Phormium Yellow Leaf Phytoplasma Is Associated with Strawberry Lethal Yellows Disease in New Zealand

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ABSTRACT

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A yellows disease of strawberry plants was identified in propagation beds in New Zealand. Affected plants were flatter to the ground, showed purpling of older leaves, reduced leaf size, yellowing of younger leaves, and sometimes plant death. A phytoplasma was observed in the phloem of affected plants. The 16S rRNA gene of the phytoplasma was amplified by polymerase chain reaction from symptomatic plants and from one asymptomatic plant, but not from 36 other asymptomatic plants. Nucleotide sequence analysis of the 16S rRNA gene showed that the phytoplasma is closely related or identical to the phytoplasma associated with the yellow leaf disease of New Zealand flax (*Phormium tenax*).

A number of diseases ascribed to phytoplasmas, rickettsialike (16), and bacteri-like (23) organisms have been reported infecting strawberry plants worldwide. The most comprehensively reported phytoplasmas include aster yellows (6) and strawberry green petal (17,18,28). Other diseases in which phytoplasmas have been implicated include bronze leaf wilt (7), lethal decline (30), lethal yellows-mycoplasmal yellows (15,16,34), big bud-little leaf (32), multiplier disease, and witches'-broom (8,27).

Of these diseases, strawberry green petal has been reported in New Zealand (2). In addition, a yellows-type disorder has been reported in runner beds propagated by specialist growers in the Katikati district, Bay of Plenty (26). Affected plants of cvs. Douglas and Tioga characteristically lacked vigor in the field. Under glasshouse conditions, affected plants rapidly degenerated, resulting in premature death (25). The disorder was shown by electron microscopy to be associated with a phytoplasma (26), but the identity of the phytoplasma was not determined. Despite changes

in strawberry cultivars (cv. Pajaro is currently preferred in New Zealand), the yellows disorder still occurs in runner beds.

This study shows that the symptoms of the yellows disorder in New Zealand are most similar to the mycoplasma yellows and lethal yellows disorders described in strawberries in Australia (16,32,34). We also show that the phytoplasma associated with the disease in New Zealand is closely related to, or identical to, the phytoplasmas associated with phormium yellow leaf (PYL), Australian grapevine yellows, and papaya dieback.

MATERIALS AND METHODS

Source of plants. Sixteen strawberry plants of cv. Pajaro were obtained from runner beds, of which 7 were symptomatic, 8 were asymptomatic, and 1 showed slight reddening of the outer leaves, possibly representing an early stage of infection. Twenty-nine asymptomatic, nuclear stock plants from meshed, insect-free greenhouses also were tested.

Electron microscopy. The petiole of strawberry plants was cut into 5-mm pieces and fixed in 2% freshly prepared paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 under vacuum for 1 h. The samples were then washed three times in buffer, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in an ethanol series, infiltrated, and embedded in Spurr's resin. The phloem region of the petiole was sectioned and stained in aqueous uranyl acetate and lead citrate. Sections were viewed on a JEM-1200EX II transmission electron microscope (JEOL, Tokyo) operating at 80 kV.

Extraction of nucleic acid from plants. DNA was extracted from plant tissue by modification of the "hot cetyltrimethylammonium bromide (CTAB)" method of Doyle & Doyle (10), combined with a high salt ethanol precipitation step (11). Approximately 1 g tissue was frozen in liquid nitrogen, then ground in a pre-chilled mortar and pestle. The powdered tissue was incubated in 10 ml pre-heated extraction buffer (0.14 M sorbitol, 0.22 M TrisHCl pH 8.0, 22 mM EDTA pH 8.0, 0.8 M NaCl, 0.8% wt/vol CTAB, 1% N-lauryl sarcosine) and incubated for 20 to 30 min at 65°C with occasional shaking. Samples were extracted twice with equal volumes of phenol and chloroform, and once with an equal volume of chloroform. NaCl was added to a final concentration of 2 M. Two volumes of ethanol were added and samples were kept at -20°C overnight. Samples were centrifuged at $7,500 \times g$ for 20 min (4°C), and the pellet washed with 2 ml 70% ethanol and dried under vacuum for 5 to 10 min. DNA pellets were resuspended in 1.0 ml distilled water.

Polymerase chain reaction. Primers used in this study are listed in Table 1. Primers R16F2 and R16R2 (20), and NGF, NGR, and NP6A (21) were designed to be "universal" phytoplasma primers. Primers M1 and M3 (21) were designed to be specific to the phytoplasmas associated with PYL, Australian grapevine yellows, and papaya dieback diseases. Primers Gd1 and Berg 54 (1) and R4 (21) were designed to be universal prokaryotic 16S rRNA gene primers. Primers Gd1 and Berg54 amplified products from both healthy and infected plants, and were used to establish the polymerase chain reaction (PCR) competence of each DNA sample.

Each PCR reaction contained 50 mM Tris-HCl pH 9.0, 20 mM ammonium sulfate, and 2.5 mM MgCl₂. Template DNA was used at 10 to 50 ng for single-step PCR reactions. For nested PCR, products of the primary amplification step were diluted 1:25 prior to reamplification. Primers were used at a concentration of 400 nM. Amplification involved an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C, 30 s; 55°C, 15 s; and 72°C, 1 min in a Corbett FTS-1S Capillary Thermal Sequencer. All sets of reactions included DNA samples from

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healthy plants and controls lacking template DNA.

Analysis of PCR amplification products. PCR products were analyzed by electrophoresis on 1.5% agarose gels in 1× Tris-borate-EDTA (TBE) buffer (67 mM Tris-HCl; 22 mM boric acid; 1 mM EDTA,

pH 8.8). Gels were stained with ethidium bromide according to standard protocols (29).

Sequencing of PCR amplification products. PCR products were electro-eluted from agarose gel slices and sequenced using an ABI Prism 377 DNA

Sequencer. PCR for DNA sequencing was performed in an ERICOMP Easy with the following conditions: 94°C 1 min, one cycle; 95°C 30 s, 50°C 15 s, 60°C 4 min, for 25 cycles; 95°C 30 s, 50°C 3 min, for 15 cycles; then 60°C 10 min for one cycle.

RESULTS

Field symptoms. Infected plants became readily apparent in the field: they were flatter to the ground than adjacent healthy plants and their foliage growth more open. As the disease progressed, the older leaves became purple-bronze in color, particularly toward the margin. Young leaves were smaller than normal, with shortened petioles and a conspicuous chlorosis, initially occurring around the leaf margin, but later covering much of the leaf area (Fig. 1A). The production of flowers and fruit was inhibited, although flowers and fruit which did develop were not distorted. Leaves on runners were small, curled, and twisted, light yellow in color, and formed rosettes (Fig. 1B). The disease was sometimes lethal, although plants often remained alive but failed to make any significant growth.

Electron microscopy. Electron microscopy was undertaken to confirm the association reported by Patel and Milne (26) between disease symptoms and presence of a phytoplasma. In our study and in the study by Patel and Milne (26), phytoplasmas were observed in immature and mature sieve cells (Fig. 2). In some cells, large concentrations of phytoplasmas were observed, while in other cells the phytoplasmas were mainly concentrated along cell walls. Five structural categories of phytoplasmas, elementary bodies, intermediate and filamentous forms, mature cells, and budding cells were observed as reported elsewhere (33). Filamentous forms were usually observed near sieve plate pores, as reported in other studies (3,31).

PCR amplification of the 16S rRNA gene. A PCR product of 1.6 kilobase (kb) was amplified from each strawberry plant using the universal 16S rRNA primers Gd1 and Berg 54, indicating that all DNA samples were competent for PCR. Products of approximately 500 base pairs (bp) were obtained by nested PCR from 7 symptomatic plants using the phytoplasma-specific primers R16F2-R16R2, and NGF-NGR (Fig 3A). PCR products were also amplified from a plant showing slight reddening of the outer leaves and from an asymptomatic plant, presumably indicating that both were at an early stage of infection. PCR products were not obtained from 8 other asymptomatic plants from the field or from 29 nuclear stock plants. All plants which tested positive using the phytoplasma-specific primers also tested positive with the PYL-specific primers M1 to M3 (Fig 3B)

Nucleotide sequence. Nucleotide sequence analysis of a 713-bp fragment am-

Fig 1. Symptoms of strawberry lethal yellows on cv. Pajaro. (A) The older leaves display purple-bronzing and the younger leaves show a pronounced marginal chlorosis. (B) Characteristic symptoms on leaves of stoloniferous plants. Leaves are twisted and curled into rosettes. A healthy sample is shown for comparison (left).

plified from a diseased strawberry plant using primers R4-NP6A revealed nucleotide polymorphisms at positions 1151 (C/T) and 1432 (A/G) (numbering according to Liefing et al.; 21). At all other positions, the nucleotide sequence of the PCR product was identical to that of PYL. The two polymorphisms correspond to the differences between operons *rrnA* and *rrnB* of PYL phytoplasma (21). Based on these results, we conclude that strawberry lethal yellows and PYL phytoplasma are either closely related or identical.

DISCUSSION

Our results confirm the earlier studies of Patel and Milne (26) that the yellows disease of New Zealand strawberry is probably caused by a phytoplasma. Further, our results show that the phytoplasma is closely related, or identical, to PYL, a phytoplasma previously recorded in New Zealand only in New Zealand flax (*Phormium tenax*, *P. cookianum*; 5). PYL disease was first recorded in New Zealand in 1908, and it contributed to the eventual demise of the once-thriving fiber industry based on New Zealand flax (5). PYL phytoplasma is transmitted by the mono-

phagous cixid plant hopper *Oliarus atkinsoni*, a species occurring only in New Zealand. Nymphs of this hopper develop in the rhizomous regions of the plant; this is the region in New Zealand flax plants with the highest concentrations of phytoplasma (35). This close association, and the existence of tolerant forms of *P. tenax*, suggest a long association between phytoplasma, host, and vector (4). The narrow host range of *O. atkinsoni* suggest that another insect species is involved in the spread of PYL into strawberry. Two possible candidates, the bramble leafhopper *Ribautiana tenerima* (Herrich-Schaeffer) and the yellow pasture leafhopper *Zygina zealandica* (Myers), have been observed on strawberry in New Zealand (25).

The symptoms on strawberry in New Zealand show some similarities to those associated with strawberry lethal decline in North America (30). Lethal decline is possibly associated with peach X-disease (12). However, peach X-disease, a serious disease of stone fruits in North America (14,19), has not been recorded in stone fruit in New Zealand (37), and the peach X-disease phytoplasma is taxonomically distinct from PYL phytoplasma (21). It

seems unlikely, therefore, that strawberry lethal yellows and strawberry lethal decline are the same disease.

The symptoms of the yellows disease in New Zealand appear to most closely resemble those described in strawberries in Australia for three similar or identical diseases, mycoplasma yellows (16), lethal yellows (34), and little leaf disease (32). There is a high degree of nucleotide sequence homology between the phytoplasmas associated with PYL in New Zealand and the phytoplasmas associated with Australian grapevine yellows and papaya dieback in Australia (13,24,36). On this basis, we have proposed that all three diseases may be caused by the same phytoplasma (Liefing et al, unpublished). The name "*Candidatus* Phytoplasma aus-

Fig. 2. Transmission electron micrograph of strawberry phloem tissue infected with pleomorphic phytoplasmas. "C" indicates the phloem companion cell. The bar represents 500 nm.

Fig. 3. Agarose gel (1.5%) electrophoresis of nested-polymerase chain reaction (PCR) products of (lanes 1-3) three infected and (lanes 4 and 5) two healthy strawberry plants amplified using nested-PCR. The universal phytoplasma primers R16F2-R16R2 were used for the primary amplification step, and universal phytoplasma primers (A) NGF-NGR or (B) "*Candidatus* Phytoplasma australiense" group-specific primers M1-M3 for the re-amplification step. Marker (M) was 100-base pair ladder (Life Technologies).

Table 1. List of primers for polymerase chain reaction

Primer name	Sequence (5' - 3')	Position ^a	Reference
Gd1	ACG GAG AGT TTG ATC CTG	3 → 20	1
Berg54	AAA GGA GGT GAT CCA GCC GCA CCT TC	1535 → 1510	1
R16F2	ACG ACT GCT AAG ACT GG	152 → 168	20
R16R2	TGA CGG GCC GTG TGT ACA AAC CCC G	1397 → 1373	20
NGF	AGG CGG CTT GCT GGG TCT T	725 → 743	21
NGR	AGC CAT TGT ATC ACG TT	1231 → 1215	21
M1	GAA GGC ATC TTT TTA TC	182 → 198	21
M3	GTA CCG TCA TAA TGG	485 → 471	21
R4	GGG TTA AGT CCC GCA ACG AG	1073 → 1092	21
NP6A	TTA CGA CTT AAC CCC AA	1490 → 1473	21

^a Positions based on the sequence of the phytoplasma associated with phyllody of *Oenothera hookeri* (22).

traliense" has been proposed for this phytoplasma (9). In view of the fact that similar or identical phytoplasmas occur in both Australia and New Zealand, it is possible that the yellows diseases of strawberry in both countries are also caused by the same phytoplasma. As infected plants develop a yellows disease, and as the disease is often lethal, we have used the name strawberry lethal yellows, first proposed by Stubbs (34), to describe the disease in New Zealand.

The lethal yellows diseases in both Australia and New Zealand are currently managed by roguing of diseased plants (15). In propagation beds in New Zealand, infection levels are usually less than 10%, and infected plants are predominantly found around the perimeter of blocks. This suggests that further spread through blocks is restricted by current insecticide spray regimes. A knowledge of the vector(s) and alternate hosts may further assist in control.

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