PCR Assay for Detection of the Phytoplasma Associated with Maize Bushy Stunt Disease

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ABSTRACT

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DNA amplification by polymerase chain reaction (PCR) was used to detect the phytoplasma associated with maize bushy stunt (MBS) disease. A pair of oligonucleotide primers was synthesized according to partial sequences of a cloned 1-kb fragment of genomic DNA of the MBS phytoplasma (Texas isolate) maintained in sweet corn. PCR performed for 30 cycles with primer annealing at 61°C amplified a DNA product of about 740-bp in reaction mixtures containing template DNA derived from symptomatic corn singly infected with MBS phytoplasma isolates from either Texas, Florida, Costa Rica, or Mexico. No comparable product was amplified from DNAs of healthy corn, plants affected by various other phytoplasmal diseases, or from *Spiroplasma kunkelii*. Forty cycles of PCR enabled detection of a Florida isolate of the MBS phytoplasma in all leaf and stalk samples tested from presymptomatic plants 12 days after plants were fed upon by inoculative vector *Dalbulus maidis* leafhoppers and in the majority of nonvector *Peregrinus maidis* planthoppers after 1 to 5 days of exposure to symptomatic plants.

Maize bushy stunt (MBS) and corn stunt (CS) are two economically important diseases of corn *(Zea mays* L.) in the southern United States, Mexico, and Central America (16). Two different phloem-colonizing mollicutes are associated with these diseases. A nonculturable phytoplasma (previously referred to as mycoplasmalike organism, MLO) is believed to be the causal agent of MBS (37) whereas *Spiroplasma kunkelii,* a cultivable mollicute with helical morphology, is the cause of CS disease (6,49). MBS can be distinguished from CS disease on the basis of symptom appearance on corn at elevated temperature, the range of insect vector species, plant host range, and length of the latent period in a natural leafhopper vector *Dalbulus maidis* DeLong and Wolcott (37, 38). Despite these distinctions, diagnosis of MBS may be confounded by mixed infections with CS or maize viruses, and symptom variations due to host genotype, pathogen strain, and environmental conditions have also been reported (3,17).

Diagnosis of phytoplasma-associated diseases based on symptoms has traditionally required confirmation by observation of phytoplasma presence in plant phloem using transmission electron microscopy (TEM) because these pathogens are too small to be adequately resolved by light microsopy (43). DNA-specific DAPI (4′,6-diamidino-2-phenylindole.2HCl) staining in conjunction with epifluorescence microscopy has also been extensively used as a more practical alternative for localizing phytoplasma infections in plants (41,42). Although quite sensitive, both TEM and DAPI staining methods are nonspecific, as they do not provide any information about causal phytoplasma identity.

Serological methods incorporating polyclonal or monoclonal antibodies have improved detection of several plant pathogenic mollicutes (4,7,9) including *S. kunkelii* (31) and the MBS phytoplasma (5). Sensitive and specific detection of numerous phytoplasmas in both their plant (27) and insect (10,24,35) hosts has also been achieved by DNA hybridization utilizing cloned random fragments of phytoplasma DNA as probes. The polymerase chain reaction (PCR) incorporating oligoprimers derived from sequences of mollicute ribosomal RNA (rRNA) genes (1,13, 19,29,36) or cloned anonymous fragments of DNA (4,14,20,25,40) has increased detection sensitivity beyond lower limits previously demonstrated with DNA probes. This most recent development has greatly facilitated detection and investigation of phytoplasma in plant hosts that typically contain only low titers of these pathogens (4,20).

We report the development of a PCR assay enabling specific detection of the phytoplasma associated with MBS disease of corn. For performance of PCR, an oligoprimer pair was designed from partial sequences of a cloned fragment of MBS phytoplasma genomic DNA. The utility of the MBS-specific PCR for detection of the pathogen in plants and insects was also evaluated.

MATERIALS AND METHODS

Sources of healthy and MBS-diseased corn. Two isolates of the MBS phytoplasma were separately maintained in Aristogold Guardian Evergreen sweet corn (*Zea mays* L. var. *saccharata* (Sturtev.) L. H. Bailey) at the University of Florida's Fort Lauderdale Research and Education Center (FLREC) by periodic insect transmission of each pathogen to healthy plants using the vector leafhopper *D. maidis,* as previously described (10). One isolate originated from Texas (MBST) and was kindly provided by L. R. Nault (The Ohio State University, Wooster). A second isolate (MBSF) was obtained from field-grown corn in Dade County, FL (10). Nine additional isolates, which included one from Costa Rica and eight others from the vicinity of Poza Rica, Texcoco, and Tlatlizapan, Mexico, were similarly maintained in corn cv. Early Sunglow at the Ohio Agricultural Research and Development Center, Wooster. Samples of freshly harvested, symptomatic corn tissues containing individual isolates were shipped by overnight courier to the FLREC where they were stored frozen at –75°C until used.

Sources of other mollicutes. A 7-year-old nonbearing cliff date palm (*Phoenix rupicola* T. Anders.) (LYPR) and a 9-year-old coconut palm (*Cocos nucifera* L.) cv. Jamaica Tall (LYJT), both exhibiting mid-stage foliar symptoms indicative of lethal yellowing (LY) disease (32), were felled on the grounds of the FLREC. Immature leaf bases surrounding the apical meristem (heart tissues), a reliable source of the LY phytoplasma (43), were excised from each palm crown. Plants affected by various

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other phytoplasma-associated diseases indigenous to Florida were maintained in shadehouses. These included Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) singly infected with pigeon pea witches'-broom (PPWB) (21), periwinkle witches'-broom (FPWB) (33), or periwinkle virescence (FPVR) disease. Seed-initiated periwinkle and coconut palm cv. Jamaica Tall were grown in containers in a screenhouse and used as sources of healthy plant tissues for comparative purposes.

Periwinkle singly infected with the following additional phytoplasmas were also kindly provided by other researchers: eastern aster yellows (EAY), J. A. Wyman (University of Wisconsin, Madison); western X, prune strain (WX), and western aster yellows (WAY), B. C. Kirkpatrick (University of California, Davis); Yucatan periwinkle witches'-broom (YPWB), M. A. Villanueva, (Centro de Investigacion Cientifica de Yucatan, Merida, Mexico). *Spiroplasma kunkelii* Florida isolate T80 (11) was cultured in C3-G medium (30).

Insect acquisition feedings and plant inoculations. Colonies of vector *D. maidis* and the nonvector planthopper *Peregrinus maidis* Ashmead were reared separately on healthy sweet corn in an insectary at the FLREC by established methods (44,45). Groups of *D. maidis* adults were given a 7-day acquisition access period (AAP) on MBSF-symptomatic plants and then transferred to a series of healthy plants for 21 days. On completion of the incubation period, 30 to 60 inoculative leafhoppers were caged upon additional 4-week-old healthy test plants (4 or 5 plants per pot) for a 3-day inoculation access period. After removal of leafhoppers, test plants were placed in a screenhouse for 12 days. In each of two experiments, eight presymptomatic plants were harvested and samples consisting of the second-most immature leaf and a segment (3 g) of basal stem were removed from each plant and used separately for DNA extraction purposes. Alternatively, inoculated plants were incubated for 3 to 4 weeks until symptom (leaf reddening) expression, at which time healthy *P. maidis* adults were caged upon them for AAPs of 1, 2, 3, or 5 days, respectively. On completion of each AAP, 20 or 24 planthoppers were removed, placed in small polypropylene vials and immediately stored at –75°C. Colony-reared insects or corn plants exposed to healthy insects only served as experimental controls.

Nucleic acid extractions. Preparations enriched with phytoplasmas were obtained from 100-g quantities of stalks of MBST-diseased corn and heart tissues of both LY-diseased cliff date and coconut palm by differential centrifugation after tissues were ground in an osmotically augmented extraction buffer (24). DNAs were extracted from each resulting preparation, as described previously (20). Likewise, sample DNAs were also extracted from comparable tissues of healthy corn by this procedure.

Total DNAs were also obtained by small-scale extractions (1 to 3 g) of fresh or frozen plant tissue samples according to the procedure of Firrao et al. (15). Samples consisted of leaves or stalk tissues from healthy and MBS-symptomatic corn and shoots from healthy or symptomatic periwinkle plants with various other phytoplasmal diseases. Cells from cultures of *S. kunkelii* were harvested by centrifugation at 20,000 × *g* for 30 min at 4°C. DNA was extracted from pelleted cells by the procedure of Dellaporta et al. (12). Resulting plant and spiroplasma DNAs were quantified by fluorometry (TKO-100 minifluorometer, Hoefer Scientific, San Francisco, CA) and stored at 4°C.

Frozen nonvector *P. maidis* planthoppers were ground individually in sterile 1.5-ml microfuge tubes containing 300 µl of DNA extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM disodium EDTA, pH 8.0, 1.4 M NaCl, 1% polyvinylpyrollidone [PVP-40], 1% mercaptoethanol). Extracts were incubated for 30 min at 65°C, cooled, and mixed with 300 µl of chloroform/isoamyl alcohol (24:1 vol/vol). Mixtures were emulsified by vortexing at high speed for 5 s then centrifuged at 12,000 × *g* for 15 min at 24°C. Each upper aqueous phase was transferred to a second 1.5-ml microfuge tube and nucleic acids were precipitated by addition of 300 µl of 2-propanol. Nucleic acids were pelleted by centrifugation at 12,000 × *g* for 15 min, washed with 70% ethanol, briefly dried in vacuo, resuspended in 50 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) buffer, and stored at 4°C.

DNA manipulations. Enriched MBST phytoplasma DNA was separated from mixtures with plant DNA by cesium chloride–bisbenzimide density gradient centrifugation, according to a previously described procedure (18). For molecular cloning, about 500 ng of gradient-enriched phytoplasma DNA was partially digested with *Eco*RI (Promega Biological Research Products, Madison, WI) for 1 h at 37°C. Resulting fragments were ligated with dephosphorylated *pUC*19 using a 10:1 insert/vector ratio and cloned in *Escherichia coli* DH5α cells (Gibco BRL Life Technologies, Gaithersburg, MD). Recombinants were identified as white colonies on Luria-Bertani agar medium containing ampicillin (100 µg ml^{-1}) and X-gal (32 µg ml^{-1}). Recombinant plasmid DNA was extracted by alkaline lysis from small-scale cultures of selected recombinant colonies (39). About 200 ng of each plasmid DNA was blotted onto duplicate nylon membranes (Nytran, Schleicher and Schuell Inc., Keene, NH) according to the protocol of Lee and Davis (26). Blots were air dried, baked at 80°C for 30 min and washed in 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0), 0.5% sodium dodecyl sulfate (SDS) at 65°C for 1 h immediately prior to prehybridization and hybridization with labeled DNA probes.

Recombinant plasmids containing MBST phytoplasma DNA inserts were tentatively identified by moderately stringent differential dot hybridization using *Eco*RI-digested healthy corn DNA or gradient-enriched MBST phytoplasma DNA as probes. Probes were labeled with [^{32}P]-dATP (NEN Research Products, Boston, MA) by using random primers (Random Primed DNA Labeling kit, Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Membranes were prehybridized at 68°C for 16 h in 6× SSC containing 10× Denhardt's solution (0.1% Ficoll, 0.1% PVP-10, 0.1% bovine serum albumin), 0.5% SDS, and 1 mg ml^{-1} denatured salmon sperm DNA. Blots were hybridized with denatured probes at 68°C for 16 h in the same solution and then washed at moderate stringency consisting of two washes in 2× SSC, 0.1% SDS at 25°C (30 min each wash), one wash in 0.2× SSC, 0.1% SDS at 55°C (30 min), and once again in 0.2× SSC, 0.1% SDS (30 min). Membranes were then sealed in plastic wrap and exposed to Konica PB7, X-ray film (Konica Medical Corp., Wayne, NJ) with an intensifier screen (Lightning Plus, DuPont, Newark, DE) for at least 16 h at –75°C. Blots were then rewashed at higher stringency (60°C third wash) and re-exposed to film.

Dot blots of recombinant plasmid DNA were stripped of initial probes by boiling each membrane in 0.1× SSC, 0.5% SDS and reprobed at moderate (55°C third wash) stringency, with [^{32}P]-dATP labeled, gradient-enriched, *Eco*RI-digested EAY phytoplasma DNA or with similarly digested DNA extracted from healthy periwinkle. Cloned DNA inserts from four recombinant plasmids that hybridized only to enriched MBST phytoplasma DNA probe were labeled, as before, and used individually as probe to screen either dot blots of DNAs derived from healthy or MBST phytoplasma–infected corn or Southern blots of DNAs derived from plants with various other phytoplasmal diseases. For dot blots, each sample DNA was applied to nylon membranes as a series of twofold dilutions beginning with 2 µg. For Southern blots, 0.5 to 1.0 g of each sample DNA was digested with *Eco*RI for a minimum of 16 h at 37°C. Resulting digests were electrophoresed in 0.8% agarose gels using 1× TAE (40 mM Tris-acetate, 1 mM EDTA) as running buffer. DNA fragments were blotted from gels onto nylon membranes by a modification of Southern's method (39). Probe hybridizations were initially evaluated at moderate (55°C) and then higher (60°C) stringency, as described above.

Oligonucleotide primers and PCR conditions. Probe MBS-C39 (1 kb), an

anonymous fragment of MBST phytoplasma genomic DNA that hybridized to DNA of MBS-diseased corn only at moderate stringency, was partially sequenced using M13/*pUC* primers and standard dideoxy nucleotide termination reactions (39). A pair of oligonucleotide primers were synthesized on the basis of the sequence data. Both sequencing and primer synthesis were performed by the DNA sequencing and synthesis Core laboratories at the University of Florida's Interdisciplinary Center for Biotechnology Research.

For PCR, template DNAs extracted from plants were first diluted to 25 ng μl^{-1} with sterile deionized water. Amplifications were performed in 25-µl final reaction volumes each containing 25 ng of sample DNA template, 25 ng of each primer, 62.5 M of each dNTP, 0.5 U of AmpliTaq DNA polymerase (AS) with recommended PCR buffer (Perkin-Elmer Cetus, Norwalk, CT) and overlaid with mineral oil. PCR was performed for 30 or 40 cycles in a programmable thermal controller (Model PTC-100, MJ Research Inc., Watertown, MA) using the following parameters: 30 s (90 s for first cycle) denaturation step at 94°C, annealing at 61°C for 50 s and primer extension at 72°C for 80 s (5 min for final cycle). Reaction mixtures containing healthy plant DNA or sterile deionized water substituted for template DNA served as negative controls in each experiment.

For analysis of nonvector *P. maidis* for presence of the MBSF phytoplasma by PCR, 1 µl (2%) of total nucleic acids extracted from single insects was used as template in each 25-µl reaction mixture. Amplifications were performed for 40 cycles as previously described. For each experiment, templates consisting of either nucleic acids from healthy, colony-reared insects, deionized water, or DNA from MBS-symptomatic corn were used as negatives and a positive control, respectively. Following all amplifications, 10 µl of each final reaction mixture was examined by electrophoresis through 1% agarose gels. PCR products in gels were stained with ethidium bromide then visualized by UV transillumination and photographed.

RESULTS

Of the 150 recombinant plasmids initially evaluated by differential dot hybridizations, 35 were judged to contain cloned fragments of MBST phytoplasma genomic DNA. Thirty-one of these hybridized with a probe consisting of gradient-enriched EAY phytoplasma DNA but not with a healthy periwinkle DNA probe at moderate (55°C third wash) stringency, indicating that they contained inserts that were phytoplasma-specific but not MBS phytoplasma-specific (data not shown). Cloned DNA inserts of sizes 3.0, 1.3, 1.0, and 0.9 kb were excised from the four remaining nonhybridizing recombinant plasmids and used as probes in reciprocal hybridizations to confirm their disease specificity. All four probes were observed to hybridize with DNA extracted from MBST-diseased corn and to varying extents with DNAs of plants with other phytoplasmal diseases on Southern blots at moderate stringency (data not shown). However, signals resulting from hybridization of probe MBS-C39 (1.0 kb) with DNAs of both EAY and FPVR phytoplasma-infected periwinkle were barely discernible (data not shown) and subsequently eliminated when the blot was rewashed at higher (60°C) stringency (Fig. 1).

Partial enzymatic sequencing of the ends of probe MBS-C39 yielded sequence data with an overall G+C content of 31.4%. Twenty-mer and 18-mer oligonucleotide sequences designated MBS-F1 and MBS-R1, with an estimated T_m of 60.1 and 60.4°C, respectively, were synthesized for use as PCR primers on the basis of sequence data (Fig. 2). The reliability of the chosen primer set was evaluated by PCR analysis of template DNAs derived from MBST-symptomatic corn, plants with various other phytoplasmal diseases, and *S. kunkelii* cells. Thirty or 40 cycles of PCR performed with an annealing temperature of 61°C repeatedly amplified a prominent DNA product of about 740 bp in size in reaction mixtures containing DNA from MBST-symptomatic corn but not from healthy corn. The size of the DNA product was consistent with its predicted size according to the distance between primer sequences within the original MBST phytoplasma DNA probe. No products were apparent in final reaction mixtures that contained DNA from healthy plants, periwinkle infected with either WAY, FPWB, PPWB, or WX phytoplasmas, coconut palm with LY disease, or *S. kunkelii* (Fig. 3).

Positive detection of the MBST phytoplasma in symptomatic plants was also accomplished by moderately stringent dot hybridization with probe MBS-C39. Hybridization signals were clearly evident for samples representing as little as 3.9 ng of DNA from a phytoplasma-enriched preparation of stalks, or 15.6 ng when samples consisted of total DNAs extracted by small-scale extraction of these tissues (Fig. 4). Decreasing quantities of the latter DNA sample as template for PCR provided a measure of the relative sensitivity of this method compared with probe hybridization for detection of the MBST phytoplasma. Positive detection of the pathogen was repeatedly achieved after 30 cycles with as little as 5 pg of total DNAs from infected stalk tissues and an annealing step at 61°C (Fig. 5). Thus, for this sample, PCR improved detection of the MBST phytoplasma by as much as 3,000-fold beyond the lower limits attainable by dot hybridization with probe MBS-C39.

The reliability of the chosen primer sequences was verified by PCR assessment of template DNAs extracted from symptomatic corn plants singly infected with isolates of the MBS phytoplasma originating from four geographic locations. A prominent 740-bp product was consistently amplified after 30 cycles from each

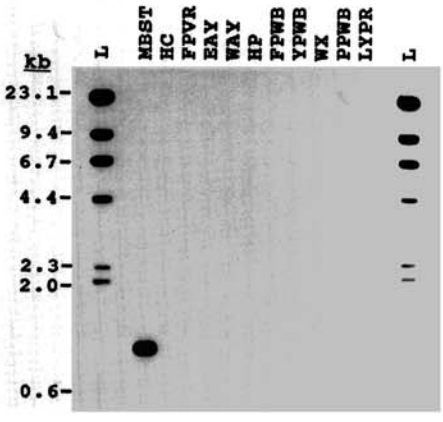

Fig. 1. Southern hybridization of probe MBS-C39, a 1.0-kb fragment of chromosomal DNA cloned from a Texas isolate of the maize bushy stunt phytoplasma, to *Eco*RI-digested DNAs derived from healthy plants and plants with various phytoplasmal diseases. Sample DNA extracted from sweet corn (*Zea mays* var. *saccharata*) with MBST, a Texas isolate of the maize bushy stunt phytoplasma; HC, healthy sweet corn. DNA from periwinkle (*Catharanthus roseus*) plants with FPVR, Florida periwinkle virescence; EAY, eastern aster yellows; WAY, western aster yellows; HP, healthy periwinkle; FPWB, Florida periwinkle witches'-broom; YPWB, Yucatan periwinkle witches'-broom; WX, western X; PPWB, Florida pigeon pea witches'-broom. DNA from lethal yellowing-diseased cliff date (*Phoenix rupicola*) palm. L, lambda DNA/*Hin*dIII fragments.

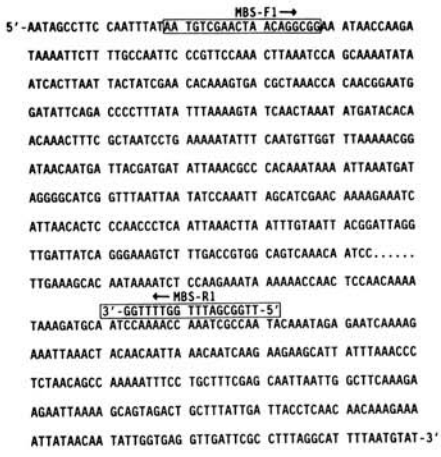

Fig. 2. Partial sequence of probe MBS-C39, a 1.0-kb fragment of chromosomal DNA cloned from a Texas isolate of the maize bushy stunt (MBS) phytoplasma. The locations of primers MBS-F1 and MBS-R1 used for performance of polymerase chain reactions are indicated in boxes.

of the 11 MBS phytoplasma isolates tested, which included eight isolates from Mexico and single isolates of the pathogen from Costa Rica, Florida, and Texas (Fig. 6).

The sensitivity of MBS-specific PCR was further evaluated by analyzing total DNAs extracted from the entire second-most immature leaf, and from basal stalk tissues of corn plants 12 days after exposure to multiple inoculative vectors. PCR performed for 30 cycles, utilizing 25 ng of host DNA as template for each reaction, readily detected MBSF phytoplasma infection of both leaf and stalk samples excised from all 16 presymptomatic test plants. Products typical of those observed as a result of PCR are illustrated in Figure 7. By comparison, the same samples were all judged negative for infection when 1 µg of DNA derived from each leaf or stalk was screened by moderately stringent dot hybridization using probe MBS-C39 (data not shown).

Subsequently, performance of PCR for monitoring acquisition of the MBSF phytoplasma by nonvector insects was also evaluated by testing adult *P. maidis* planthoppers given AAPs of 1, 2, 3, or 5 days, respectively, on symptomatic plants. Forty cycles of PCR were sufficient for consistent and reproducible detection of the pathogen within the majority of planthoppers tested in each of two experiments, including those exposed to diseased plants for 1 day only (Table 1).

DISCUSSION

The goal of this work was to develop primers for use in MBS-specific PCR. The exceptional sensitivity of PCR offers many advantages for detection of plant pathogens (22). Application of this technique to detection and investigation of phytoplasmas seems particularly appropriate due to the small size of these plant pathogens and an inability to culture them in vitro. Also, phytoplasmas occur in relatively low titers and are often inconsistently distributed, colonizing phloem tissues only, within various organs of their respective plant hosts (27). While application of both the enzyme-linked immunosorbent assay (ELISA) and DNA probe hybridizations has considerably improved detection of phytoplasmas in their respective hosts, more recent studies have demonstrated the practical superiority of PCR for this purpose (4,20,40).

To impart the desired specificity to the PCR assay, anonymous fragments of MBST phytoplasma DNA were first cloned and then analyzed for sequences unique to the pathogen that might serve as primers. We chose this particular approach to primer selection because PCR assays enabling pathogen-specific detection of phytoplasma associated with Canadian clover proliferation (14), Caribbean coconut lethal yellowing (20), and American grapevine yellows (4) have been successfully developed by this means. However, phytoplasma detection in plant tissues incorporating primer sets based upon 16S ribosomal RNA (rRNA) sequences has also been accomplished (1,19,29), although pathogen-specific detection by this alternative approach has yet to be demonstrated.

Evaluation of cloned genomic fragments of MBST phytoplasma DNA by dot hybridization using enriched DNA of an aster yellows phytoplasma isolate proved to be an appropriate method for identifying fragments representing phytoplasma-specific rather than MBS phytoplasma-specific sequences. The selection of this pathogen as a source of probe DNA was based upon previous reports of considerable genetic similarity between AY-group phytoplasmas and the MBS phytoplasma (2,23,28); a relationship further substantiated by the outcome of our preliminary screening procedure, since 31 of 35 cloned MBST phytoplasma DNA fragments hybridized with the AY phytoplasma DNA probe preparation. Likewise, three of the remaining four nonhybridizing fragments when used as probes in later reciprocal hybridizations also detected DNAs of EAY, WAY, and FPVR phytoplasmas at moderate stringency. Due to their lack of detection specificity, hybridizing (phytoplasma-specific) fragments were discounted as candidate sequences for primer development.

A primer pair derived from the remaining nonhybridizing probe MBS-C39 permitted reliable and reproducible amplification of a 740-bp DNA product from all plants, each containing one of 11 MBS phytoplasma isolates originating from four different localities throughout the geographic range of the disease. However, as anticipated from earlier probe hybridization results, pathogen-specific detection was attained with these primers only after thermocycling conditions were modified to include an annealing temperature of 61°C. Presence of the MBS phytoplasma in both leaf and stem samples from presymptomatic plants was also readily demonstrated by MBS phytoplasma–specific PCR. By

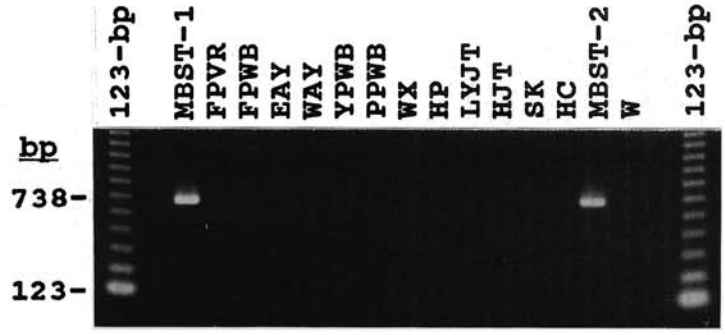

Fig. 3. Polymerase chain reaction (PCR) products amplified from total DNAs extracted from maize bushy stunt (MBS) phytoplasma–infected sweet corn (*Zea mays* var. *saccharata*), plants with various other phytoplasmal diseases and *S. kunkelii,* analyzed by 1% agarose gel electrophoresis. Template DNAs for PCR consisted of total DNAs derived from MBST, sweet corn (*Zea mays* var. *saccharata*) infected with a Texas isolate of the MBS phytoplasma; periwinkle (*Catharanthus roseus*) with FPVR, Florida periwinkle virescence; FPWB, Florida periwinkle witches'-broom; EAY, eastern aster yellows; WAY, western aster yellows; YPWB, Yucatan periwinkle witches'-broom; PPWB, Florida pigeon pea witches'-broom; WX, western X; HP, healthy periwinkle; HC, healthy corn; SK, *Spiroplasma kunkelii*. DNA derived from phytoplasma-enriched preparations of LYPR, lethal yellowing-diseased coconut (*Cocos nucifera*) cv. Jamaica Tall palm; HJT, healthy coconut cv. Jamaica Tall; MBST-2, corn with Texas maize bushy stunt; W, water control. 123, BRL 123-bp ladder.

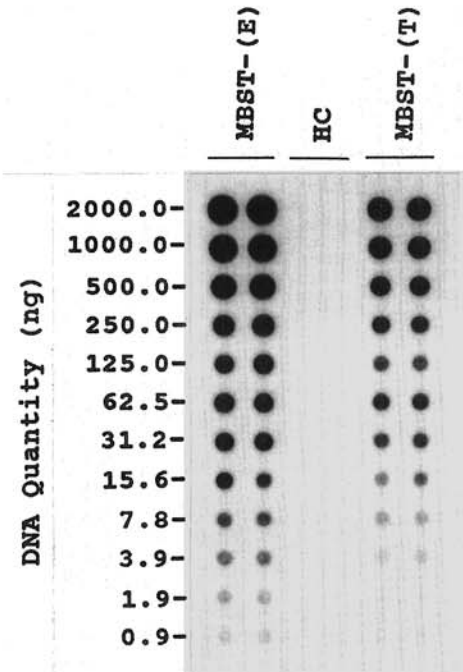

Fig. 4. Moderately stringent (55°C) dot hybridization of probe MBS-C39 (1.0 kb) to DNAs extracted from healthy and maize bushy stunt (MBS) phytoplasma-infected sweet corn (*Zea mays* var. *saccharata*). Samples consisted of DNAs derived from MBST-E, a phytoplasma-enriched preparation of stalks from symptomatic corn plants infected with a Texas isolate of the MBS phytoplasma; HC, small scale extraction of total DNAs from healthy corn stalks; MBST-T, small scale extractions of total DNAs from stalks of MBST-diseased corn.

comparison, pathogen detection was unsuccessful when these samples were screened by dot hybridization using chromosomal DNA probe MBS-C39. A similar inability to detect the MBS phytoplasma in presymptomatic corn stalks using a chromosomal DNA probe has been previously reported (34) although detection attempts were successful when cloned extrachromosomal (plasmid) DNA of the MBS phytoplasma was used as probe. However, not all isolates of the MBS phytoplasma apparently contain plasmid DNAs (10), thus limiting the utility of such probes for disease indexing of corn.

To date, PCR has seen only limited application for detection of phytoplasmas in vector and nonvector insects alike (36, 47). In this study, we evaluated the performance of MBS phytoplasma–specific PCR for pathogen detection in insects by analyzing *P. maidis* individuals following their exposure to MBSF-symptomatic corn. This particular species was chosen for investigation because it is a maize specialist and actively feeds on bushy stunted test plants. Also, *P. maidis* does not vector the MBS phytoplasma (44) or any other plant pathogenic phytoplasma (46). Thus, presence of the MBSF phytoplasma within each planthopper was clearly restricted to quantities of the pathogen ingested by this nonvector during feeding activity only. Furthermore, a limited amount (2%) of the total nucleic acids from each planthopper was included as template in reaction mixtures since use of greater quantities can result in inhibition of PCR (47). Despite the latter constraint, presence of the MBSF phytoplasma in most insects was confirmed by PCR and underscored the effectiveness of this detection technique.

The vectors of many phytoplasmal diseases remain to be identified (8,46). In searches for unknown vectors, pathogen-specific PCR should provide a practical means to survey substantial numbers of homopterans although positively testing insects need not necessarily be vectors (47). However, preliminary screenings by PCR may serve to quickly narrow down searches for candidate species that contain the pathogen of interest. Also, as was demonstrated in this study, only minimal quantities of nucleic acids from a single insect are necessary for testing by PCR, leaving residual nucleic acids available for further analysis by DNA probe hybridization. The latter technique is evidently insensitive to phytoplasma acquisition by individual insects but can detect those in which some multiplication of the pathogen has occurred (10,35,48). While neither obviates the need for conducting transmissions to unequivocally identify vectors, presence and fate of phytoplasmas in both insect and plant hosts can be precisely monitored by using these complementary techniques.

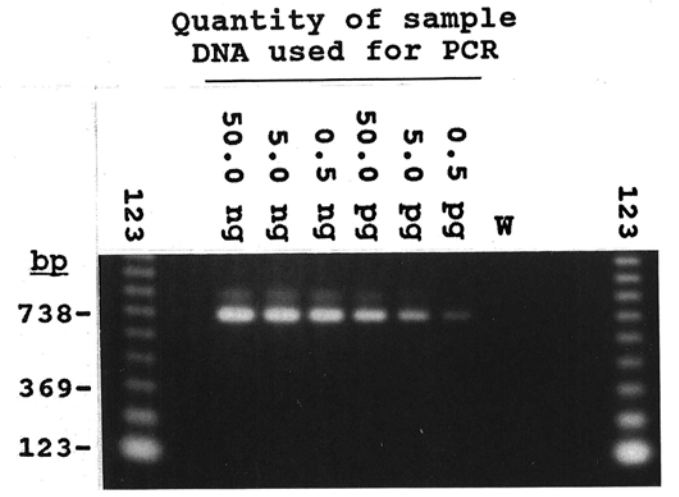

Fig. 5. Sensitivity of pathogen-specific polymerase chain reaction (PCR) for detection of the maize bushy stunt (MBS) phytoplasma in sweet corn (*Zea mays* var. *saccharata*). Following 30 cycles of amplification, 10 µl of each 25-µl final reaction mixture was analyzed by electrophoresis through a 1% agarose gel. Template for PCR consisted of total DNAs derived from stalk tissues of MBST, symptomatic plant infected with a Texas isolate of the MBS phytoplasma; W, water control; 123, BRL 123- bp ladder.

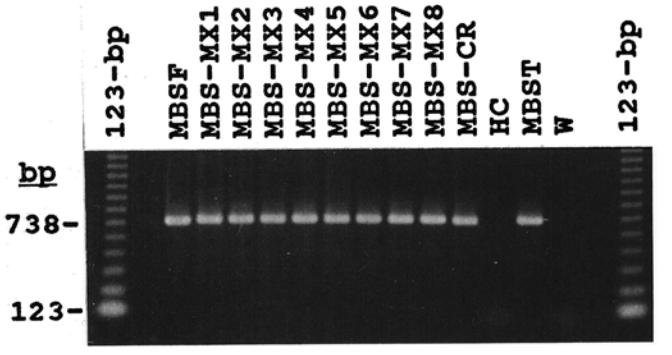

Fig. 6. Polymerase chain reaction (PCR) analysis of total DNAs extracted from stalks of sweet corn *Zea mays* var. *saccharata*) plants singly infected with isolates of the maize bushy stunt (MBS) phytoplasma originating from four geographic localities. Template DNAs for PCR consisted of MBSF, MBS phytoplasma, Florida isolate; MBS-MX1-8, eight isolates from Mexico; MBS-CR; isolate from Costa Rica; HC, healthy sweet corn MBST, Texas isolate; W, water control. 123, BRL 123-bp ladder.

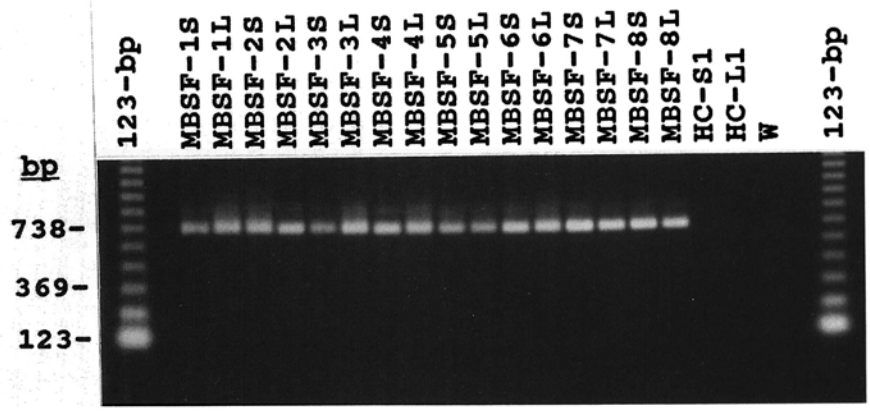

Fig. 7. Detection of Florida maize bushy stunt (MBS) phytoplasma DNA in leaves and stalks of presymptomatic sweet corn (*Zea mays* var. *saccharata*) plants after 40 cycles of MBS phytoplasma-specific polymerase chain reaction (PCR). Template DNAs for PCR analyses were derived from corn tissues 12 days after plants were exposed to multiple inoculative *Dalbulus maidis* adults for 3 days. MBSF-S1-8, basal stalk samples from eight plants inoculated with a Florida isolate of the MBS phytoplasma; MBSF-L1-8, second-most immature leaves from eight inoculated plants; HC- S1, basal stalk from healthy, uninoculated plant; HC-L1, second-most immature leaf from uninoculated plant; W, water control; 123-bp, BRL, 123-bp ladder.

Table 1. Summary of results from use of pathogen-specific polymerase chain reaction (PCR) for detection of a Florida maize bushy stunt (MBSF) phytoplasma isolate in nonvector *Peregrinus maidis* adults given acquisition access periods (AAPs) of 1 to 5 days on MBSF-symptomatic sweet corn plants

	Experiment 1[a,b]		Experiment 2	
AAP (days)	No. positive / no. tested	% positive	No. positive / no. tested	% positive
1	13/20	65.0	23/24	95.8
2	20/20	100.0	24/24	100.0
3	17/20	85.0	23/24	95.8
5	12/20	65.0	23/24	95.8
Control[c]	0/20	0.0	0/24	0.0

[a] A 0.02 volume of total nucleic acids extracted from each adult planthopper was used as template in 25-µl reaction mixture volumes during 40 cycles of amplification by MBS- specific PCR.

[b] Ten microliters of each final reaction mixture was analyzed by 1% agarose gel electrophoresis. Presence of a visible 740-bp DNA amplimer indicated those insects that had acquired detectable quantities of the MBSF phytoplasma.

[c] Adult *P. maidis* fed on healthy, uninoculated corn plants only.

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