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RESEARCH ARTICLE

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Use of PCR to detect infection of differentially susceptible maize cultivars using *Ustilago maydis* strains of variable virulence

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Abstract *Ustilago maydis* was specifically detected in infected maize plants by means of the polymerase chain reaction (PCR) using oligonucleotides corresponding to a specific region downstream of the homeodomain of the *bE* genes of the pathogen. The reaction gave rise to amplification of a ca. 500-bp product when tested with *U. maydis* DNA, but no amplification was detected with DNA from fungi not related to *U. maydis*. Using these primers, *U. maydis* was detected in infected maize plants from differentially susceptible cultivars as early as 4 days after inoculation with strains of variable degrees of virulence. Detection of *U. maydis* at early stages of infection, or in asymptomatic infected plants should assist in studies on plant–pathogen interactions.

Keywords PCR detection · *b* locus · Ustilaginales · Plant infection · Mating type

Introduction

The basidiomycete *Ustilago maydis* (DC.) Cda. is the smut pathogen of maize (*Zea mays* L.) with worldwide distribution. During the saprophytic phase, the fungus is haploid and grows as budding yeasts (sporidia). Mating of compatible sporidia leads to the formation of a dikaryotic mycelium, which invades the plant and eventually induces formation of tumors full of diploid teliospores. Teliospores germinate forming a promycelium in which meiosis occurs giving rise to the meiotic segregants, which multiply by budding and thus complete the life cycle of the fungus [2, 15].

Different events in the life cycle of *U. maydis* are regulated by the mating-type loci *a* and *b*. The *a* locus is required for cell-to-cell recognition during the mating process [5] and for the maintenance of filamentous growth [3]. The *a* locus has two idiomorphs, a_1 and a_2, and both have been cloned. Each idiomorph encodes a pheromone and a receptor for the pheromone synthesized by the compatible partner strain [5]. The *b* locus regulates the steps in sexual development that occur after fusion of haploid cells. The *b* locus has at least 25 alleles at each of two genes, *bE* and *bW*. Different *a* and *b* alleles in mating partners are necessary to trigger mating, filamentous growth, and tumor induction [10, 16].

The polymerase chain reaction (PCR) has been used to detect a number of fungal plant pathogens based on the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) or by sequences specific to the pathogen [1, 11, 17]. This technique is useful in plant infection studies when no external or characteristic symptoms of disease are apparent. For our studies on the course of experimental infection of maize plantules by virulent and avirulent strains of *U. maydis* obtained in the laboratory, we utilized the PCR method described below to detect the pathogen in infected symptomatic and asymptomatic plants. Primers whose sequence corresponds to a conserved region downstream of the homeodomain of the *bE* genes of *U. maydis* [1, 13] were used to amplify the corresponding fragment as a diagnostic tool.

Materials and methods

Fungal strains

The following haploid strains of *Ustilago maydis* were used in this study: wild-type strains FB1 (a_1b_1) and FB2 (a_2b_2) (provided by Flora Banuett, University of California, San Francisco), BX27

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(a_1b_1) and BX28 (a_2b_2). Strain BX27 was obtained from FB1 by illegitimate recombination of a cassette designed to disrupt the chitin-synthase-coding gene *Umchs6* [18], and strain BX28 by genetic recombination between BX27 and FB2 in planta, as described previously [7]. A mixture of these two strains, affected in an unknown gene, has low virulence to maize seedlings, producing minor symptoms such as chlorosis but no or only few small galls (B. Xoconostle-Cazares, J. Ruiz-Herrera. unpublished data). Strains were maintained at −70 °C in 50% glycerol. They were recovered in HCM-liquid medium [12], incubated with shaking (200 rpm) at 28 °C for 2 days and used as inoculum for subsequent experiments. The maize cultivar B95R LPC-21 moderately resistant to *U. maydis* (J.L. Pons, INIFAP-Celaya, Mexico, personal communication) was used in some experiments.

Plant inoculation

U. *maydis* strains were grown in 3 ml of HCM liquid medium for 24 h at 28 °C under continuous shaking (200 rpm). A sample (1 ml) was transferred to 50 ml of fresh HCM liquid medium and grown for 18 h under the same conditions. Cells were recovered by centrifugation at 2,000 *g*, washed with sterile distilled water by centrifugation, and resuspended in sterile distilled water to a density of 1×10^8 cells per ml. Equal volumes of suspensions of cells of opposite mating types were mixed. Aliquots (100 µl) of the mixed suspensions were then inoculated into the stem of 8-day-old maize cultivar Cacahuazintle seedlings using a small syringe [14]. As controls, plants were inoculated with the same volume of sterile distilled water. Plants were maintained in a greenhouse, and symptoms of the disease were recorded as appearance of chlorosis, anthocyanins, and the tumors or galls that are characteristic of *U. maydis* infections.

Purification of DNA

DNA from *U. maydis* was extracted using basically the protocol from Fujimura and Sakuma [9]. DNA samples from the following organisms were provided by the indicated investigators: *Fusarium moniliforme*, A. Glenn (University of Georgia, Athens, Ga., USA); *Trichoderma harzianum*, V. Rocha and A. Flores (CINVESTAV-Irapuato, Irapuato, Gto., Mexico); and *Cronartium quercuum* f. sp. *fusiforme* (the causal agent of fusiform rust of southern pines and oak trees), Dr. Sara Covert (School of Forest Resources, University of Georgia, Athens, Ga., USA). DNA from whole maize plants, lower stem, upper stem or leaves was extracted using the method described by Dellaporta et al. [8]. In all cases, DNA quality and quantity were evaluated using electrophoresis and spectrophotometry, respectively.

PCR protocol

PCR amplification was carried out in a volume of 50 µl containing either fungal or plant DNA, and specific primers. Primers 1369 (5′-CTCGAGGTTCATCAGCTCA-3′) and 1370 (5′-GCTGAGTTCTGGAGTCG-3′) correspond to sequences located in a conserved region downstream of the homeodomain of the *bE* genes from *U. maydis*; primer 1369 is located at bp 645 of *bE1* and *bE2*, and bp 489 of *bE5*; primer 1370 is located at bp 1147 of *bE1* and *bE2*, and 992 of *bE5* [1]. Amplification with these primers should result in a 502-bp product. The reaction mixture also contained 100 mM dNTPs, 2 mM $MgCl_2$, 1× PCR buffer (Gibco) and 2.5 U of DNA Taq polymerase. Cycling conditions (30 cycles) were: denaturation at 95 C, 1 min; annealing at 55 °C, 1 min; and extension at 72 °C, 1 min, followed by a final extension for 10 min at 72 °C. One tenth of the reaction volume was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and observed under ultraviolet light. Positive controls included DNA from *U. maydis* using the two primers. Negative controls included DNA from uninoculated plants, and DNA from *U maydis* or infected plants with *U. maydis* using each primer separately.

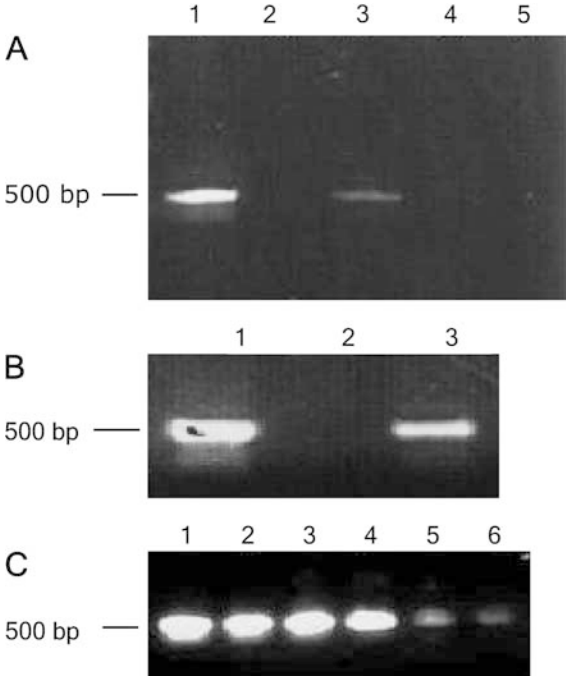

Fig. 1 **A** PCR amplification of DNA extracted from maize plants 4 days after inoculation with a mixture of sexually compatible wild-type strains of *Ustilago maydis*. *Lane 1* 50 ng *U. maydis* DNA; *lane 2* 50 ng *U. maydis* DNA using one primer only; *lane 3* 200 ng DNA from inoculated maize plants, *lane 4* 200 ng DNA from inoculated maize plants using one primer only, *lane 5*, 200 ng DNA from uninoculated maize plants. **B** PCR amplification of DNA extracted from maize plants 9 days after inoculation with *U. maydis*. *Lane 1* 50 ng *U. maydis* DNA, *lane 2* 200 ng DNA from uninoculated maize plants, *lane 3* 200 ng DNA from inoculated maize plants. **C** PCR amplification of *U. maydis* DNA. *Lanes 1–6* 500 250, 100, 50, 20 or 10 ng *U. maydis* DNA, respectively

Microscopic observations

Plant tissue was collected from the chlorotic zones as suggested by Banuett and Herskowitz [4] using a razor blade, and immediately processed into fine strips. These samples usually had a single layer of cells at one part of the section. The sections were stained with cotton blue and observed and photographed in a Leica microscope equipped with a SPOT camera.

Results

Primers 1369 and 1370 specifically amplified a DNA product of ca. 500 bp when DNA samples of *U. maydis* were used as templates (Fig. 1A, lane 1; Fig. 1B, lane 3; Fig. 1C). No amplification was observed when PCR assays were carried out with one primer only (Fig. 1A, lane 2). Also, no amplification products were detected when using DNA from other fungi unrelated to *U. maydis,* such as the corn pathogen *F. moniliforme*, the potato and tomato pathogen *P. infestans*, the normal soil inhabitant *T. harzianum,* or the oak trees pathogenic basidiomycete *C. quercuum* f. sp. *fusiforme* (not shown).

The 500-bp amplification product was clearly seen when we used 200 ng of DNA extracted from maize plants as early as 4 days after inoculation with a mixture of wild-type strains FB1 and FB2 (Fig. 1A, lane 3). The PCR product was not obtained with a single primer or DNA from uninoculated plants (Fig. 1A, lanes 4 and 5, respectively). At this stage of the infection, no signs of disease were visible in the plants, except chlorotic zones around the inoculation spot in the leaves. Nevertheless, microscopic observation showed mycelium colonizing these zones (Fig. 2A). The same type of mycelium, but less abundant, was observed in plants inoculated with a mixture of the low-virulent strains BX27 and BX28 (Fig. 2B).

Results showed that when equal amounts of DNA isolated from maize tissue at 4 or 9 days of inoculation were analyzed by PCR, the resulting band was stronger in the 9-day sample (Fig. 1B, lane 3 vs. Fig. 1A, lane 3). Figure 1C is included as a demonstration of the sensitivity of the method.

To determine the distribution of the pathogen in the plant by means of PCR, DNA was extracted from maize plants 9 days after inoculation with the wild-type strains. In these experiments we separated: (1) the basal part of the stem, (2) the upper part of the stem, which also included the base of the leaves, and (3) the upper part of the leaves only. All DNA samples were analyzed by PCR as described. The 500-bp fragment was amplified from each of the selected parts of the plants (Fig. 3, lanes 3–5). The 500-bp band was also amplified from maize plants inoculated with the *U. maydis* BX27 and BX28 strains of low virulence (Fig. 3, lanes 7–9) 9 days after inoculation. At this time, no disease symptoms were observed in plants inoculated with these strains, but only small chlorotic zones around the hole left by the inoculation needle. By contrast, plants inoculated with wild-type strains exhibited gross external symptoms of the disease at this time, such as yellowing, the presence of anthocyanins, and the beginning of tumor formation. Microscopic observation of plants inoculated with the wild-type strains revealed vigorously growing mycelium in the chlorotic zones (Fig. 2C). However, in plants inoculated with the mixture of BX27 and BX28 strains, the small chlorotic areas of the leaves showed

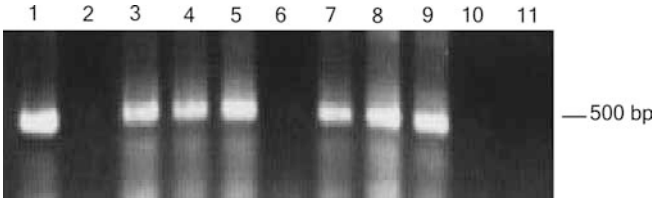

Fig. 3 PCR amplification of DNA extracted from several maize tissues inoculated with virulent and low-virulent strains of *U. maydis*. *Lane 1* 50 ng *U. maydis* DNA, *lane 2* 50 ng *U. maydis* DNA using one primer only, *lanes 3–6* 200 ng DNA from maize plants inoculated with a mixture of compatible virulent strains, *lane 3* DNA from the basal part of the stem, *lane 4* DNA from the upper part of the stem and the first part of new leaves, *lane 5* DNA from the upper part of the leaves, *lane 6* DNA from the basal stem using one primer only. *Lanes 7–10* 200 ng DNA of maize plants inoculated with a mixture of compatible low-virulent strains, *lane 7* DNA from the basal stem, *lane 8* DNA from the upper part of the stem and the basal part of leaves, *lane 9* DNA from the upper part of the leaves, *lane 10* DNA from the basal stem using one primer, *lane* 11 200 ng DNA from uninoculated maize plant

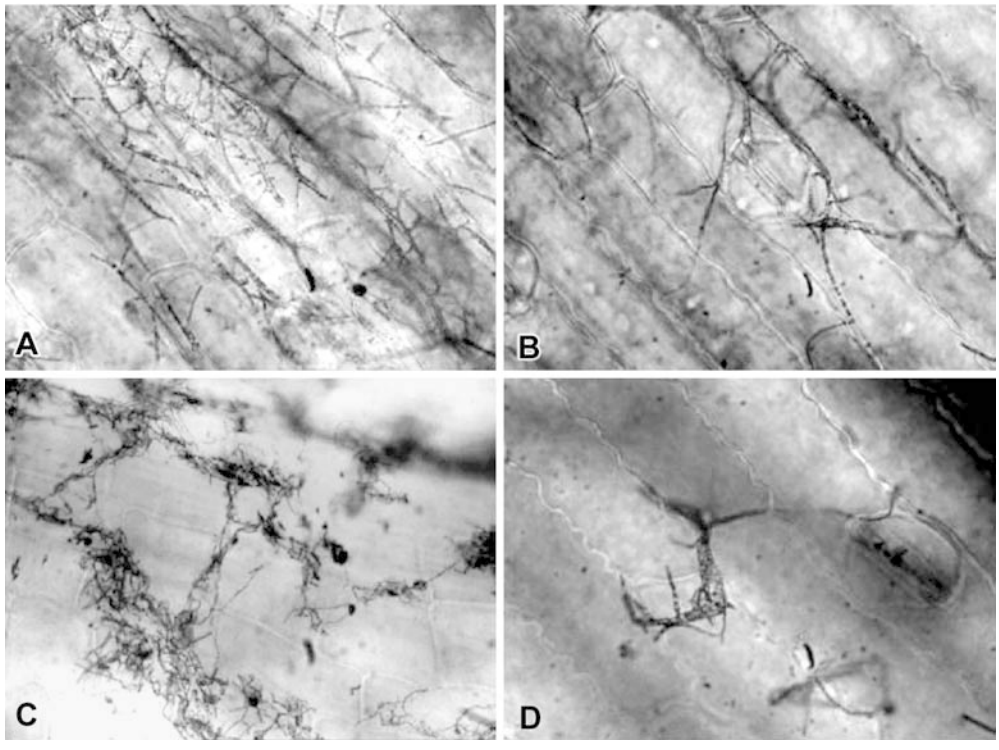

Fig. 2 Microscopic examination of maize tissue infected with *U. maydis*. **A** Mycelium present in the chlorotic zones 4 days after inoculation with a mixture of compatible wild-type strains. **B** Mycelium present in chlorotic zones 4 days after inoculation with a mixture of low-virulent strains. **C** Mass of mycelium present in the chlorotic zone 9 days after inoculation with wild-type strains. **D** Morphologically altered and vacuolated mycelium in chlorotic zones 9 days after inoculation of maize plants with low-virulent strains

only a few threads of distorted mycelium, which was vacuolated and had signs of senescence (Fig. 2D).

When the PCR reaction was run using more stringent conditions, i.e., an annealing temperature of 60 °C, the amplification product was visible in the lower and in the upper parts of the stem, but not in the symptom-free upper part of the leaves (not shown). The same was true for the low-virulent strains. Additionally, there was no difference in the detection of *U. maydis* by PCR using a susceptible cultivar (Cacahuazintle) and a presumably moderately resistant cultivar (B95R LPC-21), although the symptoms of disease were less apparent in the latter plants (data not shown). When DNA from plants infected for 4 days with a haploid FB2 strain was analyzed, a faint band of 500 bp was barely detected in some experiments, but no amplification was obtained when DNA samples of further dates were used. The positive result obtained after 4 days might have been due to remaining cells of the inoculum.

Discussion

The infection process of *U. maydis* in maize has been examined microscopically in the past [4, 6], mostly in order to analyze the completion of the life cycle in the plant. As a diagnostic technique, however, the procedure has low sensitivity and is technically challenging when asymptomatic plants are analyzed, or in plants inoculated with strains of low virulence. In the past, PCR has been successfully applied for the detection of plant pathogens in situ [1, 11, 17]. We have used this method to analyze infection of differentially susceptible maize cultivars inoculated with *U. maydis* strains with different degrees of virulence. The technique was robust and reliable for the identification of the pathogen in different parts of the plant, even when small amounts of DNA were used. Neither interference by plant DNA, nor reaction with several related and unrelated fungal species were observed.

The PCR technique used is not quantitative, but when using the same amounts of DNA and the same number of amplification cycles, a more intense band was obtained with DNA isolated 9 days after inoculation than with DNA isolated 4 days after inoculation. Note also that the specific product was amplified from plants inoculated with virulent and from plants inoculated with low-virulent strains. These results suggest that both strains can remain in the infected tissues and move as the plant grows. They also demonstrate the sensitivity of the method, since low-virulent mutants grew poorly into the inoculated plants and gave almost no external symptoms of the infection. Note also that by using higher stringency in the procedure, the results were different when analyzing different parts of the plants. The results suggest that, by changing the stringency of the PCR reaction, it is possible to follow the movement of the pathogen in the growing tissues of the infected plants. Detection of *U. maydis* by PCR is a useful method to analyze infection in maize cultivars of different susceptibility, and fungal strains with different degrees of virulence. It also allows the distribution of the pathogen in the plant to be studied.

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