Studies on Sweet Potato Little-Leaf Phytoplasma Detected in Sweet Potato and Other Plant Species Growing in Northern Australia

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ABSTRACT

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Symptoms of vein clearing, small leaves ("little leaf"), stunting of plants, proliferation of shoots, and small tuberous roots were observed in sweet potato growing in the Darwin region of the Northern Territory, Australia (12 30′ S latitude) in December 1990. This was the first report of such symptoms in sweet potato but there was anecdotal evidence of similar symptoms in other crop, ornamental, and weed host species growing in this region. Plant pathogenic mycoplasmalike organisms, or phytoplasmas, were observed in sieve tube elements of diseased sweet potato using fluorescent and electron microscopy. To screen a range of local plant host species for phytoplasmas, a region of the 16S rRNA phytoplasma gene was amplified in the polymerase chain reaction (PCR). The relatedness of phytoplasmas detected in different host species was determined by amplifying a larger 16S rRNA fragment and subjecting it to restriction fragment length polymorphism (RFLP) analysis using four restriction enzymes. No differences were detected between phytoplasmas associated with the local plant host species, and the RFLP pattern of the phytoplasmas from this region appeared to be different from those published for other phytoplasmas. This suggests that the phytoplasmas from this region of northern Australia compose a new subgroup. Phytoplasma chromosomal DNA was extracted from diseased sweet potato, and the chromosome was linearized by gamma-irradiation prior to separation by pulsed-field gel electrophoresis. The size of the full-length sweet potato little-leaf phytoplasma chromosome was 600 kb, which is one of the smallest phytoplasma genome sizes reported so far.

Additional keywords: chromosome, *Mollicutes,* PFGE, sweet potato little leaf.

Diseased sweet potato showing reduced leaf size, shoot proliferation, and small tuberous roots has been reported from the Ryukyu Islands (24), Papua, New Guinea (17,23), Tonga (9,10), the Solomon Islands (2,3,8) and the Penghu Islands, Taiwan (24). The disease in sweet potato, which is associated with a mycoplasmalike organism (MLO) (2), is commonly referred to as either "sweet potato little leaf" (SPLL) or "sweet potato witches'-broom" (SPWB). In this report MLOs are referred to as phytoplasmas, a term that is receiving increasing support (22). Sweet potato diseases attributed to phytoplasmas cause serious crop losses, particularly in the Pacific, but until recently there has been no way of screening or differentiating phytoplasmas associated with these diseases in each region. For this reason it is also not known whether the diseases of sweet potato in Australia, Asia, and the Pacific are associated with one or many phytoplasmas. Jackson et al (7) reported that the Asian Vegetable Research and Development Centre had screened 365 sweet potato cultivars for resistance to SPLL. One cultivar was found to be immune to SPLL in Taiwan, but proved to be susceptible when tested in the Solomon Islands, which suggests that more than one phytoplasma may be involved.

Little work has been done on the relationship between sweet potato phytoplasma(s) and phytoplasmas associated with other plant host species. Shen and Lin (20) reported the production of monoclonal antibodies against a phytoplasma associated with sweet potato (SPWB) collected on the Penghu Islands of Taiwan. SPWB phytoplasma was differentiated serologically from some phytoplasmas, including those associated with aster and elm yellows and rice yellow dwarf. Monoclonal antibodies to SPWB did however cross-react with peanut and asparagus bean witches'-broom phytoplasmas and with phytoplasmas associated with the weeds *Rynchosia minima* and *Alysicarpus vaginalis* (L.) D.C. found on the Penghu Islands.

This paper reports the first study of a phytoplasma associated with a disease of sweet potato in Australia. Preliminary diagnosis using electron microscopy and fluorescent staining is reported. Detection of phytoplasma DNA in sweet potato and other local host plant species using the polymerase chain reaction (PCR) is discussed and genetic relatedness using restriction fragment length polymorphism (RFLP) analysis is reported. This work has led to the development of a diagnostic tool for phytoplasmas in sweet potato that will have important implications for the crop in this region. The size of the SPLL phytoplasma chromosome was also determined using pulsed-field gel electrophoresis.

MATERIALS AND METHODS

Source of phytoplasmas. Sweet potatoes (*Ipomoea batatas* (L.) Lam.) naturally infected with a phytoplasma were collected from the field 65 km southeast of Darwin, Australia (12° 30′ S latitude), and maintained by vegetative propagation. SPLL phytoplasma–infected periwinkle (*Catharanthus roseus* (L.) G. Don) obtained by transmission through the parasitic climber *Cuscuta australis* R. Brown, was propagated by cleft grafting. Phytoplasmas from other plant species were studied in the naturally infected host and these were collected within a 60-km radius of Darwin except for *Lycopersicon esculentum* Mill. (tomato) with tomato big bud (TBB) disease, which was sent from Queensland, Australia. Maryland aster yellows (AY) phytoplasma DNA was used as a reference phytoplasma in differentiation studies.

Fluorescent microscopy. A DNA-specific fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI), was used to stain phyto-

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plasmas in the phloem of sweet potato petioles and midveins (17). Samples were examined under fluorescent illumination.

Electron microscopy. Petiole sections were fixed in cacodylate buffer (0.05 M sodium cacodylate, pH 7.2, 0.15 M sucrose, 2 mM calcium chloride) containing 2.5% gluteraldehyde, and postfixed in 1% (w/v) osmium tetroxide in 0.5× cacodylate buffer. The sections were dehydrated by passing through an ethanol series before infiltration with Spurr's medium. After polymerization, ultrathin sections were cut, collected on 200μ-mesh copper grids, and stained with saturated aqueous uranyl acetate.

Extraction of nucleic acid from plants. Nucleic acid samples from healthy and diseased plants were prepared using a previously published method (6) modified from Lee et al (11) and Neimark and Kirkpatrick (15), and repeated here for convenience. Leaf midribs, petioles, and young stems (5 g) were sliced into 0.2–0.5 cm lengths, placed in a prechilled mortar and pestle, and ground in 20 ml of ice-cold isolation medium (0.1 M Na_2HPO_4, 10% sucrose, 2% PVP-40, pH 7.6 to which 0.15% bovine serum albumin and 1 mM ascorbic acid were added just before use)(4). The extract was filtered through cheesecloth and the filtrate centrifuged (1,500 *g*, 5 min, 4 C). The supernatant was centrifuged again (18,000 *g*, 25 min, 4 C) and the pellet resuspended in 20 ml of Tris-sucrose (TS) buffer (20 mM Tris-HCl pH 8.0, 10% sucrose) by gentle pipetting. The suspension was first centrifuged (1,500 *g*, 5 min, 4 C) and the supernatant centrifuged again (18,000 *g*, 5 min, 4 C). The pellet was resuspended in 800 μl of extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 250 mM NaCl) containing proteinase K at 100 μg/ml and *n*-lauroyl-sarcosine at a final concentration of 1%. The extract was incubated for 1–2 h at 55 C with occasional mixing. Ice-cold isopropanol (0.6 volume) was added to the extract, mixed gently, and left either for 1 h or overnight at −20 C. The extract was centrifuged (7,500 *g*, 15 min, 4 C) and the pellet resuspended in 9 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) to which were added proteinase K (to give 100 μg/ml) and sodium dodecyl sulfate (to give a final concentration of 0.5%). The extract was mixed thoroughly and incubated for 1 h at 37 C after which 1,575 μl of 5M NaCl and 1,260 μl of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) were added, mixed thoroughly, and the extract incubated for 10 min at 65 C. The sample was then extracted with an equal volume of chloroform/isoamyl alcohol (24:1) followed by extraction with an equal volume of TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1). The nucleic acid was precipitated from the supernatant by the addition of 0.6 volume isopropanol at −20 C overnight and pelleted by centrifugation (7,700 *g*, 10 min, 4 C). The pellet was washed with 2 ml of ice-cold 70% ethanol and resuspended in TE buffer. Average yield was 30–40 mg of DNA per 100 mg fresh weight.

Polymerase chain reaction. The two sets of primers used were designed on the basis of 16S rRNA sequence from a phytoplasma pathogen of *Oenothera hookeri* (13). One set of primers amplified a 513-bp fragment, extending from position 738 through 1,251 (O-MLO reference number). The sequence of the forward primer (16R758F) is 5′-GTCTTTACTGACGCTGAGGC-3′ and of the reverse primer (16R1232R) is 5′-CTTCAGCTACCCTTTG-TAAC-3′. This primer pair was used in the PCR to detect phytoplasma DNA in sweet potato and a range of plant host species. The second set of primers amplified a 1,245-bp fragment, extending from position 152 through 1,397 (12). For the PCR, nucleic acid extracts from healthy or phytoplasma-infected tissues were diluted in TE buffer to give a final concentration of 20 ng/μl. For the 513-bp amplification, the reactions contained 0.2 mM each dNTP, 0.4 μM each primer, DNA polymerase buffer supplied with the enzyme, 1 U Taq DNA polymerase (Boehringer GmbH, Mannheim, Germany), and a 20-ng nucleic acid sample in a total volume of 20 μl. The reaction mixtures were taken up in a capillary tube and placed in a Corbett FTS-1 thermocycler (both from Corbett Research, N.S.W., Australia). Parameters for 35 cycles of amplification were: denaturation at 94 C for 30 s (3 min for cycles 1–5), annealing at 50 C for 30 s (1 min for cycles 1–5), and primer extension at 72 C for 30 s (1 min for cycles 1–5 and 90 s for cycle 35). For the 1,245-bp amplification, the same PCR cocktail was used in a total volume of 50 μl and overlaid with 50 μl of mineral oil (Sigma Chemical Co., St. Louis, MO) before being placed in a Corbett FTS-320 thermocycler (Corbett Research). Parameters for 35 cycles of amplification were: denaturation at 94 C for 1 min (3 min for cycle 1), annealing at 50 C for 2 min, and primer extension at 72 C for 3 min (10 min for cycle 35). Control reactions were water and healthy plant DNA. After amplification, a one-tenth volume aliquot from each sample was subjected to electrophoresis in either a 1.2% (513 bp) or 1% (1,245 bp) agarose gel and visualized by staining with ethidium bromide and UV illumination.

RFLP. Fifteen microliters of the PCR were digested separately using four restriction enzymes: *Alu* I (8U), *Mse* I (4U), *Rsa* I (10U), and *Hpa* II (6U) (New England Biolabs, Beverly, MA) in buffers supplied by the manufacturer. Digestions were incubated overnight at 37 C and the fragments were visualized by electrophoresis in an 8% polyacrylamide gel in 1 × TBE buffer followed by staining with ethidium bromide for 30 min. The gel was photographed on a UV transilluminator.

SPLL chromosome extraction. Chromosomes were extracted using a modified procedure (15). Leaf midveins and petioles from periwinkle showing little leaf symptoms were sliced into 0.5–1 cm portions and ground in an extraction buffer containing 0.1 M Na_2HPO_4, 10% sucrose, 10 mM EDTA, 0.15% BSA, and 1 mM ascorbic acid, pH 7.6, using 5 ml of buffer per gram of tissue. The brei was filtered through two layers of cheesecloth and the filtrate centrifuged at 1,500 *g* for 5 min, and the supernatant centrifuged again at 18,000 *g* for 25 min. The pellet was resuspended in a starting volume of TS (20 mM Tris-Cl pH 8, 10% sucrose) using a glass homogenizer. The low- and high-speed spins were repeated and the final pellet of cells was resuspended in a small volume of TS (50 μl/g tissue).The cell suspension was maintained at 37 C for 5 min and then mixed with an equal volume of 2% InCert agarose (FMC BioProducts, Rockland, ME) using a truncated pipette tip. The suspension was dispensed into 100 μl molds and allowed to set on ice for 10–15 min. The blocks were then pushed out of the molds using a flame-sterilized, bent pasteur pipette into a microfuge tube containing lysis buffer (0.5 M EDTA, 1% *n*-lauroyl-sarcosine, pH 8) with proteinase K (1 mg/ml final concentration). The blocks were incubated at 50 C for 3 days with three changes of lysis buffer each day to remove the green pigment.

Electrophoresis. The agarose blocks containing the enriched phytoplasma preparation after lysis were cut into 2–3 mm slices, washed for 3 × 30 min in SDW on ice, and then placed in 200 μl 0.5 × TE. The slices were irradiated for 10 min (54.8 Gray) using

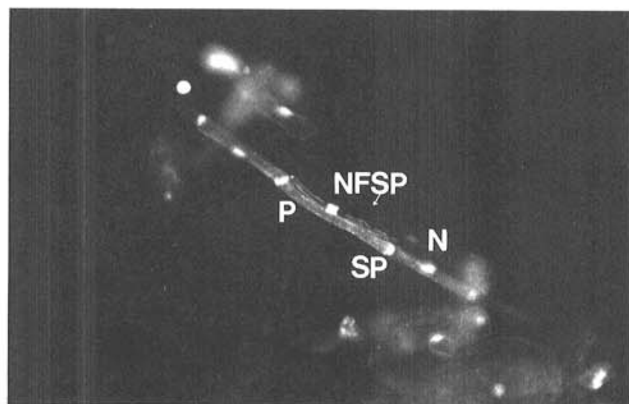

Fig. 1. Light micrograph showing typical fluorescence from phytoplasma-infected sieve tube elements (P) of sweet potato stained with a DNA-specific fluorescent dye, 4′,6′-diamidino-2-phenylindole (DAPI). Position of the sieve plates (SP) is obvious from the intense fluorescence in this region, due, it is assumed, to the presence of phytoplasmas. Sieve tube elements with a nonfluorescing sieve plate (NFSP) can be seen; this suggests that not all sieve tube elements from an infected plant contain phytoplasmas at detectable levels. Sieve tube elements from healthy plants appear similar to these nonfluorescing cells. Nuclei (N) of phloem parenchyma cells appear as out-of-focus elliptical fluorescent spots. Bar = 20 μm.

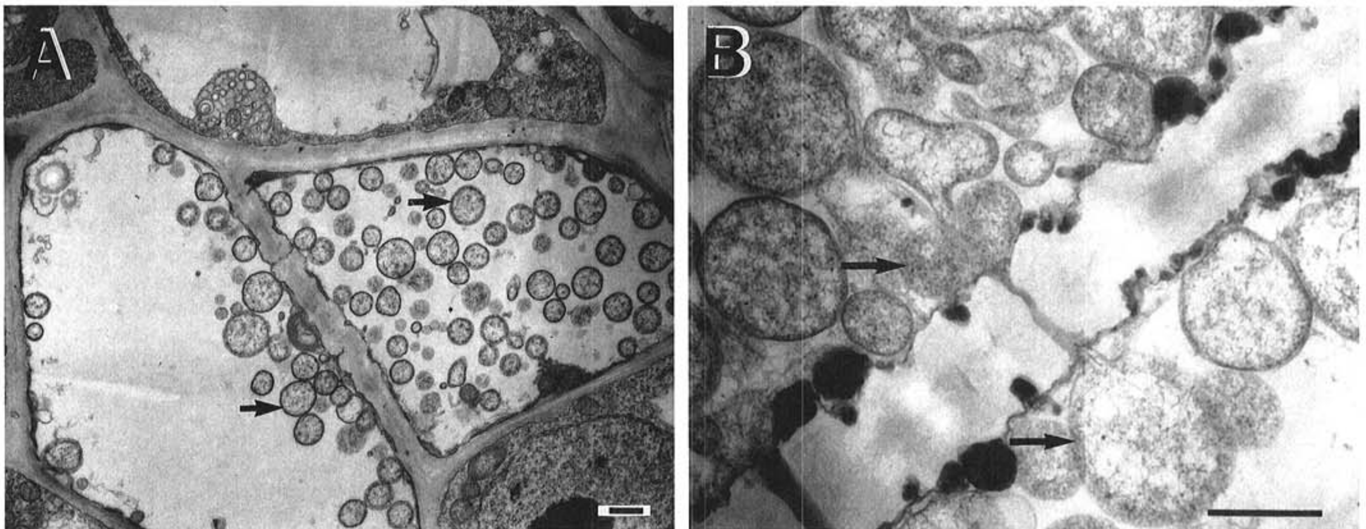

Fig. 2. Transmission electron micrograph of a cross section of a diseased sweet potato stem. **A,** Phytoplasmas (arrowheads) can be seen in the sieve elements. **B,** Higher magnification of the phytoplasmas (arrowheads) associated with the plasmodesmata. Bars = 250 nm.

a ^{60}Cobalt source to produce on average one random double-stranded break per chromosome (18). Blocks were then subjected to pulsed-field gel electrophoresis (PFGE) using a CHEF I unit (Bio-Rad Laboratories, Richmond, CA) at 200 V, 40–90 s pulse time, 12 C, for 20 h on a 1% agarose gel (Sea Plaque, FMC BioProducts, Rockland, ME). Bands were visualized by staining for 4 h with ethidium bromide, destaining in water overnight at room temperature, and then photographed. Chromosome sizes were estimated by comparing sample bands with yeast chromosome markers and the chromosome of *Mycoplasma mycoides* digested with different restriction endonucleases.

Digestion of mycoplasma DNA. Agarose blocks containing *Mycoplasma mycoides* subsp. *mycoides* (LC) GC 1176-2 and Y DNA were kindly provided by Lloyd Finch, University of Melbourne. A thin slice of the block was washed for 3 × 30 min in TE on ice and then incubated in 250 μl of restriction enzyme buffer containing 4 mM DTT and 50 μg/ml BSA for 30 min on ice before replacing this with a fresh solution containing 8U of the appropriate enzyme. Following restriction digestion and separation by electrophoresis, the mycoplasma DNA gave bands of 478, 722, and 1,380 kbp (*BssH* II), 137, 389, 616, 684, and 764 kbp (*Sma* I) and 77, 118, 144, 167, 228, 280, 322, 572, and 671 kbp (*Xho* I) (Boehringer GmbH, Mannheim, Germany).

Southern blot analysis of SPLL chromosome. Pulsed-field gels were irradiated with UV light using a Stratalinker UV Crosslinker 1880 (Stratagene, La Jolla, CA) before alkali transfer to a nylon membrane (Amersham Australia, N.S.W., Australia) using a PosiBlot Pressure Blotter (Stratagene). The membrane was hybridized using Rapid-Hyb Buffer (Amersham Australia). The probe was a 500-bp PCR product of the 16S rRNA phytoplasma DNA labeled with α-^{32}P dATP by random priming (Promega, Madison, WI). The X ray was exposed for 4 days before developing.

RESULTS

Diseases associated with phytoplasmas in the field. In the field, diseased sweet potato had little-leaf and proliferation symptoms with occasional vein clearing. A weed commonly known as pigweed had obvious little-leaf symptoms and grew as ground cover beneath the sweet potato crop. Other local plants collected because they had little-leaf symptoms were *Solanum melongena* L. (eggplant), *Alysicarpus* sp., *Passiflora foetida*, *Evolvulus* sp., and *Amaranthus* sp.

Fluorescent microscopy. Isolated sieve tube elements from diseased sweet potato plants macerated on microscope slides and stained with the DNA stain DAPI showed fluorescence at the sieve plates and occasionally along the length of the sieve tube element (Fig. 1). Some sieve tube elements in macerates from diseased plants did not show fluorescence at the sieve plates (Fig. 1), indicating that not all sieve tube elements from an infected plant contained phytoplasmas at levels detectable by this method. Nuclei of phloem parenchyma cells from both diseased and symptomless plants appeared as out-of-focus elliptical fluorescent spots (Fig. 1). Sieve tube elements from healthy plants showed no fluorescence.

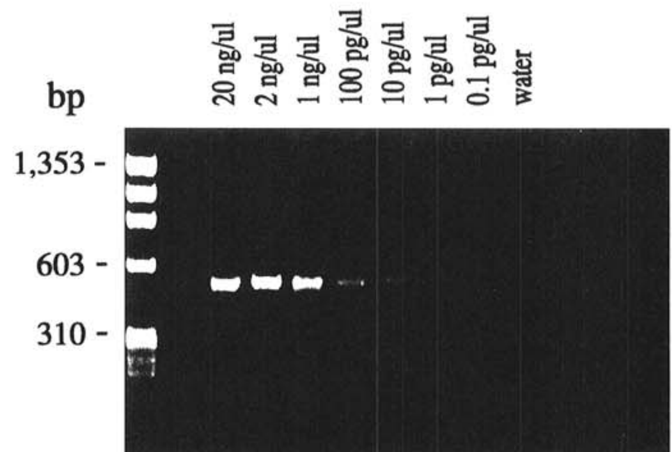

Fig. 3. Detection limit of sweet potato little leaf phytoplasma DNA by the polymerase chain reaction (PCR). Decreasing concentrations of total DNA extracted from a diseased sweet potato were used in the PCR. DNA standards are ϕx174 cut with *Hae* III (lane 1).

Electron microscopy. Elliptical bodies were observed in the sieve elements of diseased sweet potato (Fig. 2A and B) and these bodies, presumably the cross-sectional view of pleomorphic phytoplasmas, were observed associated with plasmodesmata (Fig. 2B). No such bodies were observed in the sieve elements of healthy sweet potato plants.

Polymerase chain reaction. With the primer pair 16R758F and 16R1232R, a 513-bp fragment was amplified in each of the reaction mixes containing template DNA prepared from diseased sweet potato. No PCR products were obtained with samples prepared from healthy plants. To determine the sensitivity of this assay, the 20-ng/μl nucleic acid sample used for routine PCRs was further diluted to 2,000, 1,000, 100, 10, 1, and 0.1 pg/μl and 1 μl of each dilution was used in each 20-μl PCR mix. Amplified products could be visualized on an agarose gel when as little as 1 pg of nucleic acid was used in the PCR (Fig. 3). For routine

screening of diseased plants, however, 20 ng of nucleic acid were used in the PCR.

Using the primers designed by Lee et al, a phytoplasma-specific DNA band of 1,245 bp was amplified in reaction mixes containing nucleic acid prepared from each of the local diseased plant host species collected in the Darwin region, TBB from Queensland, and the reference AY from the United States (Fig. 4). On no occasion did we observe PCR products from mixes containing nucleic acid prepared from healthy plants of these species.

Fig. 4. Polymerase chain reaction (PCR) amplification of a 1,245-bp fragment of the 16S rRNA gene. Lane 1 shows the ϕx174/*Hae* III DNA size markers. Samples used in the PCR were healthy sweet potato (lane 2), sweet potato, eggplant, pigweed, *Alysicarpus* sp., *Passiflora foetida*, *Evolvulus* sp. and *Amaranthus* sp. with little-leaf symptoms (lanes 3-9), tomato with big-bud symptoms (lane 10) and the reference aster yellows phytoplasma (lane 11).

RFLP. For RFLP analysis, the primer pair R16F2 and R16R2 was used to amplify a specific 1,245-bp fragment from nucleic acid prepared from diseased sweet potato, the diseased plant host species collected locally and interstate, and the reference AY phytoplasma. The amplified phytoplasma DNA was analyzed by separate digestion with four different restriction enzymes. RFLP patterns of the eight local and AY 16S rDNA amplified fragments are shown (Fig. 5) and no polymorphisms were observed between the Australian little-leaf phytoplasmas, or between the little-leaf phytoplasmas and tomato big bud.

SPLL chromosome. Enriched SPLL phytoplasma prepared from diseased periwinkles that were embedded in agarose and gamma-irradiated produced linear chromosomes that entered and migrated as a single DNA band in pulsed-field gels (Fig. 6). No DNA bands were detected in preparations from healthy plants. In other experiments (data not shown) bands were observed in some unirradiated preparations that probably represent chromosomes that have been linearized by physical shearing or by endonuclease activity during preparation. Yeast chromosome markers and *Mycoplasma mycoides* strain Y and GC mixture

Fig. 5. RFLP patterns of the 1,245-bp product digested with four different restriction endonucleases: *Mse* I, *Alu* I, *Rsa* I, and *Hpa* II. M1 = ϕx174/*Hae* III DNA markers; HSP = healthy sweet potato; SP = sweet potato; EP = eggplant; PW = pigweed; AL = *Alysicarpus* sp.; PF = *Passiflora foetida*; EV = *Evolvulus* sp., AM = *Amaranthus* sp.; TBB = tomato big bud; AY = aster yellows; M2 = pUC19/*Hae* III DNA markers.

digested separately with different restriction endonucleases were used to determine the size of the SPLL phytoplasma chromosome, which was 600 kb.

Southern hybridization. In a separate experiment, phytoplasma preparations from uninfected periwinkles and those with SPLL disease were subjected to pulsed-field gel electrophoresis. The identity of bands observed in the pulsed-field gels was confirmed by Southern hybridization using a phytoplasma-specific 16S rDNA probe (Fig. 7).

DISCUSSION

In this study, a disease of sweet potato that causes little-leaf symptoms was shown by fluoresence and electron microscopy to be associated with a phytoplasma. Molecular techniques were used to facilitate detection and characterization of this phytoplasma that is associated with different plant host species growing near the sweet potato crop. The PCR and RFLP methods were used because PCR amplification of the 16S rRNA gene of phytoplasmas (5) has great potential as a diagnostic tool, and RFLP analysis of amplified 16S rRNA sequences (1) is useful in combination with biological information for differentiating phytoplasmas.

The phytoplasmas studied by PCR and RFLP were extracted from field-infected host plants. Published DNA extraction methods used to extract phytoplasmas from periwinkle did not permit the reliable amplification of phytoplasma DNA from some plant host species. This may have been due to the presence of PCR inhibitors in the nucleic acid preparations. A DNA extraction method was optimized to allow the reliable amplification of phytoplasma DNA in the PCR (6). In this study, PCR amplification of a 513- and 1,245-bp region of the 16S rRNA gene was used to detect and differentiate phytoplasmas. Our experience has shown that amplification of a small product is more reliable than amplification of a larger fragment, so primers that amplify the 513-bp product were routinely used for the initial screening of plant hosts with suspected phytoplasma-associated diseases.

When using the PCR as a diagnostic tool for phytoplasmas it may be more appropriate to use more than one set of primers to ensure that all possible phytoplasma strains are detected. Toth et al (22) used ribosomal protein sequence data to show that phytoplasmas are more closely related to the true acholeplasmas than to other members of the *Mollicutes.* The forward primer (16S758F) used to amplify the 513-bp fragment has three mismatches with *Acholeplasma laidlawii*, so, if any of the phytoplasmas occurring in Australia are more "acholeplasmalike" than those previously studied, they may not be amplified efficiently using this primer. Continuing diagnostic work on phytoplasmas in this region is being done using more than one set of primers that are designed to overcome these limitations.

Using the screening method described here, phytoplasmas could be reliably detected in sweet potato, eggplant, *Evolvulus* sp., *Amaranthus* sp., tomato, *Passiflora foetida*, pigweed, and *Alysicarpus* sp. The latter two weed species may have interesting epidemiological significance because they grow in close association with the sweet potato crop, and pigweed supports high levels of *Orosius argentatus,* a leafhopper vector of phytoplasmas (M. C. Chisholm and K. S. Gibb, *unpublished data*). A study of SPWB phytoplasma from Taiwan using monoclonal antibodies also showed that the weed *Alysicarpus* sp. could be a host for phytoplasmas (20). The relationship between SPLL and SPWB has not been determined using molecular or serological techniques.

RFLP analysis of local phytoplasmas and TBB using the four restriction enzymes *Mse* I, *Alu* I, *Rsa* I, and *Hpa* II showed that these phytoplasmas were not different from each other. This is either because they are in fact the same phytoplasma, or there are subtle differences between these phytoplasmas that are not detected by this method. This study was confined to phytoplasmas collected within a small area but ongoing work on Australian phytoplasmas is using both sequence and RFLP data to develop an optimum system for differentiating these pathogens. In addition, more than one primer pair will be used to amplify phytoplasma DNA for RFLP and sequence analysis.

Since very little is known about phytoplasmas from the Australian/Asian/Pacific region, the identity of the SPLL phytoplasma in relation to phytoplasmas from North America and Europe was of considerable interest. Although a number of phylogenetic systems for phytoplasmas are emerging, each one differs in the selection of primer sequences and restriction enzymes, which makes comparisons difficult. An added problem is the lack of "type" phytoplasmas available for use as internal standards. This is mainly a reflection of phytoplasma nomenclature and the inability until now of determining whether phytoplasmas with the same name are the same or different, either biologically or at the molecular level. In this study a reference phytoplasma was the Maryland aster yellows (AY) phytoplasma. The RFLP patterns for AY, which were the same as those obtained by Lee et al, (12), were clearly different from those of the SPLL group. For three restriction enzymes (*Mse* I, *Rsa* I, and *Hpa* II), the SPLL group fitted the 16Sr II group of Lee et al, which includes peanut witches'-broom (PnWB), red bird cactus witches'-broom,

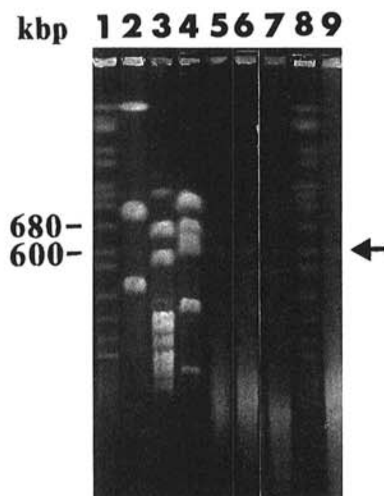

Fig. 6. Pulsed-field gel showing the SPLL phytoplasma chromosome (lanes 5, 6, and 9) and healthy plant DNA (lane 7). For size determination, yeast chromosome markers (lanes 1, 8), *Mycoplasma mycoides* digested with *BssH* II (lane 2), *M. mycoides* digested with *Xho* I (lane 3) and *M. mycoides* digested with *Sma* I (lane 4) were used. The arrow indicates the chromosome position at 600 kb.

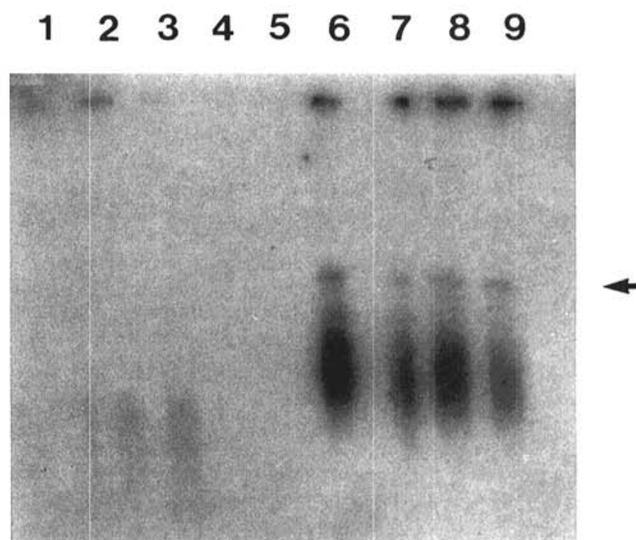

Fig. 7. Autoradiograph of a pulsed-field gel showing a 600-kb band (arrow) in the diseased samples (lanes 6–9) but not the healthy samples (lanes 1–5).

and an unidentified phytoplasma, all from Taiwan. The *Alu* I restriction pattern of the SPLL group, however, differed from that obtained for PnWB, which suggests that the SPLL group of phytoplasmas from this region of northern Australia composes a new subgroup. Schneider et al (19) analyzed a 1,500-bp region of the 16S rRNA phytoplasma gene and, using their criteria, the SPLL group best fits their group VI, which includes western X-disease, flavescence dorée, and peach yellow leaf roll. Schneider's groupings are based on two RFLP profiles using *Alu* I and *Rsa* I. Lee et al (12) placed western X-disease in group 16Sr III-A. It is also interesting to note that in the study reported here, TBB was not differentiated from the SPLL group, but Schneider et al (19) placed TBB in group I which includes American aster yellows. This illustrates the point that phytoplasmas are identified according to their plant host but the same name can represent different phytoplasmas. An alternative explanation could be that passage and propagation in periwinkle has led to changes in the nucleic acid sequence, or that there has been cross-contamination of phytoplasmas.

The development of a universal classification system for phytoplasmas would greatly facilitate the study of these poorly understood plant pathogens. Another classification system based on sequence data (14) describes a new set of groupings. It is possible that phylogenetic systems based on sequence data will give valuable additional information that may be overlooked by RFLP analysis. This is especially true for systems like the one described here, in which phytoplasmas collected from a range of plant host species in a localized area do not show genetic differences by RFLP analysis. This approach would also be useful when studying phytoplasmas from the same host species collected from a wide geographical area. Most value would be obtained, however, if RFLP and sequence data were used to support a single classification system for phytoplasmas rather than to develop two systems.

Very little is known about the phytoplasma genome and its organization. Neimark and Kirkpatrick (15) reported the isolation of full-length chromosomes from phytoplasmas using pulsed-field gel electrophoresis (PFGE). Phytoplasmas cannot be cultured so chromosome studies are done using enriched preparations of phytoplasmas from plant sap. This means that not only are the concentrations of phytoplasma chromosomes low and therefore difficult to visualize, but the presence of host plant material interferes with further genomic characterization such as restriction mapping (unpublished data). Neimark and Kirkpatrick (18) showed that gamma-irradiated chromosomal DNA in agarose plugs converted the circular phytoplasma chromosome into full-length linear molecules that migrated as sharp, discrete bands in PFGE. Neimark and Kirkpatrick (15) found that the sizes of a number of phytoplasma chromosomes ranged from 640 to 1,185 kb; at 600 kb, the SPLL chromosome is smaller than these. Although gamma-irradiation of the DNA was used to linearize the phytoplasma chromosome, full-length phytoplasma chromosomes were observed quite frequently in unirradiated preparations. This has been reported before (15,18) and the consensus is that some chromosomes are linearized by shearing or by nucleases released during the extraction process. In this study, no bands were observed in extracts from healthy plants. Restriction endonuclease digestion of chromosomal DNA embedded in the agarose blocks was successful with a limited number of restriction enzymes, but only after additonal steps were taken to remove inhibitors present in the agarose blocks (A. C. Padovan and K. S. Gibb, *unpublished data*). This work is the subject of ongoing studies, but results so far indicate that the sum of the sizes of restriction fragments determined by their mobilities in the gel is consistent with a chromosome of 600 kb. The identity of the 600-kb DNA band was confirmed by Southern hybridization using a 16S rDNA probe; in these studies no additional bands were observed, so there was no evidence to support the existence of megaplasmids.The hybridization observed as a smear below the 600-kb band is probably degraded phytoplasma chromosome. No such hybridization is seen with the healthy samples. One of the aims of ongoing work is to reduce the level of this degraded chromosomal material that interferes with the detection of small chromosomal DNA fragments present after digestion with restriction enzymes.

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