et al., 1997; Wells, 1996). Interestingly, in the 4092 bp fragment sequenced, no tandem repeats were identified, however, this does not eliminate the possibility that a repeat occurs in the region yet to be sequenced.

Bacterial plasmids contain an essential region which may contain several genes such as the origin of replication, the protein for replication initiation and plasmid-borne genes involved in the control of its replication (del Solar et al., 1998). The shorter *rep* gene in TBB eDNA suggests that replication in these plasmids involves a unique mechanism, at least for phytoplasma eDNAs. Housekeeping genes are not

generally found on plasmids (Koch, 1981) but the presence of *dnaG* in TBB eDNA suggests that in the TBB eDNA system, the *dnaG* and chromosomal genes are used for replication instead of the *rep* gene (del Solar et al., 1998). The bacterial *dnaG* is a nucleotidyltransferase that synthesises the oligoribonucleotide primers required for DNA replication on the lagging strand of the replication fork; it can also prime the leading stand and has been implicated in cell division. If *dnaG* was not located on the TBB chromosome but present only on the plasmid, the primase may be transferred to the chromosome for DNA replication possibly through horizontal transfer (Khan, 2005). However, PCR tests amplified the primase gene from all TBB samples tested, including samples that were negative for eDNA. These results suggest that the *dnaG* may also be located on the chromosome, which is also the case for peanut witches' broom phytoplasma and its associated plasmid (GenBank Accession No. AY270152 and AY270153). The full-length *rep* gene was found on the PNWB plasmid, therefore in this system the *dnaG* may not be responsible for replication. Due to incomplete sequence data of the chromosome encoded PNWB *dnaG* gene, we were unable to determine if the gene in PNWB was complete and functional. Although PCR tests of TBB samples suggest that the primase gene is also located on the chromosome, the functionality of the protein is not known. The *dnaG* gene was also encoded on the BLTVA plasmid, but whether it is also on the chromosome is unknown. However, the BLTVA plasmid also encodes the full-length *rep* gene so the primase may not be responsible for replication in this plasmid. No other plasmid-encoded *dnaG* gene was identified for other phytoplasma plasmids. Based on the 16S rRNA genes, the TBB and PNWB phytoplasmas are closely related (Seemüller et al., 1998; Wang and Hiruki, 2005) and this is affirmed by the close genetic relatedness of the plasmid-encoded *dnaG* genes.

This research has provided insight into extrachromosomal DNA from two important Australian phytoplasmas and it is the first report of eDNA from *Candidatus* Phytoplasma australiense. In this phytoplasma the full-length *rep* gene is similar to those from other phytoplasmas and contains both plasmid and geminivirus-like motifs associated with the rolling circle mechanism of replication. We also report an unusual gene arrangement and speculate upon a unique plasmid replication system for TBB eDNAs where the *dnaG* is utilised for replication in place of the truncated Rep protein. Beyond knowing the genetic code of these eDNAs, it is still unclear what roles these plasmids actually play in phytoplasma disease expression and if they are true bacterial plasmids that can replicate autonomously.

## Acknowledgments

This research was funded by the Cooperative Research Centre for Tropical Plant Protection (CRCTPP) and Charles Darwin University.

## References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bai, X., Zhang, J., Ewing, A., Miller, S.A., Radek, A., Shevchenko, D., Tsukerman, K., Walunas, T., Lapidus, A., Campbell, J.W., Hogenhout, S.A., 2006. Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. J. Bacteriol. 188, 3682–3696.

Benson, G., 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 27, 573–580.

Bertaccini, A., Davis, R.E., Lee, I.M., Conti, M., Dally, E.I., Douglas, S.M., 1990. Detection of chrysanthemum yellows mycoplasmalike organism by dot hybridisation and Southern blot analysis. Plant Dis. 74, 40–43.

Chen, J., Chang, C.J., Jarret, R., Gawel, N., 1992. Isolation and cloning of DNA fragments from a mycoplasma like organism associated with walnut witches'-broom disease. Phytopathology 82, 306–309.

Collmer, A., 1998. Determinants of pathogenicity and avirulence in plant pathogenic bacteria. Curr. Opin. Plant Biol. 1, 329–335.

Davis, M.J., Tsai, J.H., Cox, R.L., McDaniel, L.L., Harrison, N.A., 1988. Cloning of chromosomal and extrachromosomal DNA of the mycoplasma like organism that causes maize bushy stunt disease. Mol. Plant Microbe Interact. 1, 295–302.

Davis, R.E., Dally, E.L., Tanne, E., Rumbos, I.C., 1997. Phytoplasmas associated with grapevine yellows in Israel and Greece belong to the stolbur phytoplasma subgroup, 16SrXII-A. J. Plant Pathol. 79, 181–187.

Davis, R.E., Jomantiene, R., Kalvelyte, A., Dally, E.L., 2003. Differential amplification of sequence heterogeneous ribosomal RNA genes and classification of the '*Fragaria multicipita*' phytoplasma. Microbiol. Res. 158, 229–236.

De La Rue, S.J., Schneider, B., Gibb, K.S., 1999. Genetic variability in phytoplasmas associated with papaya yellow crinkle and papaya mosaic diseases in Queensland and the Northern Territory. Aust. Plant Pathol. 28, 108–114.

del Solar, G., Moscoso, M., Espinosa, M., 1993. Rolling circle-replicating plasmids from gram-positive and gram-negative bacteria: a wall falls. Mol. Microbiol. 8, 789–796.

del Solar, G., Alonso, J.C., Espinosa, M., Diaz-Orejas, R., 1996. Broad–host–range plasmid replication: an open question. Mol. Microbiol. 21, 661–666.

del Solar, G., Giraldo, R., Ruiz-Echevarria, M.J., Espinosa, M., Diaz-Orejas, R., 1998. Replication and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev. 62, 434–464.

Dellaporta, S.L., Wood, J., Hicks, J.B., 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1, 19–21.

Deng, S., Hiruki, C., 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. J. Microbiol. Methods 14, 53–61.

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus (San Francisco, Calif.) 12, 13–15.

Felsenstein, J., 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics 5, 164–166.

Gibb, K.S., Padovan, A.C., Mogen, B.D., 1995. Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plant species growing in northern Australia. Phytopathology 85, 169–174.

Harrison, N.A., Tsai, J.H., Bourne, C.M., Richardson, P.A., 1991. Molecular cloning and detection of chromosomal and extrachromosomal DNA of mycoplasmalike organisms associated with witches' broom disease of pigeon pea in Florida. Mol. Plant Microbe Interact. 4, 300–307.

Higgins, C.F., Gallagher, M.P., Mimmack, M.L., Pearce, S.R., 1988. A family of closely related ATP-binding subunits from prokaryotic and eukaryotic cells. BioEssays 8, 111–116.

Hintermann, G., Fischer, H.M., Crameri, R., Hutter, R., 1981. Simple procedure for distinguishing CCC, OC and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid 5, 371–373.

Khan, S.A., 1997. Rolling circle replication of bacterial plasmids. Microbiol. Mol. Biol. Rev. 61, 442–455.

Khan, S.A., 2000. Plasmid rolling circle replication: recent developments. Mol. Microbiol. 37, 477–484.

Khan, S.A., 2005. Plasmid rolling circle replication: highlights of two decades of research. Plasmid 53, 126–136.

Koch, A.L., 1981. Evolution of antibiotic resistance gene function. Microbiol. Rev. 45, 355–378.

Kollar, A., Seemüller, E., 1989. Base composition of the DNA of mycoplasmalike organisms associated with various plant diseases. J. Phytopathol. 127, 177–186.

Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., Bové, J.M., 1990. Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Am. Phytopathol. Soc. 80, 233–237.

Koonin, E.V., 1993. A superfamily of ATPases with diverse functions containing either classical or deviant ATP-binding motif. J. Mol. Biol. 229, 1165–1174.

Koonin, E.V., IIyina, T.V., 1992. Geminiviruses replication proteins are related to prokaryotic plasmid rolling circle DNA replication initiator proteins. J. Gen. Virol. 72, 2763–2766.

Kuboyama, T., Huang, C.C., Lu, X., Sawayanagi, T., Kanazawa, T., Kagami, T., Matsuda, I., Tsuchizaki, T., Namba, S., 1998. A plasmid isolated from phytopathogenic onion yellows phytoplasma and its heterogeneity in the pathogenic mutant. Mol. Plant Microbe Interact. 11, 1031–1037.

Kuske, C.R., Kirkpatrick, B.C., 1990. Identification and characterisation of plasmids from the western aster yellows mycoplasmalike organisms. J. Bacteriol. 172, 1628–1633.

Liefting, L.W., Shaw, M.E., Kirkpatrick, B.C., 2004. Sequence analysis of two plasmids from the phytoplasma beet leafhopper transmitted virescence agent. Microbiology 150, 1809–1817.

Marchler-Bauer, A., Bryant, S.H., 2004. CD-Search: protein domain annotations on the fly. Nucleic Acids Res. 32, W327–W331.

Nakashima, K., Hayashi, T., 1995. Extrachromosomal DNAs of rice yellow dwarf and sugarcane white leaf phytoplasmas. Annu. Phytopathol. Soc. Jpn. 61, 456–462.

Namba, S., Oshima, K., Gibb, K., 2005. In: Blanchard, A., Browning, G.s. (Eds.), Phytoplasma Genomics. Horizon Bioscience, Norfolk, pp. 97–133.

Nishigawa, H., Oshima, K., Miyata, S., Ugaki, M., Namba, S., 2003. Complete set of extrachromosomal DNAs from three pathogenic lines of onion yellows phytoplasma and use of PCR to differentiate each line. J. Gen. Plant Pathol. 69, 194–198.

Nishigawa, H., Oshima, K., Kakizawa, S., Jung, H.Y., Kuboyama, T., Miyata, S., Ugaki, M., Namba, S., 2002a. Evidence of intermolecular recombination between extrachromosomal DNAs in phytoplasma: a trigger for the biological diversity of phytoplasma? Microbiology 148, 1389–1396.

Nishigawa, H., Oshima, K., Kakizawa, S., Jung, H.Y., Kuboyama, T., Miyata, S., Ugaki, M., Namba, S., 2002b. A plasmid from a non-insect-transmissible line of a phytoplasma lacks two open reading frames that exist in the plasmid from the wild-type line. Gene 298, 195–201.

Nishigawa, H., Miyata, S., Oshima, K., Sawayanagi, T., Komoto, A., Kuboyama, T., Matsuda, I., Tsuchizaki, T., Namba, S., 2001. In planta expression of a protein encoded by the extrachromosomal DNA of a phytoplasma and related to geminivirus replication proteins. Microbiology 147, 507–513.

Oshima, K., Kakizawa, S., Nishigawa, H., Kuboyama, T., Miyata, S., Ugaki, M., Namba, S., 2001a. A plasmid of phytoplasma encodes a unique replication protein having both plasmid and virus-like domains: clue to viral ancestry or result of virus/plasmid recombination? Virology 285, 270–277.

Oshima, K., Shiomi, T., Kuboyama, T., Sawayanagi, T., Nishigawa, H., Kakizawa, S., Miyata, S., Ugaki, M., Namba, S., 2001b. Isolation and characterization of derivative lines of the onion yellows phytoplasma that do not cause stunting or phloem hyperplasia. J. Phytopathol. 91, 1024–1029.

Padovan, A.C., Gibb, K.S., 2001. Epidemiology of phytoplasma diseases in papaya in Northern Australia. J. Phytopathol. 149, 649–658.

Padovan, A.C., Gibb, K.S., Persley, D., 2000. Association of '*Candidatus* Phytoplasma australiense' with green petal and lethal yellows diseases in strawberry. Plant Pathol. 49, 362–369.

Panopoulos, N.J., Peet, R.C., 1985. The molecular genetics of plant pathogenic bacteria and their plasmids. Annu. Rev. Phytopathol. 23, 381–419.

Porebski, S., Bailey, L.G., Baum, B.R., 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Rep. 15, 8–15.

Rekab, D., Carraro, L., Schneider, B., Seemüller, E., Chen, J., Chang, C.J., Locci, R., Firrao, G., 1999. Geminivirus-related extrachromosomal DNAs of the X-clade phytoplasmas share high sequence similarity. Microbiology 145, 1453–1459.

Rodriguez, R.L., Tait, R.C., 1983. Recombinant DNA Techniques: An Introduction. Addison-Wesley Publishing Company, Massachusetts. p. 67–79.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Habor New York. p.

Saraste, M., Sibbald, P.R., Wittinghofer, A., 1990. The P-loop - a common motif in ATP- and GTP- binding proteins. Trends Biochem. Sci. 15, 430–434.

Schneider, B., Maurer, R., Saillard, C., Kirkpatrick, B.C., Seemüller, E., 1992. Occurrence and relatedness of extrachromosomal DNAs in plant pathogenic mycoplasma like organisms. Mol. Plant Microbe Interact. 5, 489–495.

Schneider, B., Gibb, K.S., Padovan, A., Davis, R.I., De La Rue, S., 1999a. Comparison and characterisation of tomato big bud and sweet potato little leaf group phytoplasmas. J. Phytopathol. 147, 31–40.

Schneider, B., Padovan, A., De La Rue, S., Eichner, R., Davis, R., Bernuetz, A., Gibb, K., 1999b. Detection and differentiation of phytoplasmas in Australia: an update. Aust. J. Agric. Res. 50, 333–342.

Sears, B.B., Lim, P.O., Holland, N., Kirkpatrick, B.C., Klomparens, K.L., 1989. Isolation and characterization of DNA from a mycoplasmalike organism. Mol. Plant Microbe Interact. 2, 175–180.

Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A., Goschl, M., 1998. Current status of molecular classification of the phytoplasmas. J. Plant Pathol. 80, 3–26.

Shaw, M.E., 1991. Biological and Molecular Characterization of Virescence Agents Infecting Herbaceous Crops in California. University of California, Davis, USA. p.

Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemüller, E., Kirkpatrick, B.C., 1996. Phytoplasma-specific PCR primers based on sequences of the 16S–23S rRNA spacer region. Appl. Environ. Microbiol. 62, 2988–2993.

Stenger, D.C., Revington, G.N., Stevenson, M.C., Bisaro, D.M., 1991. Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling circle replication of a plant viral DNA. Proc. Natl. Acad. Sci. USA 88, 8029–8033.

Streten, C., Herrington, M.E., Hutton, D.G., Persley, D.M., Waite, G.K., Gibb, K.S., 2005. Plant hosts of the phytoplasmas and rickettsia-like-organisms associated with strawberry lethal yellows and green petal diseases. Aust. Plant Pathol. 34, 165–173.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tran-Nguyen, L., Blanche, K.R., Egan, B., Gibb, K.S., 2000. Diversity of phytoplasmas in Northern Australia sugarcane and other grasses. Plant Pathol. 49, 666–679.

Vivian, A., Murillo, J., Jackson, R.W., 2001. The roles of plasmids in phytopathogenic bacteria: mobile arsenals? Microbiology 147, 763–780.

Walker, J.E., Saraste, M., Runswick, M.J., Gay, N.J., 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1, 945–951.

Wang, K., Hiruki, C., 2005. Distinctions between phytoplasmas at the subgroup level detected by heteroduplex mobility assay. Plant Pathol. 54, 625–633.

Weitzmann, M.N., Woodford, K.J., Usdin, K., 1997. DNA secondary structures and the evolution of hypervariable tandem arrays. J. Biol. Chem. 272, 9517–9523.

Wells, R.D., 1996. Molecular basis of genetic instability of triplet repeats. J. Biol. Chem. 271, 2875–2878.