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Association of '*Candidatus* Phytoplasma australiense' with green petal and lethal yellows diseases in strawberry

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The identity of phytoplasmas detected in strawberry plants with green petal (SGP) and lethal yellows (SLY) diseases was determined by RFLP analysis of the 16S rRNA gene and adjacent spacer region (SR). RFLP and sequence comparisons indicated that the phytoplasmas associated with SGP and SLY were indistinguishable and were most closely related to '*Candidatus* Phytoplasma australiense', the phytoplasma associated with Australian grapevine yellows, papaya dieback and *Phormium* yellow leaf diseases. This taxon lies within the aster yellows strain cluster. Primers based on the phytoplasma *tuf* gene, which amplify only members of the AY strain cluster, amplified a DNA product from the SGP and SLY phytoplasmas. Primers deduced from the 16S rRNA/SR of P. australiense that amplify only members of this taxon amplified rDNA sequences from the SGP and SLY phytoplasmas. Primers that selectively amplify members of the faba bean phyllody (FBP) phytoplasma group, the most commonly occurring phytoplasma group in Australia, did not amplify rDNA from the SGP and SLY phytoplasmas.

Keywords: 16SrRNA, heterogeneity, phytoplasma, strawberry

Introduction

Strawberry (*Fragaria* × *ananassa*) is affected by a number of diseases thought to be associated with phytopathogenic phytoplasmas. These include green-petal disease (Posnette & Chiykowski, 1987), multiplier disease, witches' broom, little leaf, bronze leaf wilt (Maas, 1984), lethal yellows (Andersen *et al*., 1998) and aster yellows (Chiykowski, 1987). Some of these diseases have also been reported in Australia, including green petal, mycoplasma yellows (Greber & Gowanlock, 1979) and strawberry phyllody (Davis *et al*., 1997b). In these reports, however, no phytoplasma was identified. A rickettsia-associated lethal yellows disease has also been reported, and is difficult to distinguish from mycoplasma yellows because symptoms overlap (Greber & Gowanlock, 1979).

Andersen *et al*. (1998) reported that the phormium yellow leaf (PYL) phytoplasma is associated with strawberry lethal yellows disease in New Zealand. Liefting *et al*. (1998) showed by sequence analysis of the 16S rRNA and 16S/23S spacer region (SR) that PYL, papaya dieback (PDB) and Australian grapevine yellows (AGY) phytoplasmas are all closely related. The phytoplasma associated with AGY disease has been designated *Candidatus* Phytoplasma australiense (Davis *et al*., 1997a), and Liefting *et al*. (1998) proposed that P. australiense is the phytoplasma associated with AGY, PDB and PYL diseases. Andersen *et al*. (1998) proposed that the Australian and New Zealand strawberry lethal yellows diseases can also be caused by this phytoplasma. Recently, strawberries with green petal and yellows were screened for phytoplasmas and the diseases were shown to be associated with P. australiense (Padovan *et al*., 1998).

The objectives of this study were to provide more evidence that strawberry green petal and lethal yellows phytoplasmas are caused by P. australiense; to investigate the two different symptoms in strawberry and their relationship to phytoplasmas and rickettsia-like organisms; and to increase the understanding of diversity within P. australiense isolated from different plant hosts.

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Materials and methods

Plants and phytoplasmas

In July 1998 three strawberry plants with green petal (SGP), five plants with lethal yellows (SLY), and one symptom-free plant were collected from Redlands Research Station, Cleveland, Queensland, Australia. Leaf midribs and petioles were used as source material

for phytoplasmas. Sweet potato with sweet potato little leaf strain V4 (SPLL-V4) disease was collected near Darwin, Northern Territory in 1991; tomato with tomato big bud (TBB) near Adelaide, South Australia in 1992; and grapevine cv. Chardonnay with Australian grapevine yellows (AGY) in Loxton, South Australia in 1991. The SPLL-V4 and TBB phytoplasmas were transmitted to and maintained in periwinkle. Papaya with papaya dieback (PDB) disease was collected in 1995 from Redlands, Rockhampton and Guralda in Queensland, Katherine in the Northern Territory and Kununurra in Western Australia.

Additional phytoplasmas that have been grouped on the basis of their 16S ribosomal DNA restriction patterns and nucleotide sequences (Schneider *et al*., 1993; Seemüller *et al*., 1994) were included as reference strains. The sources of stolbur of *Lycopersicon esculentum* (STOL) from France and American aster yellows (AAY) from Florida were as described previously (Schneider *et al*., 1993). The *Phormium* yellow leaf (PYL) phytoplasma DNA was a kind gift from Drs Lia Liefting and Richard Forster (The Horticulture and Food Research Institute of New Zealand, Auckland). Phytoplasmas used in this study are listed in Table 1.

Extraction of DNA

DNA from strawberries was obtained by a small-scale extraction procedure according to Doyle & Doyle (1990), using 100–200 mg of plant tissue which was ground with micropestles in microcentrifuge tubes, with silicon carbide (300) as abrasive. Nucleic acid pellets were reconstituted in 50 μL water and stored at −20°C until required.

In vitro amplification of phytoplasma DNA using a universal primer pair

DNA concentration was estimated after gel electrophoresis with DNA standards and 20–100 ng DNA was used for individual PCR reactions. The primers P1 (Deng & Hiruki, 1991) and P7 (Schneider *et al*., 1995b) were used to amplify parts of the ribosomal operon comprising the 16S rRNA gene, the SR and the 5′ end of the 23S rRNA gene. The primers fP3 (Schneider *et al*., 1995b) and P7 were used to amplify the SR. A nested PCR procedure was used with the first primer pair, P1 and P7, and a second primer pair, fU5 (Lorenz *et al*., 1995) and m23sr (Padovan *et al*., 1995). These primers were specific for a region of the 16S rDNA region in all known phytoplasmas. For nested PCR, 1 μL of the first-round reaction mix was added to the PCR cocktail mix containing the second primer pair. The PCR was performed in 50 μL reactions containing 0·4 μM of each primer, 0·2 mM of each dNTP, 1·25 U heat-stable Taq DNA polymerase (Bresatec, Adelaide, South Australia) and 1× polymerase buffer. The reaction was overlaid with 30 μL of mineral oil and cycled 35 times with the following parameters: 95°C for 1 min, 55°C for 1 min and 72°C for 1·5 min in a thermocycler (Corbett Research, Mortlake, New South Wales). PCR conditions for second-round nested PCR were the same except that the annealing temperature was 58°C. Following PCR, 3–5 μL of each reaction was electrophoresed in a 1% (w/v) agarose gel containing 0·3 μg/mL ethidium bromide in 0·5 × TBE buffer (22·5 mM Tris–borate, 1 mM EDTA pH 8·0). Total nucleic acid extracted from symptom-free plants was subjected to PCR as a negative control, and in some experiments water controls were included in which no plant nucleic acid was added to the PCR reaction mix.

Where diseased samples were negative in the universal PCR, the tests were repeated and either double the amount of DNA, or 1 μL of a 1/10 dilution of the DNA was added. In addition, positive controls were 'spiked' with 1 μL of the test DNA to determine whether the DNA was inhibitory to the PCR.

Table 1 Phytoplasmas used in this study

Strain	Phytoplasma	Host plant	Origin	Accession[a] # 16SrRNA/SR
AAY	American aster yellows	*Catharanthus roseus*	USA	X68373
AGY	Australian grapevine yellows	Grapevine	Australia	X95706
AT	Apple proliferation	*C. roseus*	Germany	X68375
AY	Aster yellows	Aster	USA	
FBP	Faba bean phyllody	Faba bean	Thailand	
PDB	Papaya dieback	Papaya	Australia	Y10095/Y08176
PYL	*Phormium* yellow leaf	New Zealand flax	New Zealand	U43571
SGP	Strawberry green petal	Strawberry	Australia	AJ243044
SLY	Strawberry lethal yellows	Strawberry	Australia	AJ243045
SPLL	Sweet potato little leaf	Sweet potato	Australia	
SPLL-V4	Sweet potato little leaf strain V4	*C. roseus*	Australia	
StLL	*Stylosanthes* little leaf	*Stylosanthes*	Australia	
STOL	Stolbur of pepper	*C. roseus*	Serbia	X76427/AF035361
SUNHP	Sunn hemp witches' broom	*C. roseus*	Thailand	
TBB	Tomato big bud	Tomato	Australia	

[a]Accession number given only for those phytoplasmas included in sequence analysis.

Restriction enzyme digests of PCR products

Five to 10 μL of P1/P7 PCR products were digested with 4 or 5 U of *Alu*I or *Rsa*I (New England Biolabs, Berverly, MA, USA), respectively, overnight at 37°C in buffer supplied with the enzymes. Five to 10 μL of *tuf* PCR products were digested with 4 U of *Hpa*II or *Hin*dIII, overnight at 37°C in buffer supplied with the enzymes. Restriction enzyme digests were separated on 8% polyacrylamide gels as described by Davis *et al.* (1997b). The DNA was visualized under UV light and photographed for documentation.

In vitro amplification of phytoplasma DNA using specific primer pairs

The primer pair fTuf Ay and rTuf Ay (Schneider *et al.*, 1997) was used to amplify a 1000 bp fragment of the *tuf* gene. This primer pair is specific to members of the AY phytoplasma group (Schneider *et al.*, 1997) including the PDB phytoplasma (Gibb *et al.*, 1998). The primer pair P1 and rSPLLS (Schneider *et al.*, 1997) was used to amplify a 1640 bp fragment of the 16S rRNA gene and the 5′ end of the 16S−23S SR of phytoplasmas belonging to the faba bean phyllody (FBP) group, which includes the SPLL and TBB phytoplasmas. The primer pair fStol (Maixner *et al.*, 1995) and AGY 2 (Gibb *et al.*, 1998) amplifies a 600 bp region of the 16S rRNA and 16S−23S SR of the PDB and AGY phytoplasmas, but not other phytoplasmas in the AY and STOL phytoplasma groups (Gibb *et al.*, 1998). PCR conditions for use with the *tuf* primers were essentially the same as for universal detection, except that the PCR was run for 35 cycles with an annealing temperature of 53°C. Similarly, PCR with the P1/rSPLLS and fStol/AGY 2 primers was run for 35 cycles with an annealing temperature of 50°C.

Relative mobility of SR phytoplasma DNA as an indicator of mixed infections

Another approach to determine whether diseased strawberry plants harboured more than one phytoplasma was to study the relative mobilities of PCR amplification products obtained using primers that amplify the phytoplasma SR (Palmano & Firrao, 1998). All three SGP and five SLY samples, as well as 11 PDB phytoplasmas, were tested. Additional reference samples were SPLL-V4, TBB, PYL, STOL and AAY phytoplasmas. Phytoplasma DNA was amplified using the P3/P7 primer pair, and PCR amplified products were separated on an 8% polyacrylamide gel for 15 h at 110 V. The DNA was visualized under UV light and photographed for documentation.

Sequence analysis

PCR products were purified using PCR clean-up columns (Qiagen, Clifton Hill, Victoria, Australia) and the sequencing reactions were performed using the Big Dye Terminator kit as described by the manufacturer (Perkin-Elmer, Foster City, CA, USA). Sequences from the 16S rRNA gene and SR of the SGP and SLY phytoplasmas were compared with those of other phytoplasmas using the GCG 8·1.0 (Genetics Computer Group, Madison, WI, USA) program PILEUP accessed through the Australian National Genomic Information Centre, Sydney, Australia, and manual adjustments were made where necessary. Sequence similarities between the strawberry phytoplasma and other key phytoplasmas were calculated using the GCG program GAP. Phytoplasma 16S rDNA sequences used for GAP analysis, with accession numbers, are given in Table 1.

Results

In vitro amplification of phytoplasma DNA using universal primer pairs

All three of the SGP and two of the five SLY diseased samples gave the expected 1800 bp product when amplified by PCR with the fP1/rP7 universal primers. No products were amplified from the symptom-free strawberry sample. The three SLY diseased samples that did not give an amplification product were subjected to repeat PCR tests in which the DNA concentration was varied, but the result was still negative. The DNA was also used to spike positive controls, but there was no evidence of inhibition because the controls were still positive (data not shown). When these three SLY samples were subjected to nested PCR, an amplification product of 1000 bp was observed. The same-sized product was observed with SGP and SLY samples that had tested positive in the single-round PCR (results not shown).

Restriction enzyme digests of P1/P7 PCR products

When the P1/P7 PCR products were digested with the enzymes *Alu*I and *Rsa*I, the SGP and SLY samples gave the same restriction pattern as that for the reference PDB, PYL and AGY samples (Fig. 1). Similarly, when the nested PCR product was digested with *Alu*I and *Rsa*I, the restriction patterns were indistinguishable from PDB (data not shown).

In vitro amplification of phytoplasma DNA using the *tuf* primer pair

The *tuf* (f/rTuf Ay) primers amplify members of the AY phytoplasma group including the PDB, AGY and PYL phytoplasmas. To obtain further evidence that the SGP and SLY phytoplasmas also belong to this group, they were subjected to PCR using the *tuf* primers. All strawberry samples shown to be positive in the universal P1/P7 PCR, and the reference PDB phytoplasma, gave the expected 1000 bp product (Fig. 2). No product was amplified from either the reference SPLL-V4

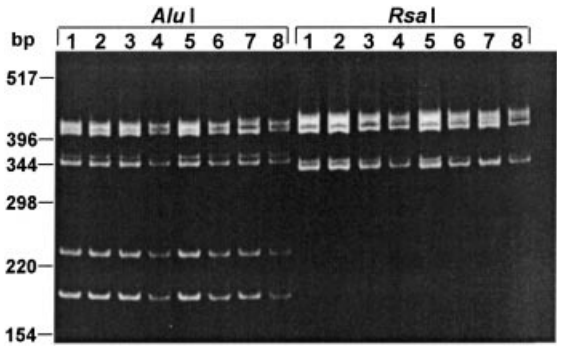

Figure 1 Restriction digestion of PCR products amplified using the primer pair fP1/rP7. The restriction enzymes are shown on the figure. DNA markers are not shown but are indicated by the sizes given in base pairs. For each restriction enzyme: lanes 1–3, SGP; lanes 4–5, SLY; lane 6, PDB; lane 7, PYL; lane 8, AGY.

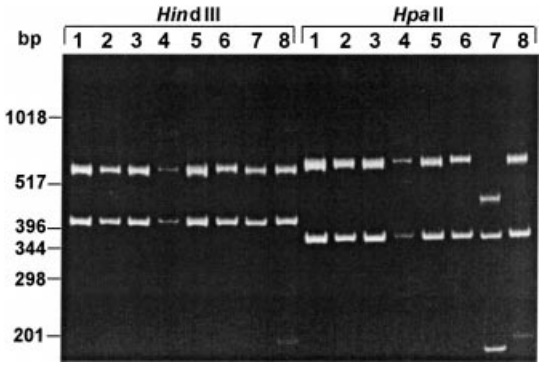

Figure 3 Restriction digestion of PCR products amplified using the primer pair fTufAY/rTufAY. The restriction enzymes are shown on the figure. DNA markers are not shown but are indicated by the sizes given in base pairs. For each restriction enzyme: lanes 1–3, SGP; lanes 4–5, SLY; lane 6, PDB; lane 7, PYL; lane 8, AGY.

phytoplasma (Fig. 2), which does not belong to the AY phytoplasma group, or from the symptom-free strawberry sample (not shown).

Restriction enzyme digests of *tuf* PCR products

When the 1000 bp *tuf* PCR products were digested with the enzymes *Hpa*II and *Hin*dIII, the SGP and SLY samples gave the same restriction pattern as the reference PDB and AGY samples (Fig. 3). The PYL sample gave a different restriction pattern for *Hpa*II.

Differential detection of phytoplasma DNA using specific primer pairs

None of the three SGP and five SLY diseased strawberries gave a product when subjected to the PCR using the primer pair fP1 and rSPLLS, which is specific to members of the Sunn hemp witches' broom (SUNHP) group, including the SPLL and TBB phytoplasmas. The PDB reference sample did not give a product, but the SPLL-V4 reference sample gave the expected 1640 bp product (Fig. 4).

The three SGP diseased strawberries and the PDB reference sample gave the expected 600 bp product when amplified by PCR using the fStol/rAGY 2 primer pair designed to amplify the PDB and AGY phytoplasmas. Of the five SLY samples, the two that were positive in the universal PCR test were also positive in the PCR test using fStol/rAGY 2. The SPLL reference sample and the three SLY samples shown to be negative in the universal test were also negative in the PDB/AGY-specific test (Fig. 4).

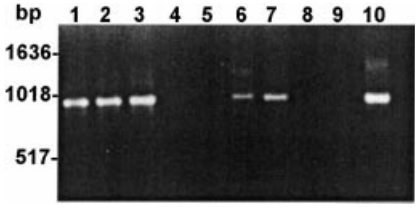

Figure 2 Amplification of phytoplasma DNA from strawberry with GP and LY diseases by PCR using the primer pair fTufAy/rTufAy which amplified the phytoplasma *tuf* gene. DNA markers are not shown but are indicated by the sizes given in base pairs. Lanes 1–3, SGP; lanes 4–8, SLY; lane 9, SPLL-V4; lane 10, PDB.

Relative mobility of SR phytoplasma DNA as an indicator of mixed infections

Following polyacrylamide gel electrophoresis of PCR products amplified using the SR primers fP3/rP7, all of the SGP and SLY diseased samples gave the expected 280 bp product. This included the three SLY samples that were negative in the single-round PCR tests. No products were amplified from symptom-free strawberry (Fig. 5).

The PCR amplified products from the PDB and PYL samples were the same size as the SGP and SLY products (Fig. 5). PYL gave a single band, but all the strawberry and papaya diseased samples gave a smaller, fainter band that ran just above the first main (280 bp) band (Fig. 5). STOL gave a single band slightly larger than 280 bp but smaller than the additional band seen for SGP, SLY and PDB samples (Fig. 5). The single AAY product was much larger at approximately 360 bp, and a single band was seen for both SPLL-V4 and TBB at approximately 330 bp (Fig. 5).

Of the three SLY samples that were negative in all other single-round PCR tests, one gave the typical double bands for the SR, but the other two had an additional larger band which had the same mobility as the SPLL-V4 and TBB phytoplasma SR (Fig. 5). Two PDB samples, one from Kununurra (Western Australia) and one from Katherine (Northern Territory), also gave the expected double bands and an additional band the same size as SPLL-V4 and TBB (Fig. 5).

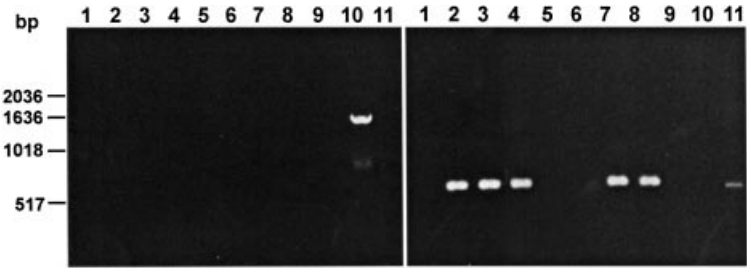

Figure 4 Amplification of phytoplasma DNA from strawberry with GP and LY diseases by PCR. The gel on the left is for the primer pair fP1/rSPLLs; on the right for the primer pair fSTOL/rAGY2. DNA markers are not shown but are indicated by the sizes given in base pairs. For both gels: lane 1, symptom-free strawberry; lanes 2–4, SGP; lanes 5–9, SLY; lane 10, TBB; lane 11, PDB.

Sequence analysis

Sequence analysis of the 16S rDNA gene indicated that the SGP and SLY phytoplasmas were most closely related to each other, and to the AGY, PDB and PYL phytoplasmas (Table 2). The next closest relative was the STOL phytoplasma. SR sequences gave the same results (data not shown).

Discussion

In single-round PCR tests using the universal P1/P7 primers, all three SGP samples were phytoplasma-positive compared with only two of the five SLY samples. Padovan *et al.* (1998) also reported that phytoplasmas were detected in strawberry plants with SGP and SLY diseases using a universal phytoplasma PCR test. The plants with SLY disease were severely affected and, at the time of extraction, were yellow and necrotic. This is in contrast to the plants with SGP, which were still green. It is possible that the poor state of the plant resulted in poor quality DNA that was not optimum for PCR testing. When the DNA was subjected to nested PCR, the remaining three SLY samples were shown to be phytoplasma-positive. While it is possible that the phytoplasma titre was low in these plants, or the DNA quality was not optimum, the added sensitivity afforded by nested PCR allowed amplification of phytoplasma DNA. This indicated that all of the SLY plants contained phytoplasma DNA.

Padovan *et al.* (1998) also reported that the phytoplasmas associated with SGP and SLY diseased strawberry plants were indistinguishable from P. australiense. These results are confirmed in the current study, in which the RFLP patterns of the P1/P7 PCR products were indistinguishable from P. australiense using AGY, PDB and PYL as reference samples. Sequence analysis of the 16S rDNA and adjacent SR also indicated that the phytoplasmas in diseased strawberry plants were most closely related to P. australiense.

For further investigation of the identity of the phytoplasma in strawberry, the primers used were based on the phytoplasma *tuf* gene, which encodes the elongation factor Tu and can be used for specific amplification of members of the aster yellows group (Schneider *et al.*, 1997). Gibb *et al.* (1998) showed that the PDB phytoplasma was amplified by the *tuf* primers, but phytoplasmas belonging to the FBP strain cluster (Schneider *et al.*, 1995a) were not amplified. The FBP strain cluster includes the TBB phytoplasma which occurs in a wide range of plant host species in Australia, and is associated with phyllody and bud distortion (Davis *et al.*, 1997b). Amplification of the appropriately sized PCR product using these *tuf* primers indicated that the diseased strawberry plants were infected by an aster yellows-type phytoplasma. The close relationship between the SGP, SLY, PDB and AGY phytoplasmas is further supported by the similarity of the *tuf* gene in RFLP analysis using the restriction enzymes *Hin*dIII and *Hpa*II. The PYL phytoplasma can be distinguished by *Hpa*II, as reported previously (Schneider *et al.*, 1997).

Gibb *et al.* (1998) showed that the fStol/rAGY2

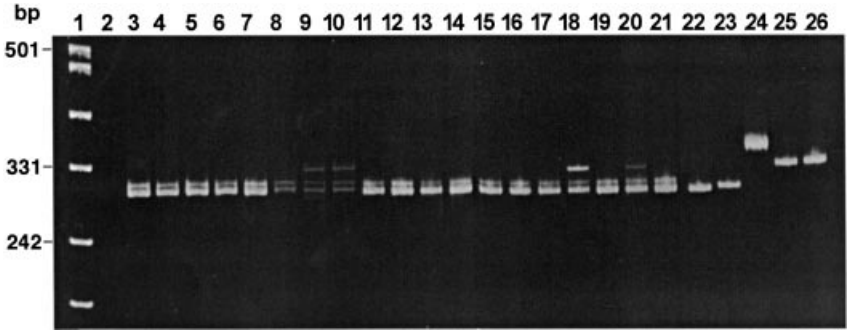

Figure 5 Amplification of phytoplasma DNA by PCR using the primer pair fP3/rP7. Lane 1, DNA marker pUC19/*Hpa*II (Bresatec); lane 2, symptom-free strawberry; lanes 3–5, SGP; lanes 6–10, SLY; lanes 11 and 21, PDB from Redlands; lanes 12, 14 and 15, PDB from Rockhampton; lane 13, PDB from Guralda; lanes 16 and 18, PDB from Kununurra; lanes 17, 19 and 20, PDB from Katherine; lane 22, PYL; lane 23, STOL; lane 24, AAY; lane 25, SPLL-V4; lane 26, TBB.

Table 2 Pairwise similarities (%) between SGP and SLY 16S rDNA sequences and a selection of reference phytoplasmas

	SGP	SLY	AGY	PDB	PYL	STOL	AAY
SLY	99·7						
AGY	99·8	99·6					
PDB	99·8	99·8	99·7				
PYL	99·6	99·6	99·5	99·8			
STOL	97·7	97·7	97·6	97·9	97·9		
AAY	95·5	95·5	95·5	95·7	96·4	95·1	
AT	91·6	91·7	91·6	91·9	91·7	91·5	91·6

Gap weight 5·0; gap weight length 0·3.

primer pair amplified an rDNA product from papaya with dieback and from grapevine with AGY. This primer pair, however, did not amplify products from plants infected by other members of the stolbur group (Gibb *et al*., 1998), a subgroup of the aster yellows group. The successful amplification of a product from SGP and SLY plants provides additional evidence that the phytoplasma associated with these diseases in strawberry is P. australiense.

One theory to explain the two symptom types in strawberry, green petal and lethal yellows, is that one disease may have been caused by a mixed infection with a different phytoplasma. The most likely candidate would have been the TBB phytoplasma, which is widespread in Australia (Davis *et al*., 1997b). A papaya with dieback, and positive for P. australiense, was found to have a mixed infection with the TBB phytoplasma only after the group-specific P1/rSPLLs primers were used (Gibb *et al*., 1998). This, however, did not seem to be the case for the strawberry samples, none of which gave a PCR product when amplified using the P1/rSPLLs primer pair.

To study the possibility of mixed infections further, the SR of SGP, SLY, PDB, PYL, STOL, AAY, SPLL-V4 and TBB samples were subjected to PCR and separated by polyacrylamide gel electrophoresis to resolve small size differences in SR lengths. From the authors' experience, the SR is easier to amplify than the 16S rRNA gene, so any recalcitrant phytoplasmas might be amplified using this method. All of the SGP and SLY diseased strawberries gave a product indicating they were infected with phytoplasma, and the plants without symptoms gave no product. Initially the two bands of approximately 270 and 280 bp observed for both SGP and SLY samples suggested a mixed infection. However, all the PDB phytoplasmas from different areas in Queensland and the Northern Territory also gave the same two bands. The PYL, STOL, AAY, SPLL-V4 and TBB phytoplasmas gave only one band. Phytoplasmas have two sets of ribosomal genes (Schneider & Seemüller, 1994; Liefting *et al*., 1996), and Liefting *et al*. (1996) reported sequence heterogeneity in the two 16S rRNA genes of the PYL phytoplasma. It is possible that the two bands seen for the SGP, SLY and PDB phytoplasmas indicates heterogeneity in the two SR genes. Liefting *et al*. (1996) showed no sequence differences in the two PYL SRs, and the present results support this because only one band was observed for PYL and the other reference phytoplasmas. The smaller of the two bands was sequenced in this study and it would be useful to sequence the larger band. In addition, Southern blot hybridizations could be performed on digested genomic DNA from infected strawberries using both P3/P7 bands as probes to identify whether intra-operon heterogeneity or the more unlikely obligate association of a second phytoplasma with P. australiense exists.

The three SLY samples found to be negative in first-round P1/P7 PCR were positive for phytoplasmas when subjected to PCR using primers that amplified only the SR. In addition, two of these samples gave a third, larger band that was the same size as the SPLL-V4 and TBB phytoplasmas. Two PDB samples also gave this third band. This indicated a mixed infection involving SPLL-V4 or TBB and P. australiense. More samples and a closer scrutiny of symptom development and progression in the field will be needed before any relationship can be shown between mixed infections and disease expression.

Sequence analysis of the SGP and SLY 16S rDNA gene showed they shared almost 100% similarity to each other and to AGY, PDB and PYL, confirming their designation as P. australiense. Both SGP and SLY showed about 98% similarity to the stolbur phytoplasma, a representative of the next closest phylogenetic group, with similarities reduced with more distant phytoplasmas such as American aster yellows (96%) and apple proliferation (92%). Similarly, analyses based on SR sequences showed the close relationship of SGP and SLY to each other and to other members of the P. australiense group (results not shown).

Honetslegrova *et al*. (1996) reported that SGP disease in the Czech Republic was associated with a phytoplasma from the aster yellows group (16SrI) subgroup I-C, of which the type strain is the clover phyllody/strawberry green-petal phytoplasma. Harrison *et al*. (1997) reported three phytoplasmas associated with diseased strawberry plants in Florida belonging to the aster yellows group 16SrI subgroups I-C, I-I and a new subgroup. Jomantiene *et al*. (1998) confirmed the association between the 16SrI group phytoplasmas and strawberry diseases, but neither phytoplasma detected in this study belonged to subgroups C or I. Instead, one phytoplasma was classified as belonging to a new subgroup K, of which the type strain is the strawberry multiplier phytoplasma, and the other belonged to subgroup J.

Davis *et al*. (1997a) classified the AGY phytoplasma as a representative of a new subgroup J, within the aster yellows 16SrI group, and reported that its closest relative was the European stolbur phytoplasma, subgroup 16SrI-G. Other workers have also confirmed that the PDB, AGY and PYL phytoplasmas are most closely related to the German grapevine yellows and STOL phytoplasmas (Liefting *et al*., 1996; Padovan *et al*.,

1996; White *et al*., 1997; Gibb *et al*., 1998). More recently, Davis & Sinclair (1998) represented the AGY phytoplasma as a member of the stolbur 16SrXII group, subgroup B, and designated the *Candidatus* species name, '*Candidatus* Phytoplasma australiense'. Liefting *et al*. (1998) showed that P. australiense is the phytoplasma associated with AGY, PDB and PYL diseases. Andersen *et al*. (1998) showed that the PYL phytoplasma is associated with strawberry lethal yellows in New Zealand. The present results support the view that the Australian and New Zealand strawberry lethal yellows diseases are caused by the same phytoplasma (Andersen *et al*., 1998). It is interesting that Davis *et al*. (1997a) originally placed the AGY phytoplasma in the new 16SrRNA group I-J, which is where Jomantiene *et al*. (1998) placed one of the phytoplasmas detected in diseased strawberry plants in Florida. If this phytoplasma in strawberry is indeed similar to *Candidatus* P. australiense then the notion that this phytoplasma species is endemic to New Zealand and/or Australia may be questionable. Is it possible that a cultivated host such as strawberry, varieties of which are distributed throughout the world, is the key to the original distribution and then the relative isolation of P. australiense?

Liefting *et al*. (1998) reported that the phytoplasmas within the *Candidatus* P. australiense species, as defined by 16S rRNA gene sequences, can be further differentiated by analysis of the *tuf* gene sequences. The present findings support this, but we also found differences in the 16S/23S SR, indicating that PYL phytoplasma from New Zealand is not identical to PDB, SGP and SLY phytoplasmas from Australia. It may be true that the same phytoplasma species gave rise to the species now associated with AGY, PDB, PYL, SLY and SGP diseases. It is also possible that the phytoplasma species now in New Zealand has been separated from the species in Australia for some time, long enough for these differences to arise and suggesting that no recent re-infections have occurred between the two countries. In this situation it would be useful to have a subspecies concept in phytoplasma taxonomy to accommodate these observed differences. Although this group, along with other workers, is proposing that the PYL, AGY, PDB, SLY and SGP diseases are all associated with the *Candidatus* P. australiense group 16SrXII (stolbur group) subgroup B, there are no means for showing that the species associated with PYL is slightly different.

Strawberry plants used in this study showed two different symptoms: green petal and lethal yellows, and there is no indication that the two diseases are associated with different phytoplasmas. The evidence for mixed infections cannot be related to any particular symptom type. A rickettsia-associated lethal yellows disease has also been reported (Greber & Gowanlock, 1979), but electron micrographs showed no rickettsia particles in the diseased strawberry plants used in this study that tested positive for phytoplasma. Recently, strawberry plants with SLY collected from the field were PCR-negative when phytoplasma-specific primers were used. Degenerative rickettsia particles were, however, observed in the sieve tubes of plants with symptoms (D.H. Gowanlock, personal communication). This suggests that the symptoms characteristic of SLY disease in strawberry can be associated with two different organisms. It is also possible that one phytoplasma may cause very different symptoms; factors that may account for the differences include the age of the plant at time of infection, the level of phytoplasma inoculated at infection, and environmental factors including temperature. It is also possible that symptoms of SGP and SLY represent disease progression. To answer some of these questions it will be important to screen a large number of SGP- and SLY-diseased plants using phytoplasma and rickettsia-specific diagnostics, to monitor symptoms on these plants throughout a growing season and search for insect vectors.

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