PCR Amplification from a Homolog of the *b*E Mating-Type Gene as a Sensitive Assay for the Presence of *Ustilago scitaminea* DNA

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ABSTRACT

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Ustilago scitaminea, the causal agent of sugarcane (*Saccharum* spp.) smut, has a bipolar mating system in that the haploid stage of the life cycle has sporidia of two mating types (designated plus and minus). Only haploid sporidia of complementary mating types can fuse to form the infectious, mycelial dikaryon. With the use of primers based on the *U. maydis b*E mating-type gene, DNA segments from sporidia were polymerase chain reaction (PCR)–amplified from both minus and plus mating types of *U. scitaminea*. These DNA fragments were sequenced and found to be approximately 70% identical in nucleotide sequence to the corresponding region of the *b*E gene in *U. maydis* and *U. hordei*. The mating behavior of *U. scitaminea* is similar to that of other bipolar *Ustilago* spp. This and the similarity in DNA sequence between the *b* genes suggests that mating-type genes of *U. scitaminea* are similar in structure and function to those of other *Ustilago* spp. previously studied. Use of one of the cloned fragments as a probe in Southern analysis of *U. scitaminea* revealed specific hybridization to single *Bam*HI fragments of different sizes in the two mating types, confirming mating type–specific differences at this locus. Primers made to the cloned sequence have been used to amplify by PCR a DNA fragment of the predicted size from a 2,000:1 mixture of sugarcane and *U. scitaminea* dikaryon DNA.

Ustilago scitaminea Syd. & P. Syd., the causal agent of sugarcane (*Saccharum* spp.) smut, and some other *Ustilago* spp. have a bipolar mating system in which two alleles at a single locus control sporidial fusion and dikaryon formation. Other smut fungi including *U. maydis* (DC.) Corda are tetrapolar, producing haploid sporidia of four different mating types (11). Tetrapolar species have different alleles at two segregating loci, the *a* and *b* mating-type loci (12,13). Despite this apparent difference in mating systems, numerous bipolar *Ustilago* spp. (*U. scitaminea* was not tested in the cited work) contain genes homologous to both the *a* and *b* loci of tetrapolar *U. maydis* (2). The most likely explanation is that the *a* and *b* loci are present in both bipolar and tetrapolar species, but are tightly linked in the bipolar species (2). Comparisons of *b* mating-type genes from bipolar *U. hordei* (Pers.) Lagerh. and tetrapolar *U. maydis* reveal regions of highly conserved nucleotide sequence and an overall similarity of structure and function (3). In *U. maydis* the *a* locus controls cell fusion between haploid sporidia, while the *b* locus controls continued development and growth of the infectious, dikaryotic hyphae (11,14). Data presented in this paper show that *U. scitaminea* also has DNA sequences homologous to the *U. maydis b*E genes. It appears likely that these genes in *U. scitaminea* perform the same functions as the homologous *U. maydis* genes.

The life cycle of *U. scitaminea* resembles that of several other *Ustilago* spp. Airborne diploid teliospores are produced from "whips" (long, unbranched sori) and serve to spread the fungus from plant to plant (10). Teliospores germinate on wet plant surfaces to form a promycelium that in turn produces haploid sporidia. Meiosis takes place during this process; of the four initial sporidia, two are plus (+) and two are minus (–) mating types (1). Single haploid sporidia continue to multiply by budding in a yeastlike manner on the plant surface and sporidia of complementary mating types can fuse to form a dikaryon mycelium. Unlike the sporidia, the dikaryon is capable of systemically infecting sugarcane through the "eyes" (buds) at each cane node. Colonies grown from single haploid sporidia as well as dikaryon cultures may be maintained indefinitely in vitro, but do not become diploid or form teliospores.

Smut disease of sugarcane, caused by *U. scitaminea*, was first reported in South Africa in 1877 (17) and has since spread so that today it is found in nearly all sugarcane-growing areas of the world except Papua New Guinea and Australia. Smut disease of sugarcane first appeared in Hawaii in 1971 (6) and has proven to be costly to control.

Heritable resistance to smut exists in sugarcane germ plasm (21), but evaluation of cultivars for resistance is time consuming. Resistance to smut is quantitative, so field trials are used to rate resistance on a scale of 1 (highly resistant) to 9 (highly susceptible), rather than as discrete resistant and susceptible classes. Cultivars are routinely tested by dipping setts (stem pieces containing a nodal bud that are used for vegetative propagation) in a suspension of teliospores before planting, and then scoring for resistance by observing the number and time at which whips (if any) form. In some cases infected plants do not develop whips during the first growing season, so the plants are grown a second season and again scored for the development of whips.

Sugarcane stalks are frequently shipped between countries to enhance the local germ plasm base. The importation of sugarcane vegetative cuttings poses the risk of introducing smut, a particularly serious threat for countries presently free of sugarcane smut disease. For plant quarantine agencies, and for sugarcane breeders, a rapid and reliable procedure to detect the presence of smut in sugarcane plants or cuttings would be a useful tool. We present here a method that sensitively and specifically detects the presence of small quantities of smut DNA mixed with sugarcane DNA. Future experiments will determine the value of this method in predicting smut resistance under natural conditions.

MATERIALS AND METHODS

Fungal cultures. Haploid cultures of *U. scitaminea* grown from single sporidia were initiated by collecting teliospores from whips in the field and rinsing them three times in sterile water containing 500 ppm streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) before germinating them for 18 h on V8 juice agar (20 ml of V8 juice, 3 g of $CaCO_3$, 0.5 g of streptomycin sulfate, 20 g of agar, and distilled water to make 1,000 ml) containing 500 ppm streptomycin sulfate. Serial dilutions of the spores and sporidia were made in sterile water and streaked on V8 juice agar. Single-sporidia colonies were transferred individually to other V8 juice agar culture dishes and maintained in pure culture. Cultures were maintained at room tem-

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perature (21 to 30°C) with or without light. The single-sporidia colonies could be easily differentiated from colonies formed from fused pairs of sporidia because the latter were mycelial instead of yeastlike in appearance. The cultures were determined to be of either same or opposite mating type (+ or –) by cross-streaking them on agar plates. Streaks of two colonies of complementary mating type formed the white, mycelial dikaryon at the point where they cross. More than 60 single-sporidium colonies were isolated in this manner and crosses between them confirmed the results of Saxena and Singh (19) that *U. scitaminea* has only two mating types.

Inoculation of buds. As a young sugarcane shoot grows, the growing point is enclosed in whorls of leaves. The smut dikaryon is able to penetrate and colonize meristem tissue including the shoot growing point or, naturally in the field, the lateral buds on the stalk. For these experiments, the base of newly germinated buds was inoculated. Sugarcane vegetative cuttings from highly susceptible cultivar H50-2036 were germinated in plastic-covered foil trays on damp paper towels at 30°C in a growth chamber with continuous light. Buds usually germinated and grew to form shoots 1.5 to 4 cm in height in 2 to 4 days. At this time, the shoots were surface sterilized with 0.52% sodium hypochlorite and the outer bud scales removed. A dense, pastelike mixture of (+) and (–) *U. scitaminea* spores was applied with a small paint brush to the surface of the shoot base, and the shoots returned to the growth chamber. This method applied an estimated 8×10^7 to 1×10^8 sporidia per bud. A temperature of 30°C and 100% relative humidity was maintained for at least 24 h. These conditions were reported to be optimum for infection (4). Different inoculation procedures give varying percent infected plants in the same sugarcane cultivar, depending upon the severity of the technique (10). When stalks of H50-2036 with germinated buds inoculated by our method were planted in field plots, 90% (27 of 30) of the resulting shoots produced whips within 2 months. The other three shoot-growing points tested negative with polymerase chain reaction (PCR). Neither H78-292 (moderately susceptible) nor H65-7052 (resistant) produced any whips during the same period.

DNA extraction. DNA was extracted from both (+) and (–) haploid sporidial cultures following the method of Elder et al. (8) for total nucleic acid extraction from yeast. The two sporidial cultures were each 2 to 3 days old and growing on V8 juice agar. Sporidial cells were shaken in a buffer/phenol/chloroform mixture with glass beads in a microfuge tube. After a second extraction, the aqueous phase was removed and total nucleic acid was precipitated with 95% ethanol at –20°C.

DNA was extracted from fungal dikaryon cultures growing in vitro on V8 juice agar and from smut-infected sugarcane tissue, following the method of Saghai-Maroof et al. (18). When DNA from smut-infected sugarcane was extracted, the resulting sample contained the total nucleic acids from both the plant and fungus. Smut-infected sugarcane tissue included growing points of shoots from buds inoculated as described above. When DNA was extracted from growing points, shoots were cut off at the base, leaves removed, and DNA extracted from the growing point, which was about 1 cm in height. Great care was taken to surface sterilize the buds and to remove the exterior bud scales and leaves in order to avoid contamination of the interior meristem tissue. From each excised sample, 50 µl of DNA solution was obtained that had a concentration from 50 to 400 ng/µl. DNA was also extracted from smut whip tissue at a point just below the area of spore production. The immature smut whip contains plant tissue as well as a large percentage of fungal tissue.

Preparation of positive and negative controls. As a negative control of the procedure, sugarcane stalk pieces were cut from a very susceptible sugarcane cultivar (H50-2036) that usually produces whips when infected. The stalk pieces were given a hot water dip at 50°C for 2 h, which is known to eliminate the fungus without killing the stalks (10). They were planted in pots in a greenhouse where they would not be exposed to reinfection. Within 8 weeks the stalks had grown to shoots 30 to 45 cm tall. None had produced a smut whip. Growing points of 10 plants were sectioned, cleared with lactophenol, and stained with methyl blue. The growing points of another 10 plants were excised, and DNA was extracted and PCR-amplified with *U. scitaminea* *b*E4 and *b*E8 primers (see below).

For the positive control, stalks were chosen in the field that had visible smut whips. Stalk pieces were cut and surface sterilized by washing in detergent and then spraying with 95% ethanol and flaming. Outer bud scales were carefully removed before the meristematic tissue was excised and DNA extracted. One or two buds from each stalk adjacent to the excised bud were sectioned, cleared, stained, and examined microscopically.

PCR amplification and cloning of *U. scitaminea* *b*E locus. PCR amplification was carried out in a total volume of 50 µl containing 20 ng of *U. scitaminea* (–) mating type haploid DNA, 25 pmoles each of the primers *U. maydis* *b*E4 (5′-CTCGA GGTTCATCAGCTCA-3′) and *b*E8 (5′-GCTGAGTTCTGGAGTCG-3′) supplied by J. Kronstad, University of British Columbia, Vancouver, BC. The reaction mixture also contained 200 µM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1% Triton X-100, and 2.5 U *Taq* DNA polymerase. The reaction was overlaid with mineral oil and run for 30 cycles of 94, 52, and 72°C, 1 min each. The major PCR product (≈0.5 kb) was purified from an agarose gel and cloned into the *Srf*I site of the plasmid pCR-Script SK+ (Stratagene, La Jolla, CA). This clone is referred to as UsbE1–.

U. scitaminea *b*E4 (5′-CGCTCTGGTT CATCAACG-3′) and *b*E8 (5′-TGCTGTC GATGGAAGGTGT-3′) primers were synthesized based on the sequence of the (–) allele and located internal to site of the *U. maydis* primers. These primers were used to amplify a product of approximately 0.45 kb from (+) mating type *U. scitaminea* DNA. PCR conditions for this amplification were as outlined above, except that the duration of each step was adjusted to 30 s. Again, the amplification product was isolated from an agarose gel and cloned into the *Srf*I site of pCR-Script SK+. This clone is referred to as UsbE1+.

DNA sequencing. Cycle sequencing reactions were read on an Applied Biosystems 373A sequencing apparatus at the University of Hawaii Biotechnology – Molecular Biology Instrumentation and Support Facility. The sequences have been entered to GenBank as accession numbers U61290 (– mating type) and U61291 (+ mating type). Sequence assembly and comparison were performed with the Genetics Computer Group ("Wisconsin Package") programs.

Southern analysis. One microgram of genomic DNA from both mating type haploids, from uninfected sugarcane, and from the saprophytic fungus *Arthrobotrys*

Table 1. "Bestfit" comparisons of deduced peptide sequences from *b*E mating-type genes of *Ustilago* spp.[a]

	Ustilago spp.					
***Ustilago* spp.**	**UsbE1–**	**UsbE1+**	***U.m.b*E5**	***U.m.b*E7**	***U.h.b*E1**	***U.h.b*E2**
UsbE1-	100	...	...	...	...	...
UsbE1+	98/97	100	...	...	...	...
*U.m.b*E5	77/65	73/62	100	...	...	...
*U.m.b*E7	75/64	72/61	96/94	100	...	...
*U.h.b*E1	80/65	79/63	77/66	75/64	100	...
*U.h.b*E2	76/63	77/63	76/67	74/64	97/96	100

[a] % similarity / % identity. *U. maydis b* East locus, allele 5 (*U.m.b*E5) and *U. maydis b* East locus, allele 7 (*U.m.b*E7) are GenBank accession numbers X54069 and X54070, respectively (14); *U. hordei b* East locus, allele 1 (*U.h.b*E1) and *U. hordei b* East locus, allele 2 (*U.h.b*E2) are GenBank accession numbers Z18532 and Z18531, respectively (3).

musiformis was digested with *Bam*HI, separated on a 0.7% agarose gel and transferred to a positively charged nylon membrane (Amersham, Arlington Heights, IL) by alkaline capillary blotting. Hybridization and washes were carried out at 65°C by the method of Church and Gilbert (7). Probes were ^{32}P-labeled by the method of Feinberg and Vogelstein (9).

PCR assays of DNA mixtures. The PCR product from these primers is predicted to be 0.45 kb. Reactions (50 µl) were carried out as described above, but contained 100 ng of sugarcane DNA and 5 ng to 5 pg of *U. scitaminea* DNA. The thermal profile was 94, 60, and 72°C, 30 s each, for 30 cycles. Reactions were "hot started" at 80°C with the addition of *Taq* DNA polymerase and reaction salts in a volume of 10 µl. As an internal positive control, 12.5 pmoles of two primers that amplify a 0.2-kb fragment of a sugarcane ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (rbcs) gene (20) were included in the same reaction. In some experiments, the presence of the rbcs primers appeared to result in decreased amplification of the *U. scitaminea* *b*E locus band (data not shown).

RESULTS

PCR amplification of *U. scitaminea* (–) DNA with primers located in the conserved region downstream of the homeodomain of the *b*E gene of *U. maydis* resulted in a major product of ≈0.5 kb. Nucleotide sequence of this PCR product (UsbE1–) was found to be 71% identical to the *U. maydis* *b*E (allele 5) mating-type gene (data not shown). Primers based on the (–) mating type *U. scitaminea* *b*E gene were used to amplify a similar fragment from the (+) mating type. The (+) mating type *b*E fragment (UsbE1+) was 68% identical to the corresponding region of the *U. maydis* *b*E (allele 5) mating-type gene (data not shown). A comparison of the deduced peptides from these *U. scitaminea* *b*E sequences and two alleles each from *U. maydis* and *U. hordei* are shown in Table 1. UsbE1– was used as a probe on Southern blots of genomic DNA from both haploid mating types, and hybridized with a single *Bam*HI band in each case: ≈12 kb in the (+) mating type, ≈7.5 kb in the (–) mating type. No hybridization was observed with uninfected sugarcane DNA, or with DNA from *Arthrobotrys musiformis*, a soilborne saprophyte commonly found in Hawaiian sugarcane fields (Fig. 1).

The *U. scitaminea* *b*E primers were used in mock infection experiments in which DNA extracted from smut dikaryon was mixed in various proportions with sugarcane DNA; this PCR assay was able to detect as little as 50 pg of *U. scitaminea* DNA in a sample containing 100 ng of sugarcane DNA, or one part in 2,000 (Fig. 2, lanes D and J). DNA from smut whip tissue yielded an intense 0.45-kb band, far above our threshold of sensitivity (Fig. 2, lane N). This was expected since whip tissue contains a very large amount of fungal mycelium. DNA from germinated sugarcane buds that had been inoculated with smut sporidia gave clearly detectable bands in some cases (Fig. 2, lane O) while other inoculated buds produced no visible 0.45-kb band (Fig. 2, lane P). Failed reactions were made evident by the absence of the

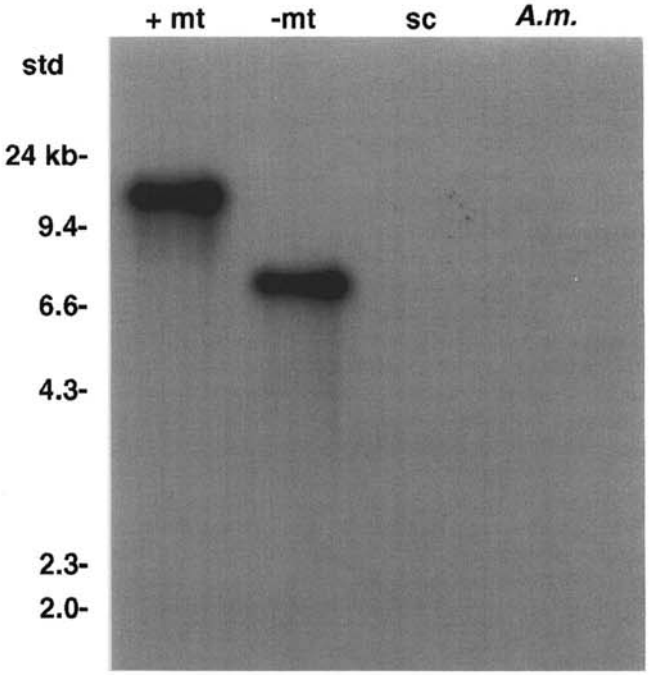

Fig. 1. Southern hybridization analysis of DNA from both mating types of *Ustilago scitaminea* and uninfected sugarcane, with *U. scitaminea* *b*E mating-type locus probe. One microgram of each genomic DNA was digested with *Bam*HI, separated on a 0.7% agarose gel, and transferred to a positively charged nylon membrane by alkaline capillary blotting. Hybridization and washes were carried out at 65°C. +mt = (+) mating-type, -mt = (–) mating-type, sc = sugarcane, *A.m.* = *Arthrobotrys musiformis*, a soilborne saprophyte commonly found in Hawaiian sugarcane fields.

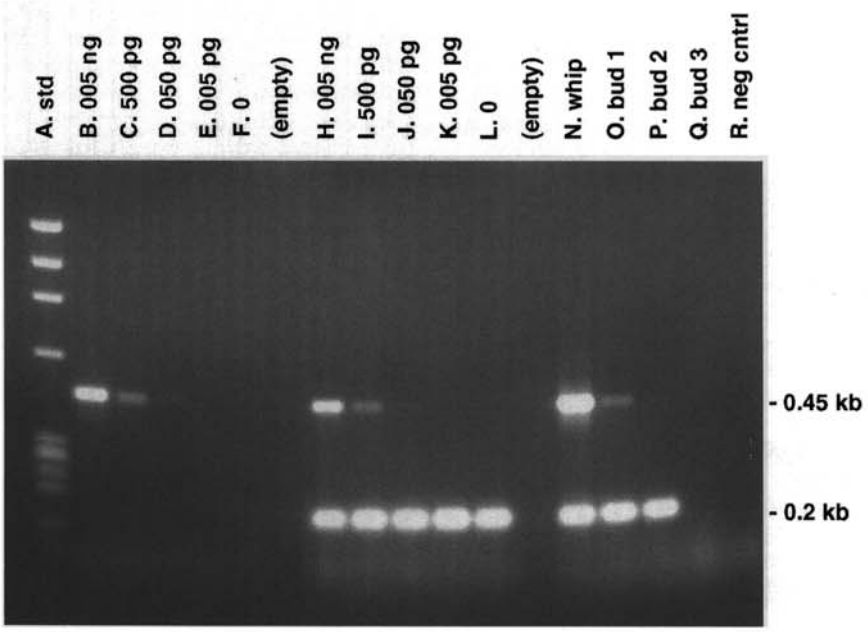

Fig. 2. Polymerase chain reaction assay of mock smut infections. Reactions B through L contained 100 ng of sugarcane DNA plus the indicated amount of *Ustilago scitaminea* DNA. Reactions N through Q contained 100 ng of DNA from sugarcane stalk whip tissue or from buds that had been inoculated with *U. scitaminea* spores. The three buds (O through Q) are examples of the three possible outcomes. All reactions contained primers for *U. scitaminea* *b*E mating-type locus, reactions H through R additionally contained primers for sugarcane ribulose1,5-bisphosphate carboxylase/oxygenase small subunit (rbcs) gene.

0.2-kb rbcs internal control band (Fig. 2, lane Q) where the presence or absence of smut DNA could not be determined. The correlation, if any, between the detection of smut DNA in these samples from young seedlings, and the later appearance of smut whips in plants, is not yet known. No PCR bands were observed in the 10 DNA samples from shoots from hot water–treated stalks (negative control). These negative controls were confirmed as uninfected by microscopic examination of 10 additional stalks. DNA samples from lateral buds of 13 field-grown stalks with whips (positive control) gave PCR bands in all but one case. Mycelium was detected microscopically in adjacent buds from all 13 stalks. No PCR products were observed when these primers were tested for amplification of DNA of three other microorganisms pathogenic to sugarcane: *Bipolaris sacchari* (E. J. Butler) Shoemaker, *Ceratocystis paradoxa* (Dade) C. Moreau, and *Xanthomonas albilineans.* All three had been isolated from infected sugarcane in Hawaii. *B. sacchari* and *C. paradoxa* are fungi with airborne spores that might contaminate the surface of sugarcane samples. The bacterium, *X. albilineans,* is a xylem-limited parasite that is carried systemically in the plants.

DISCUSSION

Bakkeren et al. (2) showed that the *a* and *b* loci of tetrapolar *U. maydis* hybridized with DNA sequences from several other bipolar and tetrapolar smut fungi on DNA gel blots. The present research indicates that *U. scitaminea,* a bipolar fungus, also has homologous mating-type genes. The cloned region of the *b*E gene homolog is about 70% identical to *U. maydis.* In addition, mating experiments between haploid sporidia of *U. scitaminea* with *U. maydis* and *U. hordei* showed partial recognition (J. Kronstad, personal communication). Since the *a* genes govern mating, there is likely to be similarity between the *a* genes as well as the *b* genes. Together, these data support the view that *U. scitaminea* has a mating system and mating-type genes similar to those of other *Ustilago* spp.

Primers made to the UsbE1– sequence direct PCR amplification of this gene fragment from a mixture of sugarcane and *U. scitaminea* dikaryon DNA in a mass ratio of 2,000:1. They also amplified the *U. scitaminea* gene fragment from a mixture of fungal and plant DNA extracted from infected buds. Negative PCR results from DNA of three other microorganisms pathogenic to sugarcane (*B. sacchari, C. paradoxa,* and *X. albilineans*) suggest this assay is quite specific for *U. scitaminea* DNA. This PCR assay could potentially be adapted as a diagnostic tool for plant quarantine.

It might also be possible to develop a PCR assay that could be used to score young plants for smut resistance. Smut resistance in sugarcane is thought to involve several different factors (5). Two of these, bud scale morphology and chemical inhibitors found in bud scales, act to block the fungus from penetrating and infecting the plant (15). Another, termed "physiological" resistance, acts to inhibit growth of the fungus within plant tissues (16). An assay that sensitively and specifically detects the presence of the pathogen in young test seedlings might be useful in determining early in the screening program whether new cultivars are very susceptible by identifying those with a high percentage of infected shoots. The method would more likely be successful if resistance depended mainly on barriers to infection.

This molecular assay may assist in determining the nature of systemic development of smut infections in sugarcane as well as the genetics of *U. scitaminea* itself. A clearer understanding of the course of infection and more reliable scoring of resistance could allow the genetic mapping of the resistance trait. If resistance is due to several factors, each of which may result from the action of more than one gene, resistance will prove very difficult to map. If this assay allows the dissection of the factors involved, it would make mapping of the individual components much more feasible. For example, if fungal cells do penetrate and grow in cultivars with only "physiological" resistance, but do not produce outwardly visible symptoms, the PCR assay could distinguish this from bud scale mediated resistance.

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LITERATURE CITED

1. Alexander, K. C., and Srinivasan, K. V. 1966. Sexuality in *Ustilago scitaminea* Syd. Curr. Sci. 35:603-604.
2. Bakkeren, G., Gibbard, B., Yee, A., Froeliger, E., Leong, S., and Kronstad, J. 1992. The *a* and *b* loci of *Ustilago maydis* hybridize with DNA sequences from other smut fungi. Mol. Plant-Microbe Interact. 5:347-355.
3. Bakkeren, G., and Kronstad, J. W. 1993. Conservation of the *b* mating-type gene complex among bipolar and tetrapolar smut fungi. Plant Cell 5:123-136.
4. Bock, K. R. 1964. Studies on sugar-cane smut (*Ustilago scitaminea*) in Kenya. Trans. Br. Mycol. Soc. 47:403-417.
5. Burner, D. M., Grisham, M. P., and Legendre, B. L. 1993. Resistance of sugarcane relatives injected with *Ustilago scitaminea.* Plant Dis. 77:1221-1223.
6. Byther, R. S., Steiner, G. W., Ladd, S. L., and Heinz, D. J. 1973. Smut disease of sugarcane in Hawaii. Plant Dis. Rep. 57:966-968.
7. Church, G. M., and Gilbert, W. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81:1991-1995.
8. Elder, R. T., Loh, E. Y., and Davis, R. W. 1983. RNA from the yeast transposable element TY1 has both ends in the direct repeats, a structure similar to retrovirus RNA. Proc. Natl. Acad. Sci. USA 80:2432-2436.
9. Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling restriction endonuclease fragments to high specific activity. Anal. Biochem. 13:6-13.
10. Ferreira, S. A., and Comstock, J. C. 1989. Smut. Pages 211-229 in: Diseases of Sugarcane, Major Diseases. C. Ricaud, B. T. Egan, A. G. Gillaspie, Jr., and C. G. Hughes, eds. Elsevier, New York.
11. Froeliger, E. H., and Kronstad, J. W. 1990. Mating and pathogenesis in *Ustilago maydis.* Dev. Biol. 1:185-193.
12. Froeliger, E. H., and Leong, S. A. 1991. The *a* mating-type alleles of *Ustilago maydis* are idiomorphs. Gene 100:113-122.
13. Kronstad, J. W., and Leong, S. A. 1989. Isolation of two alleles of the *b* locus of *Ustilago maydis.* Proc. Natl. Acad. Sci. USA 86:978-982.
14. Kronstad, J. W., and Leong, S. A. 1990. The *b* mating-type locus of *Ustilago maydis* contains variable and constant regions. Genes Dev. 4:1384-1395.
15. Lloyd, H. L., and Naidoo, G. 1983. Chemical assay potentially suitable for determination of smut resistance of sugarcane cultivars. Plant Dis. 67:1103-1105.
16. Lloyd, H. L., and Pillay, M. 1980. The development of an improved method for evaluating sugarcane resistance to smut. Proc. S. Afr. Sugar Technol. Assoc. 54:168-172.
17. McMartin, A. 1945. Sugarcane smut: Reappearance in Natal. S. Afr. Sugar J. 29:55-57.
18. Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014-8018.
19. Saxena, K. M. S., and Singh, K. 1966. The mating pattern in *Ustilago scitaminea.* Indian Phytopathol. 19:286-289.
20. Tang, W., and Sun, S. S. M. 1993. Sequence of a sugarcane ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene. Plant Mol. Biol. 21:949-951.
21. Wu, K. K., Heinz, D. J., and Meyer, H. K. 1983. Heritability of sugarcane smut resistance and correlation between smut grade and yield components. Crop Sci. 23:54-56.