Association of “*Candidatus Phytoplasma australiense*” with Sudden Decline of Cabbage Tree in New Zealand

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ABSTRACT

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Sudden decline of the New Zealand cabbage tree (*Cordyle australis*) results in the rapid death of affected plants within months of first external symptoms becoming apparent. Symptoms, which have been observed in saplings and mature trees, include vascular discoloration and leaf yellowing followed by leaf desiccation and eventual plant collapse. Previous work failed to link the disease with any causal agent. A phytoplasma has now been detected in all symptomatic saplings and some symptomatic trees tested, using one-step and nested polymerase chain reaction (PCR) to amplify portions of the 16S rRNA gene. This phytoplasma was not detected in nonsymptomatic plants. Phytoplasma DNA was found in shoot and rhizome apices, leaves and wood tissue of saplings, and in the rhizome apex and trunk tissues of adult trees. Sequencing of the PCR products from selected samples indicated that the phytoplasma is “*Candidatus Phytoplasma australiense*.” Phytoplasma cells were detected by transmission electron microscopy in phloem sieve tubes of the rhizomes of affected saplings. One sapling with early symptoms recovered after injection with tetracycline antibiotic, but two saplings with advanced symptoms did not recover. It is concluded that “*Candidatus Phytoplasma australiense*” is present in symptomatic plants and is the cause of sudden decline.

The cabbage tree (ti kouka, *Cordyle australis*) is an arborescent tufted monocotyledon in the liliaceous family Lomandraceae. It is endemic to New Zealand, where it is widespread throughout, occurring on forest margins and in wetlands. Additionally, it is now widely cultivated as an ornamental both in New Zealand and in temperate regions throughout the world. During the late 1980s, large numbers of cabbage trees began dying in the North Island and northern South Island of New Zealand, causing widespread concern (25). The disease was named sudden decline in recognition of the rapid death of affected trees. It was subsequently distinguished from rural decline, a slower decline in plant vigor occasionally associated with

plant death, and it was proposed that sudden decline is a biotic disease and rural decline a complex disease, or “decline disease” *sensu* Manion (19), caused by various biotic and abiotic agents interacting with an aging plant population (5). However, while this initial work suggested that a biotic agent was responsible for sudden decline, it failed to implicate any of a range of pathogens including viroids, viruses, culturable bacteria, fungi, or nematodes. Because of this, and because of the similarities between the symptoms of the disease and a range of recognized phytoplasma diseases, it was hypothesized that sudden decline was a phytoplasma disease, despite the absence of direct evidence (5,25). The present study reports the presence of a phytoplasma in affected plants and shows that this phytoplasma is “*Candidatus Phytoplasma australiense*,” a taxon described from grape in Australia (9) and known in New Zealand on a number of hosts (4,16). We conclude that this phytoplasma is the cause of sudden decline.

MATERIALS AND METHODS

Symptom observations and plant material. Most observations of disease

symptoms in saplings were made in a block of 240 plants grown from seed and planted at 2-m spacings at Unitech Institute of Technology, Auckland, New Zealand (latitude 36° 53' S, longitude 174° 43' E). These were augmented by observations of saplings throughout the North Island. Observations on trees, defined as adult plants that have flowered for the first time, usually evidenced by trunk branching at 1 to 3 m, were made on cultivated and naturally occurring plants throughout the North Island and northern South Island.

Tissue samples were excised from leaves, shoot apices, branches, trunks, roots, and rhizomes. In some instances, a plant was removed from the ground and dissected; in other instances, restricted sampling allowed the ongoing health of the plant to be monitored. Samples of leaf petiole were comprised of petiolar tissue extending 2 to 3 cm up from the point of attachment; leaf lamina samples were comprised of 2- to 3-cm-wide portions taken across the whole leaf approximately 10 cm from the stem attachment; shoot samples were comprised of shoot apical meristematic tissue extending 1 to 2 cm from the apex, or a sample of the stem cylinder at the distance indicated back from the shoot apex; wood samples were comprised of portions of tissue 2 to 3 cm square by 0.5 cm deep sampled from the outermost portion of the vascular cylinder at the distances indicated from the shoot apex; rhizome samples were comprised of the rhizome apical meristematic tissue extending 1 to 2 cm from the apex, or at the distance indicated from the rhizome apex; root samples were comprised of the apical 5- to 10-cm corticated portions of three to five roots. Severely stained and necrotic tissues likely to contain saprotrophic organisms were avoided. Tissue samples of symptomatic and nonsymptomatic saplings were from plants at the Unitech site or nearby (plant COR6). In addition, nonsymptomatic saplings grown in an insect-proof shadehouse were sampled. Symptomatic and nonsymptomatic trees were from the Auckland city area. Plants sampled for analysis are numbered using the prefix COR (Cordyle).

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Extraction of nucleic acid. DNA was extracted according to the protocol of Gardiner et al. (10). Approximately 0.3 g of tissue was ground with a mortar and pestle to a powder in liquid nitrogen, mixed with 2.5 ml of extraction buffer (140 mM sorbitol, 220 mM Tris, pH 7.5, 20 mM EDTA, 0.8 M NaCl, 0.8% wt/vol CTAB, 1% wt/vol *N*-lauroylsarcosine), and 400 µl of CHCl₃:octanol (24:1) was added. After incubation at 65°C for 30 min, samples were chilled on ice for 10 min and microfuged at 13,000 × *g* for 10 min. The nucleic acids in the supernatant (1 ml) were precipitated with an equal volume of ice cold isopropanol. Samples were left on ice for 30 min (or overnight at -20°C) then microfuged at 13,000 × *g* for 5 min. The resulting pellet was washed with 1 ml of 70% ethanol, drained, dried under vacuum, then resuspended in 100 µl of H₂O. The DNA was further purified using an S300HR column as specified by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ).

Polymerase chain reaction (PCR). The "universal" prokaryotic 16S rRNA gene primers Gd1 and Berg 54, which are expected to amplify organelle DNA from both healthy and infected plants, were used with all DNA samples to establish that they were PCR competent (1). All samples reported in Tables 1 to 3 were PCR competent. Primer pairs R16F2 + R16R2, and NGF + NGR are universal phytoplasma primers designed to amplify all phytoplasma 16S rDNA producing products of 1.2 kbp in one-step PCR and 500 bp in nested PCR, respectively (1).

Each PCR reaction contained 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂, 50 mM KCl, 200 µM dNTPs (Life Technologies, Rockville, MD) and 0.5 U *Taq* polymerase (Amersham) in 20 µl final volume. Template DNA was used at 10 to 50 ng for one-step PCR reactions. For nested PCR, products of the primary amplification step were diluted 1:25 before reamplification. Primers were used at a concentration of 400 nM. Amplification conditions were 92°C for 2 min, 51°C for 1 min, and 72°C for 2 min for 1 cycle, followed by 35 cycles of 92°C for 45 s, 51°C for 1 min, and 72°C for 2 min in a Corbett FTS-1S Capillary Thermal Sequencer.

Analysis of PCR amplification products. PCR products were analyzed by electrophoresis on 1.5% agarose gels in 1× 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 (26). All sets of reactions contained numerous controls lacking template DNA. While special care was taken to avoid contamination, minus DNA controls occasionally gave a band after nested PCR. In these instances, all samples in the set were disregarded. All samples giving an appropriately sized band only after nested PCR were retested, and

only samples giving bands in at least two independent reactions were scored as positive. In some samples, the band visible after one-step PCR was very faint and was difficult to record in our data capture system. Such samples were recorded as (+).

Selected PCR products were sequenced using a dye terminating kit (ABI) and resolved on an ABI Prism 377 DNA Sequencer according to the manufacturer's protocols. PCR for DNA sequencing was performed in an ERICOMP Easy with the following conditions: 94°C for 1 min, one

cycle; 95°C for 30 s, 50°C for 15 s, 60°C for 4 min, for 25 cycles; 95°C for 30 s, 50°C for 3 min, for 15 cycles; then 60°C for 10 min for one cycle.

All nucleotide positions given in this paper correspond to the numbering of *Oenothera* aster yellows (OAY) phytoplasma (17). Samples were compared with the 16S rRNA operons of the *Phormium* yellow leaf (PYL) phytoplasma (GenBank accession numbers U43569 and U43570) (14) and Australian grapevine yellows (AUSGY) phytoplasma (9) using Gap,

Table 1. Detection of phytoplasma DNA in tissues from two sapling cabbage trees at different stages of symptom expression^a

	COR1 (early stage)		COR2 (middle stage)	
	PCR 1 ^b	PCR 2 ^c	PCR 1	PCR 2
Leaf petiole	—	+	+	+
Leaf lamina	—	—	+	+
Shoot apex	(+)	+	+	+
Shoot (5 cm)	—	+	NT ^d	NT
Wood (20 cm)	—	—	+	+
Wood (60 cm)	—	+	+	+
Rhizome A apex	(+)	+	+	+
Rhizome A (10 cm)	—	+	+	+
Rhizome B apex	—	+	NA ^e	NA
Roots	+	+	+	+

^a Polymerase chain reaction (PCR) product present "+", absent "—", or "(+)" present but faint.

^b One-step PCR with phytoplasma primers R16F2 and R16R2.

^c Nested PCR with R16F2/R2 as primers for the initial step and NGF/NGR for the second step.

^d Not tested.

^e Not available as only one rhizome present on plant.

Table 2. Detection of phytoplasma DNA in tissues from sapling cabbage trees at different stages of symptom expression^a

Plant no.	Shoot apex		Rhizome apex	
	PCR 1 ^b	PCR 2 ^c	PCR 1	PCR 2
Symptomatic (stage)				
COR1 (early)	(+)	+	(+)	+
COR2 (middle)	+	+	+	+
COR3 (middle)	—	+	(+)	+
COR4 (middle)	—	+	+	+
COR5 (late)	—	+	+	+
COR6 (late)	—	—	+	+
Nonsymptomatic from field				
COR7	—	—	—	—
COR8	—	—	—	—
COR9	—	—	—	—
Nonsymptomatic from insect-proof shadehouse				
COR10	—	—	—	—
COR11	—	—	—	—
COR12	—	—	—	—

^a Polymerase chain reaction (PCR) product present "+", absent "—", or "(+)" present but faint.

^b One-step PCR with phytoplasma primers R16F2 and R16R2.

^c Nested PCR with R16F2/R2 as primers for the initial step and NGF/NGR for the second step.

Table 3. Detection of phytoplasma DNA in tissues from symptomatic adult cabbage trees at different stages of symptom expression^a

Symptom stage	Plant no.	Shoot apex		Wood		Rhizome apex	
		PCR 1 ^b	PCR 2 ^c	PCR 1	PCR 2	PCR 1	PCR 2
Early	COR13	—	+	NT ^d	NT	NT	NT
	COR14	—	—	—	+	NT	NT
	COR15	—	—	—	+	NT	NT
	COR16	—	—	—	+	NT	NT
Middle	COR17	—	+	NT	NT	—	+
	COR18	—	—	—	—	—	+

^a Polymerase chain reaction (PCR) product present "+", or absent "—".

^b One-step PCR with phytoplasma primers R16F2 and R16R2.

^c Nested PCR with R16F2/R2 as primers for the initial step and NGF/NGR for the second step.

^d Not tested.

Fig. 1. Symptomatology of sudden decline of cabbage tree at various stages. **A**, Early-stage sapling showing yellowing of some leaves and tipping of the apex. **B**, Early-stage sapling showing bands of yellowing extending along the leaves. **C**, Crown of leaves of a mid-stage sapling showing intensely yellowed leaves surrounding central tuft of green leaves. **D**, Mid-stage sapling dug from ground showing yellowed leaves and vigorous root growth. **E-F**, Advanced-stage sapling showing desiccated leaves. **G**, Dead sapling. **H**, Longitudinal section of an early-stage sapling trunk in the vicinity of yellowing leaves, showing discoloration of the vascular traces. **I**, Longitudinal section of the apex of a mid-stage sapling showing vascular discoloration. **J**, Longitudinal section of the rhizome of mid-stage sapling showing vascular discoloration and cortical browning. **K-L**, Tree photographed in September 1992 (early stage) and 2 months later (late stage) showing rapidity of leaf loss. **M**, Subsequently affected branch of mid-stage tree showing intense yellowing and subsequent desiccation of older leaves. **N**, Tangential longitudinal section of branches of mid-stage tree showing unaffected wood (left) and water-stained wood (right). **O**, Tangential longitudinal section of the lower trunk of a mid-stage affected tree showing discoloration of the vascular tissue. **P**, Lower trunk of advanced-stage tree with bark removed showing cortical damage.

Bestfit, and Pileup programs (Wisconsin Package Version 10.1, Genetics Computer Group, Madison, WI).

Tetracycline injection. Holes (1 cm diameter and 3 cm deep) were drilled into the trunk at 5 cm and 30 cm above ground level, and 50 ml of tetracycline solution (oxytetracycline at 3.75 g/liter in 1% citric acid) was injected into each hole using a hand-operated pressure gun, giving a total of 375 mg per plant, and the holes were plugged with corks.

Electron microscopy. Tissue was cut into $5 \times 1 \times 1$ mm pieces and fixed in freshly prepared 2% 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, postfixed in 1% osmium tetroxide for 1 h, dehydrated in an ethanol series, infiltrated and embedded in Spurr's resin. The vascular traces were sectioned and stained in aqueous uranyl acetate and lead citrate. Sections were viewed on a JEOL (Tokyo) JEM-1200EX II transmission electron microscope operating at 80 kV.

RESULTS

Symptoms. The pattern of disease symptoms previously reported (5,25) was based on observations of trees growing naturally in forest and shrublands and as remnants in highly modified pastureland, as well as in cultivation in urban gardens. Elucidation of the pattern of symptom expression was hampered by the sporadic occurrence and varied size of affected trees. Relatively few affected saplings were observed in this initial work, and the nature of symptoms in such plants was not clarified. The availability of large numbers of uniformly growing saplings has allowed clarification of the symptoms in plants at this stage. A total of 32 saplings were observed dying at the Unitech site over the period from late 1997 to early 2000, at which time they were between 3 and 6 years old. Affected plants were scattered throughout the block. The first visible symptom recognized in saplings was an abnormal tipping of the primary shoot apex to one side, at least sometimes associated with a slowing of length growth of newly expanding leaves. Leaves subsequently turned abnormally yellow, usually from the tip, often beginning with leaves on the inward side of the trunk (Fig. 1A); in some affected leaves, yellowing was restricted to one side of the leaf (Fig. 1B). In many instances, leaf yellowing spread through most of the older leaves (Fig. 1C and D). Affected leaves eventually turned brown and desiccated, with browning often extending in longitudinal stripes and sometimes initially affecting one side of the leaf (Fig. 1E and F). In plants affected during periods of summer water stress (January to March), the yellowing phase was relatively transient (Fig. 1E). Most of the affected leaves remain attached to the dead trunk

(Fig. 1G). The period of time from detection of the first yellowing symptoms to plant death ranged from 1 to 3 months. Examination of sapling trunk tissue in the vicinity of the first-affected leaves showed discoloration of the vascular bundles (Fig. 1H). This vascular discoloration spreads both up the trunk to the apex (Fig. 1I), where lenses of reddish brown interstitial tissue sometimes occur, and down the trunk. The trunk cortical tissue becomes brown and somewhat slimy, initially in the region of the first-affected leaves but later spreading to the petiole bases of the leaves, and subsequently throughout the trunk. Saplings have one major rhizome, which occasionally branches to give some subsidiary rhizomes and bears a large number of radiating roots. No distinct symptoms were consistently detected in this part of the plant in the early stages of the disease, but cortical browning and staining of vascular bundles and adjacent interstitial tissues occurred, especially in plants showing advanced stem symptoms (Fig. 1J). We recognize three stages of symptom expression in saplings based on leaf symptoms: early, when leaf yellowing and/or browning is restricted to less than half the leaves present; middle, from this stage until more than half the leaves are affected but the leaves near the apex remain green; and late, when most of the apical leaves are also affected.

The symptoms in trees (Fig. 1K to P) (5,26) are similar to those of saplings. The symptoms of the initially affected tuft of trees closely resemble those of the sapling trunk, with leaf yellowing, vascular discoloration, and occasional interstitial tissue browning. However, unlike the sapling, the affected leaves of trees abscise prematurely leaving bare apices (Fig. 1L). The leaves of the subsequently affected tufts on trees also become yellow and fall (Fig. 1M), although the internal tissues of the tuft apices remain macroscopically unaffected. In trees, the wood in the branch below the initially affected tuft becomes pale yellow (water-soaked) (Fig. 1N), and this water-soaking extends down the trunk but not up into adjacent branch systems. The front of the yellowing zone in larger branches and the trunk is irregular, comprising occasional flecks of yellowing tissue within apparently unaffected tissue. The outer wood of the trunk of affected trees becomes yellowed and the vascular strands discolored (Fig. 1O), and the cortical tissue subsequently becomes browned and necrotic, causing the dead bark to become detached (Fig. 1P). Cortical browning begins initially near ground level and extends up into the major branches, but not down into the rhizome, which shows no visible symptoms even when the disease is well advanced. We recognize three stages of symptom expression in trees based on leaf symptoms as observed in all the leafy tufts: early, when leaf yellowing and/or

browning is restricted to the initially affected tuft and a few adjacent tufts and water-soaking of the trunk has not reached ground level; middle, from this stage until about three-quarters of the tufts are affected and the trunk wood is all water-soaked; and late, when more than three-quarters of the tufts are affected.

PCR amplification of 16S rRNA gene.

PCR products of appropriate size were detected in a range of tissue types in two intensively sampled saplings (Table 1). In sapling COR2, sampled at the middle stage of disease expression with abundant yellowed leaves surrounding a central crown of green leaves (Fig. 1C), phytoplasma DNA was detected in all tissue types sampled using both one-step and nested PCR. In sapling COR1, sampled at the early stage of disease expression, with yellowed leaves only present on one side of the axis (Fig. 1A) and with some affected leaves only partially yellowed (Fig. 1B), phytoplasma DNA was detected using nested PCR in all tissues sampled except the leaf lamina. However, it was only occasionally detected using one-step PCR. On the basis of these results, shoot and rhizome apical tissues were targeted for sampling in additional plants. Root tissue was not chosen, as it is difficult to sample because the roots extend radially 1 m or more from the rhizome into the surrounding soil, and root samples often proved recalcitrant for PCR. Phytoplasma DNA was detected in all four additional symptomatic saplings, but not in six nonsymptomatic controls, three from the field and three from an insect-proof shade house (Table 2). Representative results of PCR from six symptomatic and three asymptomatic trees are shown in Figure 2. Sapling COR6 was unusual in that it had four rhizomes; PCR products were detected after one-step PCR in two, and after nested PCR, in all four. Based on this observation and on the finding that rhizome apex tissue from four out of the six plants examined gave a product by one-step PCR and all gave a product by nested PCR, we suggest that this tissue is the best to target in saplings to demonstrate the presence of the phytoplasma in symptomatic plants, except perhaps those at the early stage of infection when the initially affected shoot apex may be more appropriate.

In preliminary studies, tissue samples from many symptomatic trees were tested unsuccessfully for phytoplasma DNA using a range of DNA extraction methods and primers (unpublished data). Using the methods described here, tissue from the shoot apex (17 independent samples), leaf petiole (5 samples), rhizome apex (3 samples), and roots (2 samples) of some 29 symptomatic trees were examined. Products of appropriate size were detected occasionally in a range of tissue types in six of these symptomatic trees using the nested PCR procedure. However, in no instance

was a product detected by one-step PCR (Table 3). Products after nested PCR were obtained with samples of shoot, wood, and rhizome, but no obvious pattern in distribution was found. The two shoot apices that gave positive reactions were both initially affected tufts, and no positive reactions have been obtained with samples from subsequently affected apices. In the instances where positive reactions were obtained from wood samples, the samples were from a relatively narrow zone near the front of the yellowing water-soaked region and extending into the zone of yellow flecking. In tree COR15, for example, samples taken at 0.5 and 0.7 m (yellow flecked), 1.0 and 1.5 m (yellow water-soaked) above ground level were positive, whereas apparently normal tissue samples at 0.25 and 0.3 m, and yellow water-soaked

tissue at 2.0 and 2.5 m, were negative. These findings suggest that the rhizome apex is the best tissue to target in trees to demonstrate the presence of the phytoplasma in symptomatic plants, as it is difficult to recognize initially affected tufts confidently at the early stage of symptom expression, and the advancing front of staining in the wood tissue can only be sampled during the early stage. However, rhizome tissue is difficult to sample, as its apex lies up to 2 m or more deep in the ground. Phytoplasma DNA was not detected in shoot (six from three individuals), or rhizome (five from three individuals) samples from nonsymptomatic trees.

Nucleotide sequence analysis. PCR products of the 16S rRNA gene derived from saplings COR1, COR2, COR4, and tree COR14 were sequenced using primers

R16F2, R16R2, NGF, and NGR. This resulted in 1,130 bases of sequence information between positions 198 and 1328. The sequences were identical to that of the PYL phytoplasma, including the three polymorphisms described at positions 371, 543, and 1159 (14), with the exception of one base (Table 4). The one difference in sequence occurred at position 392, where instead of the cytosine (C) reported for PYL and AUSGY, a polymorphism of C or T was detected. Position 392 aligns with variable position 371 (polymorphism G or A) in a stem region of the secondary structure proposed for the PYL phytoplasma (14) and presumably allows full base pairing in this region. All five signature sequences for "*Ca. P. australiense*" defined by Davis et al. (9) were present in the sapling sequences.

Tetracycline injection. Three saplings were injected with tetracycline antibiotic, one at the early stage of symptom expression (Fig. 1A) and two at the late stage. The yellowing of leaves of the plant injected at the early stage was arrested within 3 weeks, and the plant continued to show normal active growth with production of green leaves during the subsequent 1.5 years of observation. The two plants injected at the late stage of symptom expression died within 1 to 2 months, with no indication of any alteration in the pattern of symptom expression.

Electron microscopy. In previous studies, a variety of tissues in numerous trees was examined for phytoplasma cells without success (5). In the present study, guided by our PCR findings, we examined rhizome tissue from three saplings (COR1, COR3, COR6, Table 2) and one adult (COR17, Table 3), and stem apex tissue from two saplings (COR3 and COR6). Cells characteristic of phytoplasmas were detected in tissue obtained from the rhizomes of saplings COR3 and COR6, both of which were positive by one-step PCR. Phloem sieve tubes in the apical tissue of these two saplings were collapsed. The phytoplasmas were pleomorphic, comprising round, elongate, dumbbell, and ring shaped elements, mostly 150 to 250 nm across but extending in length to 1,000 nm (Fig. 3A and B), and were characterized by a peripheral zone of ribosome-like granules around a central nucleoplasmic net, presumably including DNA (Fig. 3C and D). The phytoplasma elements were confined to phloem sieve tubes and were never seen in large numbers.

DISCUSSION

The previous studies of the symptoms of trees affected by sudden decline (5,25) are extended in the present study by observations on saplings. The symptoms in both trees and saplings are similar and resemble those of known phytoplasma diseases of large monocotyledons such as lethal yellowing of coconut and other palms (3) and

Fig. 2. Gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA extracted from cabbage tree saplings using one-step PCR with primers R16F2/R16R2 **A**, or nested-PCR with primers R16F2/R16R2, followed by primers NGF/NGR **B**. Lane 1, COR1 apex; lane 2, COR1 rhizome; lane 3, COR2 apex; lane 4, COR2 rhizome; lane 5, COR3 apex; lane 6, COR3 rhizome; lane 7, COR4 apex; lane 8, COR4 rhizome; lane 9, COR5 apex; lane 10, COR5 rhizome; lane 11, COR6 apex; lane 12, COR6 rhizome; lane 13, COR7 apex; lane 14, COR7 rhizome; lane 15, COR8 apex; lane 16, COR8 rhizome; lane 17, COR12 apex; lane 18, COR12 rhizome.

Table 4. Comparison of polymorphisms between the two 16S rRNA operons of Phormium yellow leaf phytoplasma (PYL) with those of Australian grapevine yellows (AUSGY) and *Cordylina* sudden decline (CSD) phytoplasmas

Phytoplasma	Nucleotide position ^a			
	371	392	543	1159
PYL <i>rrmA</i> ^b	G	C	G	C
PYL <i>rrmB</i> ^c	A	C	A	U
AUSGY ^d	G	C	A	U
CSD ^e	R ^f	Y ^g	R	Y

^a Nucleotide positions correspond to those of *Oenothera* aster yellows (OAY) phytoplasma (17).

^b GenBank accession number U43569 (14).

^c GenBank accession number U43570 (14).

^d GenBank accession number L76865 (9).

^e CSD DNA was sequenced from polymerase chain reaction (PCR) products, and hence it was not possible to assign the differences to a specific operon.

^f Ambiguity code R = either A or G.

^g Ambiguity code Y = either C or U.

yellow leaf of *Phormium tenax* (New Zealand flax) (7,23). *P. tenax*, endemic to New Zealand and Norfolk Island, bears large, leafy tufts on a woody, horizontal rhizome. It was once grouped in the same family as *Cordyline* but is now placed in the Phormiaceae.

Evidence implicating phytoplasma involvement in sudden decline. The present study describes two lines of direct evidence showing the presence of a phytoplasma in sudden decline-affected plants. We propose that this phytoplasma be referred to by the common name *Cordyline* sudden decline (CSD) phytoplasma. The first direct evidence is the detection of phytoplasma 16S DNA in tissues of affected plants (Tables 1 to 3), and the second is the identification of phytoplasmas within the phloem of affected plants by electron microscopy (Fig. 3). Both techniques indicate that CSD phytoplasma is present at low titer and is transient and localized in its distribution within the plant. While phytoplasma DNA was detected after one-step PCR in at least some tissue samples of all symptomatic saplings examined, only nested PCR provided positive results with symptomatic trees. Furthermore, it was found only in some of the

tissues examined and was not found in many symptomatic trees. Likewise, phytoplasmas were detected by electron microscopy in the rhizomes of only two saplings, despite examining a range of tissues in over 20 symptomatic saplings and trees. The morphology of the round, elongate, and dumbbell shaped phytoplasma cells illustrated in Figure 3 are similar to those reported for other phytoplasmas (21). The density of phytoplasmas in cabbage tree is, however, much less than that found in other plants infected by "*Ca. P. australiense*." In *Phormium tenax*, sieve plate elements densely packed with phytoplasmas can be regularly found in rhizomes of yellow leaf-affected plants (28; our unpublished results), and in strawberry, phytoplasmas are readily found in petioles of plants with lethal yellows symptoms (2). The ring-shaped cells shown in Figure 3B have not been previously recognized in "*Ca. P. australiense*"-infected plants but are common in, for example, phytoplasma-infected *Silene vulgaris*, where serial sectioning showed they are sections through invaginated cells (6). Phytoplasmas have proved difficult to detect by electron microscopy in other woody hosts infected by "*Ca. P. australiense*." In grape plants af-

fected by Australian grapevine yellows, phytoplasmas have been occasionally detected in leaf petiole and/or vein tissue (18). However, they have not yet been reported in papaya with papaya dieback symptoms, despite intensive searching (27).

The existence of a phytoplasma in sudden decline-affected plants does not per se demonstrate that it is the disease agent. However, we conclude that it is based on the absence of the phytoplasma from non-symptomatic plants, on the disease symptomatology, on the epidemiology which is consistent with insect-transmission, and on the absence of other possible disease agents (5). This conclusion is supported by the observation of disease remission following tetracycline treatment of a sapling treated at the early stage of symptom expression. Sensitivity to tetracycline antibiotics is recognized as supportive evidence of phytoplasma etiology (21). We suggest the absence of disease remission in saplings treated at the middle and late stages of symptom expression reflects reduced antibiotic transport as a result of damage to the vascular system, as has been reported for coconut palms infected with the lethal yellowing phytoplasma (20).

Fig. 3. Transmission electron micrographs of phytoplasma cells within a vascular bundle of a rhizome of a sudden decline-affected sapling cabbage tree (plant COR3). **A**, sieve tube element with sieve plate. (p) Selected dumbbell-shaped phytoplasma cells are arrowed. **B**, sieve tube element with ring-shaped cells arrowed. **C** and **D**, phytoplasma cells at higher magnification showing denser peripheral cytoplasm and central fibrillar material. Bar represents 500 nm in **A** and **B**, and 200 nm in **C** and **D**.

Sudden decline joins a relatively small group of phytoplasma diseases that do not produce floral reversion symptoms but are characterized by necrotic shock reactions that result in rapid decline and death of the infected plants (22). These include lethal yellowing of coconut (3), like cabbage tree a monocotyledonous tree, and papaya affected by dieback (27). In both these diseases, the phytoplasma is very localized in its distribution and present at low titer. In lethal yellowing-affected palms, the phloem-rich leaf bases are considered the richest source (12), and in papaya, at the early stage of infection, phytoplasma was detected by PCR most readily in the expanding leaves, flowers, and upper stem (27).

Transmission of sudden decline. Attempts to transmit sudden decline by grafting and dodder have been unsuccessful (5), a result that is not unexpected given cabbage tree is a monocotyledon. The only proven phytoplasma vector in New Zealand is the flax planthopper (*Oliarus atkinsoni*) (8,15), a species endemic to New Zealand and known to transmit PYL phytoplasma. It is unlikely to transmit CSD phytoplasma, as it is confined to *Phormium* species (8,13), where the nymphs feed on the base of the leaves and the rhizomes. Two introduced polyphagous planthoppers found on many hosts including cabbage tree are candidates for transmission of CSD phytoplasma, but experiments to date have been unsuccessful (5). A number of leafhopper species found in the vicinity of cabbage tree are also candidates.

"Ca. P. australiense" in New Zealand and Australia. The 16S sequence data indicate that CSD phytoplasma belongs to "Ca. P. australiense," a species described from grape infected with Australian grapevine yellows in Australia (9). The best characterized disease caused by this species is yellow leaf of *Phormium tenax*. This disease was first recorded in 1908, and the symptomatology was clarified by 1953 (7,23), planthopper transmission was demonstrated in 1953 (8,15), an association with a phytoplasma was made in 1969 (28), and its two rRNA genes were sequenced in 1996 (14). As well as these two hosts, "Ca. P. australiense" has also been shown to be associated with strawberry lethal yellows in New Zealand (2), strawberry lethal yellows and strawberry green petal in Australia (24), and papaya dieback (11,29). There is also a preliminary report of it from the endemic shrub *Coprosma robusta* (family Rubiaceae) in New Zealand, and symptoms suggest other plants in New Zealand may also be affected (4). Elsewhere we have proposed that "Ca. P. australiense" is endemic to New Zealand and was transported by humans to Australia where it is known only from exotic hosts (16). While cabbage tree is cultivated commonly throughout temperate regions, sudden decline is not known with certainty from any other country, although occa-

sional trees with some of the symptoms have been observed in Australia (5).

The existence of a polymorphism at position 392 in all three CSD phytoplasma samples sequenced, and its absence from the PYL phytoplasma samples sequenced by Liefiting et al. (14), suggest the possible existence of distinct populations of "Ca. P. australiense."

Description of disease progression. On the bases of the locations of tissue found to contain phytoplasma DNA and of symptom expression, we speculate that the phytoplasma is injected into the phloem stream of leaves by the postulated insect vector and follows the phloem sap down the trunk and up to the apex of the adjacent shoot (the single apex in saplings, the initially affected shoot in trees). As it moves down the trunk, it triggers a tissue response leading to water-soaking and apparently the destruction of phytoplasma cells, as it is only transiently detected in the trunk tissue near the water-soaking front. However, removal of affected branches ahead of the front does not prevent subsequent death, indicating rapid phytoplasma movement (three unpublished observations). In trees, it does not move from the trunk up into other branches. While the leaves on these other branches show symptoms, presumably as a result of the damage to the vascular tissue and possibly involving toxins of some sort, the wood tissue of the branches does not show water-soaking, and cuttings made from secondarily affected leafy tufts grow and are apparently disease free (5). Following the damage to the vascular system of the trunk, the cortex tissue exterior to it becomes necrotic, leading eventually to collapse of the bark. Belowground, the phytoplasma then moves to the apex of the massive rhizome and out in the roots. Despite the apparent concentration of phytoplasmas in the rhizome apex, the rhizome is relatively less affected than the trunk tissue; water-soaking is not obvious, the cortex remains intact, and occasional dormant shoots are stimulated to grow out (5). The absence of the phytoplasma from the leaves of secondarily affected tufts of trees, and its limited occurrence even in leaves of saplings, suggest that there will only be a very short period when a leaf-feeding insect vector might acquire phytoplasma from infected cabbage trees. In turn, this suggests that cabbage tree may be a secondary host to the phytoplasma and that the vector acquires the phytoplasma elsewhere. Of the other known hosts of "Ca. P. australiense" in New Zealand, the most likely candidates as source plants known at present are *Phormium tenax* and *Coprosma robusta*, both of which have a distribution that overlaps that of affected cabbage trees. However, more knowledge of the population structure of the phytoplasma, as well as its transmission, is needed to confirm this hypothesis.

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