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Use of an A-T-Rich DNA Clone for Identification and Detection of *Peronosclerospora sorghi*

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A recombinant plasmid, pMLY12-1, screened from a *Peronosclerospora sorghi* library hybridizes only to DNA of *P. sorghi*, or to DNA from leaves infected with *P. sorghi*, not to DNA of *P. sorghi* Thailand isolate, *P. philippinensis*, *P. sacchari*, or *P. maydis*. The terminal sequences of the 1.3-kb insert, which appears to contain mitochondrial DNA, are 85% A and T. No polymorphisms were detected when the probe was hybridized to Southern blots containing DNA from *P. sorghi* pathotype 1, pathotype 3, or a Botswana isolate digested with any of the eight restriction endonucleases tested. The banding patterns were the same whether DNA was extracted directly from the fungus or from infected leaves.

Sorghum downy mildew disease, caused by *Peronosclerospora sorghi* (Weston & Uppal) C. G. Shaw, is one of the most serious diseases of corn and sorghum in tropical and subtropical regions. Although it has been reported from dozens of countries in Asia, Central America, South America, North America, and Africa, there are still many countries where this pathogen has not been found. Disease incidence in Texas has exceeded 30% and can be much higher in regions favorable to disease development. The recent epidemic of this disease in Egypt indicates that it still poses a threat to sorghum and maize production around the world. Three other *Peronosclerospora* species, *P. sacchari*, *P. philippinensis*, and *P. maydis*, can also infect maize or sorghum. The major morphological criteria that separate different species in the genus *Peronosclerospora* are differences in the size and shape of conidia and conidiophores, which are often difficult to discern, especially since conidial dimensions vary with different host species, cultivars, and the environmental conditions at the time of sporulation (3, 12, 15, 19). This makes the identification and detection of *P. sorghi* very difficult, as do its biotrophic and seed-borne characteristics. Development of rapid, sensitive, and accurate means of detection of this pathogen are critical to the success of quarantine efforts to prevent the introduction and establishment of *P. sorghi* in new regions.

DNA probes are being used increasingly to identify and detect plant pathogens (5–9). Being aware of the problems in identifying and detecting *P. sorghi*, we were prompted to apply DNA hybridization methods to detection and diagnosis of this pathogen. Success in using DNA hybridization to detect downy mildew in tissues of infected hosts (21) and in using cloned probes to differentiate species of *Peronosclerospora* (22) suggested that it may be possible to identify highly specific probes. We report here the isolation and partial characterization of a clone that can be used as a species-specific hybridization probe for the detection and identification of *P. sorghi*.

MATERIALS AND METHODS

Sample collection. Isolates of *P. sorghi*, *P. sacchari*, *P. philippinensis*, and *P. maydis* and *P. sorghi* Thailand isolate were obtained from different collectors; species names, isolate names, locations and dates of collection, and the name of the collectors are listed in Table 1.

DNA isolation and manipulation. A minipreparation method developed by Lee et al. (11) for isolating total genomic DNA was used, with minor modifications (22). Standard manipulations were performed by the method of Sambrook et al. (17), unless otherwise stated. Total genomic DNA of *P. sorghi* pathotype 3 was digested with *Eco*RI, ligated into *Eco*RI-digested plasmid pUC19, and used to transform competent cells of *Escherichia coli* DH5α. White colonies containing recombinant plasmids were selected based on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside screening procedures. The white colonies were transferred onto GeneScreen Plus membrane and screened by colony hybridization to total genomic DNA labeled with [^{32}P]dATP by nick translation. Sources of genomic DNA were *P. sorghi* pathotype 3, *P. sorghi* Thailand isolate, *P. sacchari*, *P. philippinensis*, and *P. maydis*. Clones that hybridized to ^{32}P-labeled DNA of *P. sorghi* pathotype 3 but not to DNA from *P. sacchari*, *P. philippinensis*, *P. maydis*, or *P. sorghi* Thailand isolate were selected and grown in LB broth, and plasmid DNA was isolated by a minipreparation procedure developed by Zhou et al. (23). Separation of *P. sorghi* DNA into high- and low-density bands followed the procedure of Garber and Yoder (4).

DNA blots. All DNA concentrations in this work were determined by DNA fluorometry (model TKO 100 fluorometer; Hoefer Scientific Instruments, San Francisco, Calif.). The DNA concentration of each sample for dot blots was adjusted to 25, 10, 2.5, and 1 ng/μl by dilution. The procedures for preparing dot blots were the same as described previously (21).

Restriction enzymes were purchased from Promega, and reactions were carried out as recommended by the manufacturer. On the basis of preliminary experiments, 0.75 μg of DNA extracted from pathotypes 1 and 3 was digested with 5 U of enzyme, whereas 1.5 μg of DNA isolated from maize and sorghum leaves infected with *P. sorghi* pathotype 1, pathotype 3, or the Botswana isolate was treated with 10 U of enzyme for 4 h at 37°C. Digested DNA was subjected to electrophoresis in a 0.9% agarose gel in 1× Tris-borate-EDTA buffer (17) at 40 V overnight. Alkaline blotting of DNA from the gel to GeneScreen Plus hybridization membrane utilized the method developed by Reed and Mann (16).

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TABLE 1. *Peronosclerospora* species used in this study

Species	Isolate name	Collection		Source
		Place	Yr	
P. sorghi	Pathotype 1	Texas	1988	R. A. Frederiksen
	Pathotype 3	Texas	1988	R. A. Frederiksen
	Botswana	Botswana	1989	G. N. Odvody
	Thailand 1	Pak Chang	1975	B. Renfro
P. maydis	Indonesia	Malang		H. Vermeulen
P. sacchari	New Guinea	Papua		
Peronosclerospora sp.	China 1	Guangxi	1990	S. Y. Zhou
	China 2	Yunnan	1990	S. X. Zhen
P. philippinensis	Philippines	Luzon Expt Station	1984	J. M. Bonman

After transfer, the membrane strips were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min and then dried in a vacuum oven at 80°C and −80 kPa for 2 h.

Hybridizations and probe preparation. The membrane strips were prehybridized at 65°C for 24 h in a sealed plastic bag in a solution made by combining 12.6 ml of double-distilled water, 5.0 ml of 20× SSC, 0.4 ml of 100× Denhardt solution, 1.0 ml of 1 M Tris, 0.2 ml of 20% sodium dodecyl sulfate (SDS), 0.4 ml of 0.5 M EDTA, and 0.4 ml of denatured salmon sperm DNA (10 mg/ml). Nick translations of 100 ng of selected recombinant plasmid DNA were made with a kit provided by Promega, as instructed, using [^{32}P]dATP as a label. After prehybridization, the labeled probe was added to the prehybridization solution, and the bag was resealed and placed at 65°C for a further 24 h. The GeneScreen Plus membrane strips were then removed and washed at 65°C for 30 min each in 500 ml of 1× SSC–1% SDS and then 0.01× SSC with constant agitation. After washing, the membrane strips were exposed to Kodak Blue Brand X-ray film for 24 h for dot blots or for 3 days for Southern blots at −70°C with an intensifying screen.

DNA sequencing. A 16-μl miniprep sample containing 3.2 μg of DNA from pMLY12-1 was first treated with 4 μl of 4 M NaCl, and then 20 μl of 13% polyethylene glycol 8000 was added to the mixture. After the mixture sat on ice for 30 min, DNA was precipitated with ethanol and dried in a speed vacuum for about 5 min. The polyethylene glycol-precipitated miniprep pMLY12-1 DNA was then resuspended in 18 μl of water and treated with 2.0 μl of 2 N NaOH–2 mM EDTA for 5 min at room temperature. After 25 μl of ice-cold 5 M NH_4 acetate was added to the solution, DNA was precipitated with ethanol and dried in a speed vacuum. The DNA pellet was resuspended in 7 μl of water for sequencing. Nucleotide sequencing was performed by the dideoxy-chain termination method (17, 18), using [^{32}P]dATP, forward and reverse sequencing primers for pUC19, and the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). The DNA sequence was determined by using a semiautomated DNA sequencing gel reader (Bio-Rad Laboratories, Richmond, Calif.).

Sequence analysis. Restriction sites within the sequenced regions were located with the aid of MacVector (IBI). The sequenced regions were compared in both orientations with the entire set of sequences available in GenBank (release 66) and EMBL (release 25) by using the FASTA comparison algorithim under GCG protocols (2).

RESULTS

A total of 987 recombinant colonies were screened by colony hybridization. One recombinant plasmid, pMLY12-1, which contains a 1.3-kb *P. sorghi* pathotype 3 DNA insert, was identified that showed a hybridization signal only with DNA from *P. sorghi*. When DNA from this plasmid was labeled by nick translation to a specific activity of approximately 10^8 cpm/μg and used as a probe under standard conditions, DNA hybridization was detected with DNA extracted from each of the three available isolates of *P. sorghi* whether DNA was extracted directly from the fungus or from host tissues infected with *P. sorghi*. Samples containing 1 ng of *P. sorghi* pathotype 3 DNA can be readily detected under the hybridization conditions used (Fig. 1). No detectable hybridization occurred between the probe and DNA from *P. sacchari*, *P. philippinensis*, *P. maydis*, and *P. sorghi* Thailand isolate. pMLY12-1 did not show polymor-

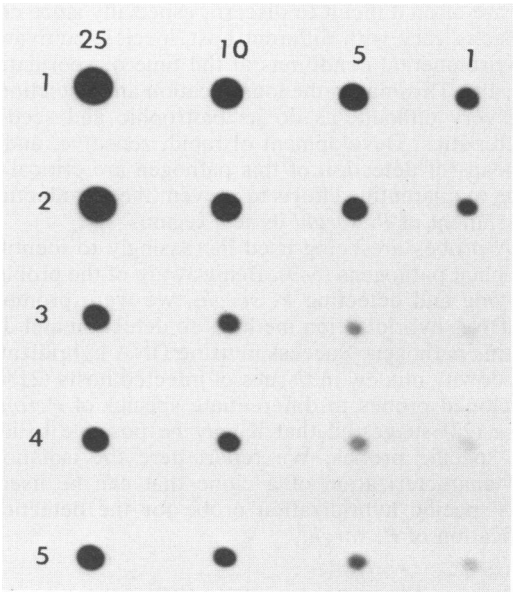

FIG. 1. Dot blot autoradiograph of DNA extracted from various isolates. Row 1, *P. sorghi* pathotype 1; row 2, *P. sorghi* pathotype 3; row 3, sorghum leaves infected with *P. sorghi* pathotype 1; row 4, sorghum leaves infected with *P. sorghi* pathotype 3; row 5, maize leaves infected with *P. sorghi* Botswana isolate, hybridized to ^{32}P-labeled pMLY12-1 DNA probe. Numbers at the top are DNA concentrations, in nanograms.

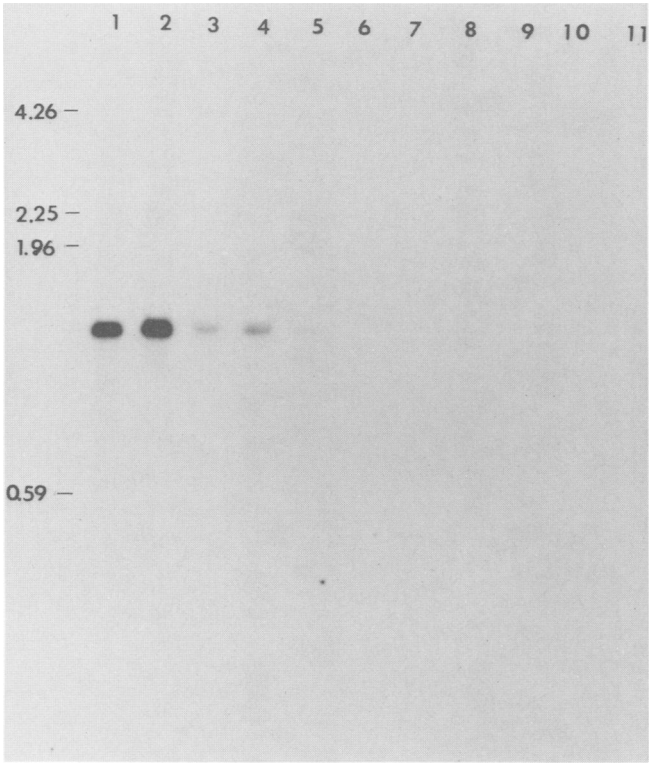

FIG. 2. Southern blot autoradiograph of DNA extracted from various isolates. Lanes 1 and 2, *P. sorghi* pathotypes 1 and 3, respectively; lanes 3 and 4, sorghum leaves infected with *P. sorghi* pathotypes 1 and 3, respectively; lane 5, maize leaves infected with *P. sorghi* Botswana isolate; lanes 6 and 7, sugar cane leaves infected with *P. sacchari* and *P. philippinensis*, respectively; lanes 8 and 9, maize leaves infected with *Peronosclerospora* sp. isolates Yuannan and Guangxi, respectively; lane 10, *P. maydis*; lane 11, *P. sorghi* Thailand isolate 1 digested with restriction enzyme *Eco*RI and hybridized to ^{32}P-labeled DNA of plasmid pMLY12-1. Numbers to the left of the figure indicate the molecular sizes, in kilobases.

phisms when hybridized to Southern blots containing *Eco*RI-, *Pvu*I-, *Hin*dIII-, *Bam*HI-, *Dra*I-, *Ssp*I-, *Sau*3A-, or *Pst*I-digested DNA from *P. sorghi* pathotype 1, pathotype 3, or the Botswana isolate. The banding patterns were the same whether DNA was extracted directly from the fungus or from infected leaves (Fig. 2 shows the *Eco*RI blot). No hybridization can be detected between the probe and DNA extracted from leaves of uninfected plants.

Among 544 bases determined from sequencing gels, >85% are A and T (Fig. 3). A computer search (MacVector) for restriction sites in the sequenced regions produced the maps shown in Fig. 4. The absence of restriction sites for *Eco*RI, *Kpn*I, *Sma*I, *Bam*HI, *Pst*I, *Sph*I, or *Pvu*I in the unsequenced portion of the clone was verified experimentally by showing that the insert remained intact following digestion, whereas digestion with *Dra*I produced numerous small fragments, indicating that there are additional TTTAAA sites in the center of the clone.

No identical or nearly identical sequences were found when either the 236- or the 308-base sequence was compared with the available published sequences. However, the lists of best potential matches were dominated consistently by sequences available from three organisms: the slime mold *Dictyostelium discoideum*, yeast mitochondrial DNA (mtDNA), and *Plasmodium parasiticum*.

DISCUSSION

In screening a relatively small number of clones from an *Eco*RI library of *P. sorghi* DNA, one clone was identified which gave a strong signal when hybridized to labeled genomic DNA from the donor but no detectable hybridization when probed with labeled DNA from closely related species. Strong signals suggest that the cloned sequence might contain a copy of a gene for rRNA or another highly repeated sequence present in the nuclear DNA of the donor. Examples among fungal plant pathogens include the "MGR" sequence, distinctive for isolates of *Magnaporthe grisea* that are rice pathogens (7), and two *Phytophthora citrophora* clones that also distinguish formae speciales (6). In each of these cases, hybridization of the clone to Southern blots of genomic DNA following digestion by restriction enzymes produces numerous bands or even a smear, reflecting the dispersed occurrence of the repeated sequence throughout the genome. In contrast, hybridization of pMLY12-1 to genomic DNA of *P. sorghi* gave simple banding patterns for each of several enzymes tested, thus requiring an alternative explanation. A clue to an alternative was gained by partial sequence analysis and comparison to known sequences. Both ends of the probe were extremely AT rich; failure to digest the unsequenced region by restriction endonucleases that include GC base pairs in the recognition site but digestion to numerous small fragments by *Dra*I (specific for TTTAAA sites) implies that the entire clone is AT rich. When the cloned sequences were compared with those available in GenBank and EMBL, relatively high initial and optimized scores were obtained with numerous sequences from *D. discoideum*, a slime mold that has 85% AT base pairs in its genomic DNA and is especially AT rich in intergenic regions (10); *P. falciparum*, the cause of human malaria, which has 82% AT (13); and yeast mtDNA, which also has about 82% AT (1). Thus, even when 65% of the bases in paired sequences matched, the matches fell in short runs of A or T scattered throughout the length of the region of homology with interspersed mismatches and/or gaps so that DNA hybridization would not be expected. The analysis did suggest that the clone could be an mtDNA fragment; this possibility was strengthened by showing that the probe preferentially hybridizes to a low-density band recovered after density gradient centrifugation. Though mtDNA in fungi typically has a lower density than nuclear DNA, this has not been verified for *P. sorghi*. Since numerous copies of mtDNA are present per nucleus, and the hybridization of AT-rich sequences should be especially sensitive to mismatches during hybridization and washing under standardly used temperature and salt conditions, AT-rich mtDNA clones may be ideal for diagnostic probes. An mtDNA probe has also been described that can be used to identify the take-all pathogens of cereals (8).

The cloning and identification of a DNA probe specific to *P. sorghi* provides a new tool for accurate detection and identification of this biotrophic fungus. This is important since identification of *P. sorghi* can be very difficult with currently available methods, especially when it occurs on maize, on which oospores are less likely to be produced, and other *Peronosclerospora* species (including *P. sorghi* Thailand isolate, *P. sacchari*, *P. philippinensis*, and *P. maydis*) which have morphological dimensions similar to those of *P. sorghi* are likely to be found. Although Micales et al. (14)

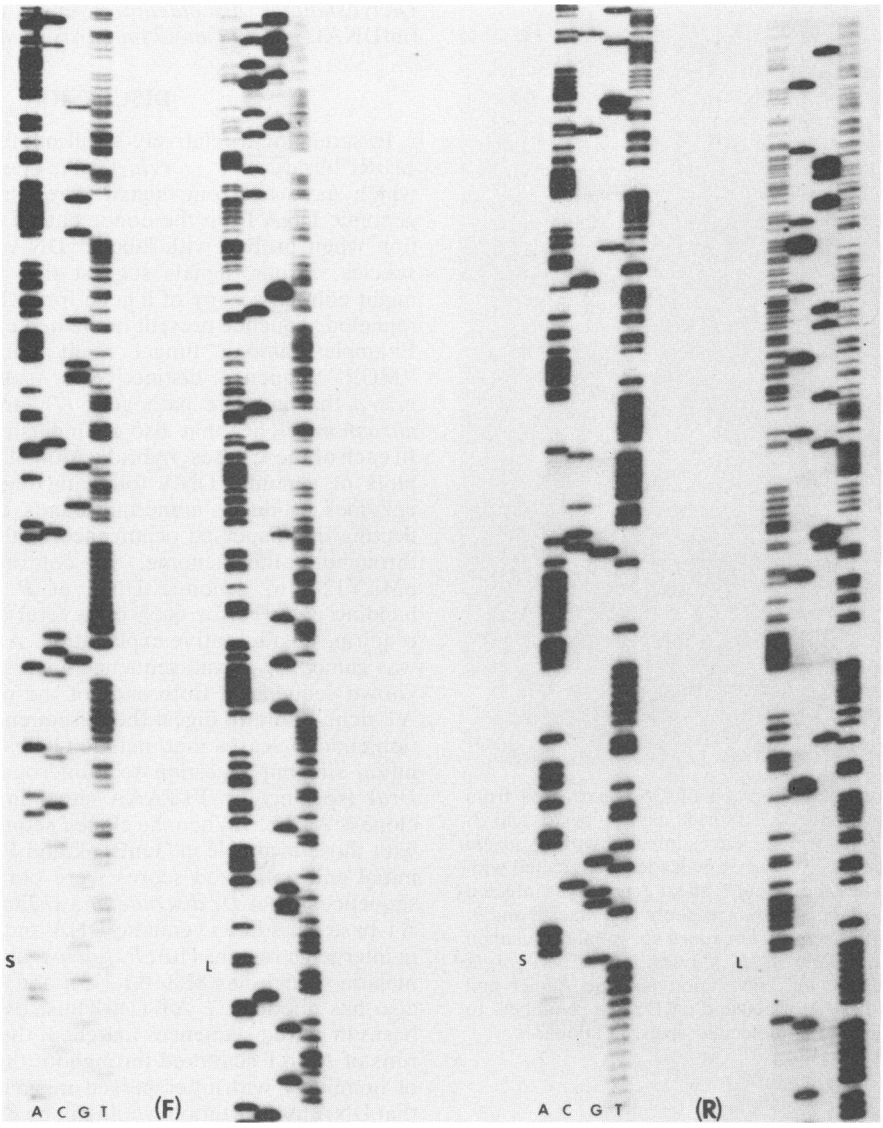

FIG. 3. DNA sequencing autoradiograph of plasmid pMLY12-1. The outer lanes in each set of four are A and T. F, forward primer [5′-d(GTTTTCCCAGTCACGAC)-3′]; R, reverse primer [5′-d(CAGGAAACAGCTATGAC)-3′]; S, short gel; L, long gel.

have used isozyme analysis to identify four *Peronosclerospora* species, *P. sorghi*, *P. maydis*, *P. philippinensis*, and *P. sacchari*, the great difficulties in collecting and maintaining living *Peronosclerospora* species, preventing their mix-up and spread, and obtaining enough living spores for enzyme extraction limit the practicality of using isozymes for identifying *Peronosclerospora* spp. We have demonstrated that there is no hybridization between the probe and DNA extracted from uninfected maize or sorghum tissues under our hybridization conditions. However, when DNA was isolated from *P. sorghi*-infected leaves, hybridization to the labeled probe was readily detected. Plasmid pMLY12-1 appears to be species specific, so it should make a useful probe for identifying and detecting *P. sorghi* in plant tissues and perhaps even in soil. If so, it could be very useful to regulatory agencies charged with preventing the introduction of *P. sorghi* in new regions. With standard dot blotting techniques, 1 ng of *P. sorghi* DNA can be detected readily by this DNA probe. For even greater sensitivity, sequence information from the probe is being used to develop primers for use in polymerase chain reaction assays.

The cloned probe hybridized well to DNA from the three most diverse isolates of *P. sorghi* available. Southern blots of electrophoretically separated fragments produced by treating DNA extracted from *P. sorghi* pathotype 1, pathotype 3, and the Botswana isolate with restriction enzymes *Dra*I, *Ssp*I, *Sau*3A, *Eco*RI, *Pvu*I, *Hin*dIII, *Bam*HI, and *Pst*I did not show polymorphisms when hybridized to ^{32}P-labeled pMLY12-1. Since only eight enzymes and three isolates of *P. sorghi* were used, it may be possible to find polymorphisms by examining more isolates and by using more restriction enzymes. Consistent restriction fragment length polymorphism differences would be helpful in epidemiological studies if they would serve to identify existing pathotypes of *P. sorghi* and thus aid in deploying appropriate resistant cultivars in different regions.

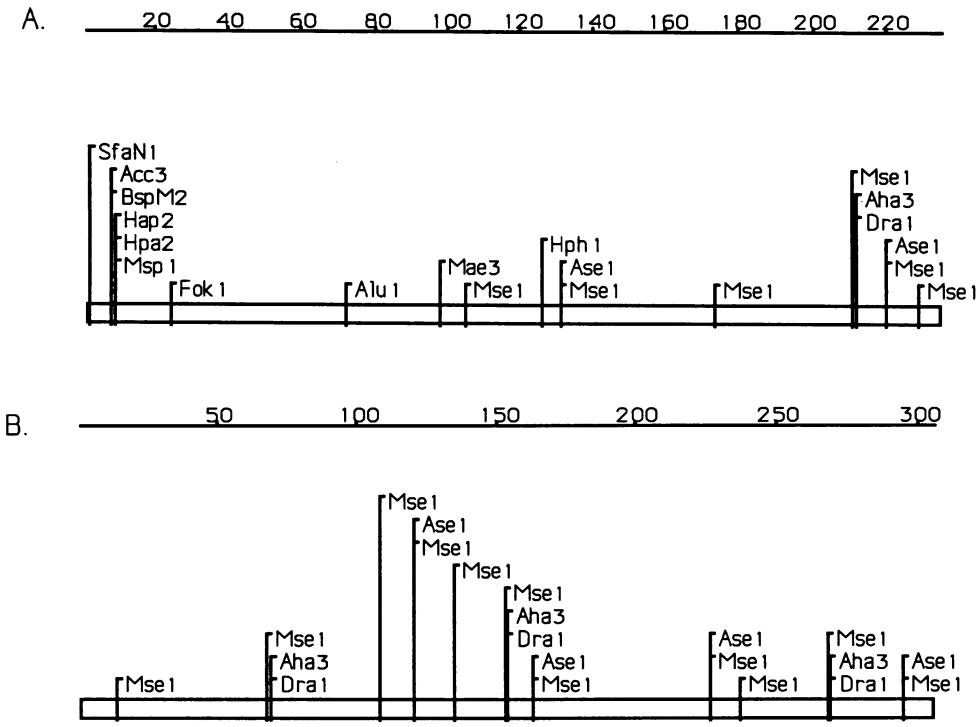

FIG. 4. Computer-generated restriction map of the sequenced ends of pMLY12-1: (A) 236-base terminus; (B) 308-base terminus. The recognition sequences are as follows: *Acc*III, TCCGGA; *Aha*III and *Dra*I, TTTAAA; *Alu*I, AGCT; *Ase*I, ATTAAT; *Bsp*M2, TCCGGA; *Fok*I, GGATG; *Mse*I, TTAA; *Mae*III, GTNAC; *Hph*I, GGTGA; *Hpa*II, *Msp*I, and *Hap*II, CCGG; *Sfa*NI, GCATC.

The finding that the same Southern blot banding patterns are obtained from DNA extracted both directly from conidia and from *P. sorghi*-infected leaves is very important for identification of this biotrophic fungus, because in most cases it is not easy to collect enough fungal tissues for DNA isolation. Since the DNA isolated from dried leaves collected from young infected plants provides consistently good results when used for Southern blots and the dried conidia are no longer infective, it should be possible to collect and compare samples from around the world without the risk of introducing a serious pathogen.

That probe pMLY12-1 did not show detectable hybridization with DNA of the *P. sorghi* Thailand isolate was not unexpected, since *P. sorghi* Thailand isolate has been found to differ from other *P. sorghi* isolates in many ways, including host range, symptom expression, oospore production, isozyme patterns (14), and restriction fragment length polymorphism patterns (20, 22). All of these data suggest that *P. sorghi* Thailand isolate should be named as a separate species to clarify the confusion in the taxonomy of *Peronosclerospora* species.

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