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Full Length Research Paper

PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data

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We have developed two taxon-selective primers for quick identification of the *Fusarium* genus. These primers, ITS-Fu-f and ITS-Fu-r were designed by comparing the aligned sequences of internal transcribed spacer regions (ITS) of a range of *Fusarium* species. The primers showed good specificity for the genus *Fusarium*, and the approximately 389-bp product was amplified exclusively. PCR sensitivity ranged from 100 fg to 10 ng for DNA extracted from *Fusarium oxysporum* mycelium. No amplification products were detected with PCR of DNA from *Rhizoctonia solani* and *Macrophomina phaseolina* isolates using these primers. The assay is useful for rapid identification of *Fusarium* spp. cultures. The application of these PCR methods for early diagnosis of the seedling and wilt disease of cotton needs to be studied further.

Key words: rDNA, taxon-specific primer, *Fusarium* genus, *Gossypium barbadense*.

INTRODUCTION

Seedling disease in cotton is a worldwide problem; particularly the wilt and root rot disease are causes loss to farmers (Hillocks, 1992). The most common fungi associated with cotton disease are *Fusarium* spp. (Roy and Bourland, 1982; Hillocks, 1992). *Fusarium* spp. occurs frequently among the fungal microflora associated with seedling disease, and *Fusarium* wilt is a major cause of seedling death in some countries (Minton and Garber, 1983). In Egypt, *Fusarium oxysporum* is frequently isolated from cotton seedlings infected with damping-off (Ashour, 1957; Mohmed, 1962; Aly et al., 1996; El-Samawaty, 1999).

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns et al., 1991; Yao et al., 1992) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan et al., 1995). Therefore we focused on the ITS regions of ribosomal genes (Figure 1) for the construction of primers that can be used to identify *Fusarium* spp. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. Taxon-selective ITS amplification has already been used for detection of the fungal pathogens such as *Fusarium* (O´Donnell, 1992) and *Verticillium* spp. (Nazar et al., 1991) . Moller et al. (1999) also developed polymerase chain reaction (PCR) to identify *Giberrella fujikuroi* anamorphs in maize kernels using primer pairs based on sequences of RAPD fragments, and were specific for *Fusarium moniliforme* and *Fusarium subglutinans*.

Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiologic techniques, as a number of constraints are removed. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. The objective of our investigation was to develop a reliable and sensitive PCR assay for the selective detection of pathogenic *Fusarium* species on cotton.

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Abbreviations: AG, anastomosis groups; ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

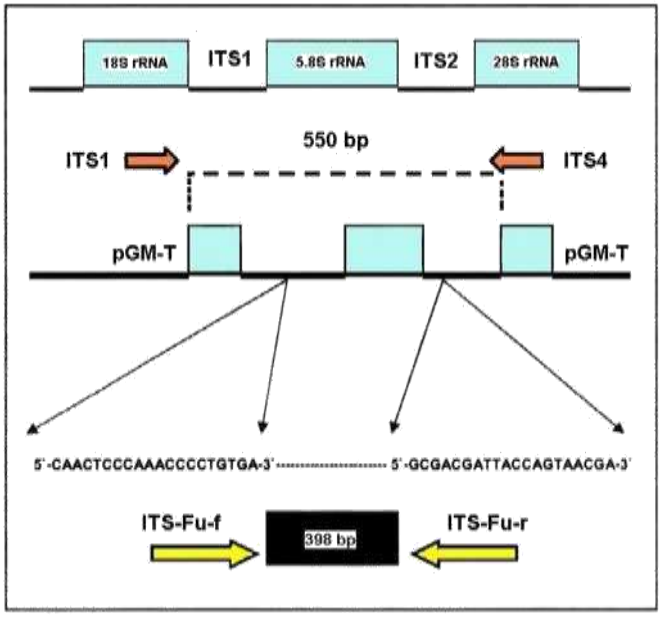

Figure 1. Oligonucleotide primers, ITS-Fu-f and ITS-Fu-r, specific for the *Fusarium* genus were constructed from the internal transcribed spacer (ITS) region.

MATERIALS AND METHODS

Fungal isolates

The following isolates were used in this experiment: three *F. oxysporum* f. sp. *vasinfectum*, three *F. oxysporum*, three *F. moniliforme*, three *F. solani*, and three *Rhizoctonia solani* (AG-4) isolates in addition to three *Macrophomina phaseolina.* The isolates originated from cotton-producing areas in Egypt.

DNA isolation

Total genomic DNA was isolated from fresh mycelium according to a miniprep protocol described by Cenis (1992). Briefly, 500 µl of liquid potato dextrose medium (Difco Laboratories Ltd., Surrey, United Kingdom) was inoculated with fungal hyphal threads and left at room temperature for 72 h. The resulting mycelial mat was pelleted by centrifugation at 13,000 rpm (Eppendorf microcentrifuge 5415c; Eppendorf UK Ltd., Cambridge, United Kingdom) for 5 min and was washed with 500 µl of Tris-EDTA (pH 8.0). The mat was then homogenized by hand in 300 µl of extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate) for 5 min. One hundred and fifty micro liters of 3 M sodium acetate (pH 5.2) was added, and the mixture was cooled to 20°C for 10 min. Fungal debris was pelleted by centrifugation at 13,000 rpm for 5 min, the supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 13,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, and DNA was resuspended in Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

PCR amplification of ribosomal DNA regions

The ITS1 and ITS2 and the inverting 5.8S coding rDNA were amplified by PCR using the primers ITS1 and ITS4 as described by White et al. (1990). Each PCR reaction mixture contained 5-10 ng of genomic DNA, 1µM each of the primers ITS1 and ITS4, reaction buffer (50 mM KCl, 50 mM Tris-HCl; [pH 8.3] 0.1 mg/ml bovine serum albumin), 3 mM $MgCl_2$, 200µM each of dNTP and 2.5 U of *Taq* DNA polymerase (Promega, Mannheim, Germany) in a total volume of 50 µl. The PCR profile was enetration at 95°C for 2 min, followed by 30 cycles of 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min. ITS bands of interest were excised from agarose gels and re-amplified by PCR using the same primer pair that was used for generating the ITS bands.

Cloning and sequencing of ITS markers

For cloning of the ribosomal DNA regions, the purified amplification product was ligated into the *Eco*RV site of the pGM-T vector system (Promega) according to the manufacturer's instructions. The boiling method (Sambrook et al., 2001) was used to isolate plasmid DNA from the bacterial host. Inserts of twelve isolates (three *F. oxysporum* f. sp. *vasinfectum*, three *F. oxysporum*, three *F. moniliforme* and three *F. solani*) were sequenced using the SequiTherm EXCEL II DNA Sequencing Kit with fluorescently labeled M13 primers (Epicentre, Biozyme, Germany) in a LI-COR Sequencer (LI- COR Inc., Lincoln, Neb.). Double-strand sequencing was performed for each clone.

Primer Design

Using the MegAlign software package (DNAStar Inc., Madison, Wis.), two primers were designed based on sequence information obtained to amplify specifically fragments within the ITS regions of *Fusarium* spp. The initial tests for specificity revealed that the primer pair ITS-Fu-f and ITS-Fu- r were highly specific for *Fusarium* genus. The nucleotide sequence of these primers are 5`-CAACTC CCAAACCCCTGTGA-3` and 5` -GCGACGATTACCAGTAACGA -3` (Figure 1). A guideline on PCR primer designing as described by Singh and Kumar (2001) was also followed.

Specificity of PCR amplification

Specificity of the PCR amplification for major *Fusarium* spp. using primers ITS-Fu-f and ITS-Fu-r was tested on a collection of four *Fusarium* species (three isolates per species), three *R. solani* (AG-4) isolates and three *M. phaseolina* isolates. Amplification of the 28S rDNA with taxon-specific PCR primer pairs was performed by the PCR method described above, except that the annealing temperature was increased to 58°C, 1.75 mM for $MgCl_2$ concentration, and 5 pmol for each primer concentrations. PCR fragments (389 bp) amplified with the two taxon-specific primer pairs were size-fractionated in 1.5% agarose gels (length, 25 cm; width, 20 cm).

Sensitivity of *Fusarium*-specific detection assays

To determine the minimum amount of fungal DNA that can be detected by the established PCR assays, variables quantities of *F. oxysporum* genomic DNA ranging from 10 ng to 100 fg were used as DNA template. All PCR products were size fractionated in agarose gels (1.5%) and visualized using ethidium bromide staining.

RESULTS

Using the universal fungal primers (ITS1/ITS4), PCR products (550-570 bp) were generated from all of the

typed species. Figure 2 illustrates the different sizes obtained for the full ITS region amplified from a selection of different *Fusarium* species.

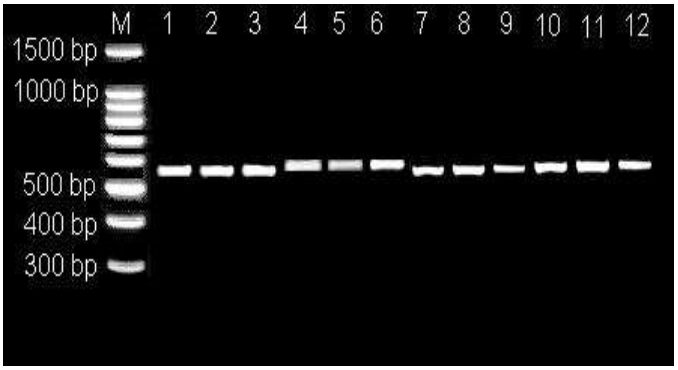

Figure 2. Full-ITS PCR products amplified from different *Fusarium* spp. with ITS1/ITS4 primers. Lanes 1-3, DNA amplified from *F. oxysporum* f. sp. *vasinfectum*; lanes 4-6, *F. oxysporum;* lanes *7-9, F. moniliforme*; lanes 10-12, *F. solani*; M, molecular-weight markers (100 bp DNA ladder, Promega).

Oligonucleotide probes were designed from ITS sequences available in this study; the full ITS region was sequenced from three isolates of each species. One sequencing reaction was performed on each strand. The primers showed good specificity for the genus *Fusarium*, and the approximately 389-bp product was amplified exclusively (Figure 3). The optimized PCR parameters for the specific amplification using ITS-Fu-f and ITS-Fu-r were 58 C for annealing temperature, 1.75 mM for $MgCl_2$ concentration, and 5 pmol for each primer concentration.

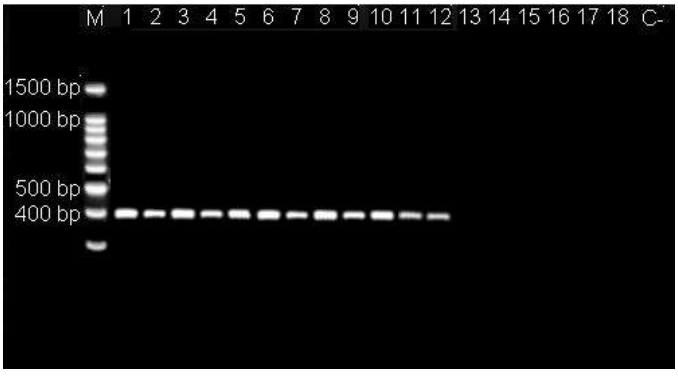

Figure 3. Specificity of the PCR assay (ITS-Fu-f and ITS-Fu-r primer pair) with genomic DNA from different fungal species. Lanes 1- 3, DNA amplified from *F. oxysporum* f. sp. *vasinfectum*; lanes 4-6, *F. oxysporum;* lanes *7-9, F. moniliforme*; lanes 10-12, *F. solani*; lanes 13-15*, R. solani*; lanes 16-18, *M. phaseolina*; C, negative control (H_2 O); M, molecular-weight markers (100 bp DNA ladder, Promega).

A range of DNA concentration of fungal culture was tested to determine the sensitivity of PCR assay. For DNA from *F. oxysporum* mycelium, 100 fg to 10 ng was sufficient for reliable amplification of the specific PCR with our primers (Figure 4). Negative controls processed simultaneously were consistently negative.

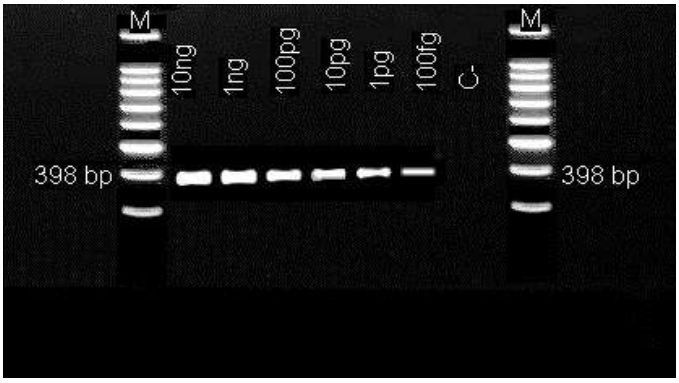

Figure 4. Sensitivity of the PCR assay (ITS- Fu-f and ITS-Fu-r primer pair) with *F. oxysporum* genomic DNA. Lane C, negative control (H_2 O); M, molecular-weight markers (100 bp DNA ladder, Promega).

DISCUSSION

The rRNA genes, commonly used in identification and taxonomic studies, were confirmed in the present study to be particularly appropriate for the purpose of providing target sequences for molecular detection. Differences in the nucleotide composition of the variable ITS region have been successfully employed to design specific primer sets that amplify DNA selectively among and within species of plant pathogens (Nazar et al., 1991; Moukhamedov et al., 1994; Schilling et al., 1996; Moricca et al., 1998). O`Donnell (1992) found a surprising level of divergence for ITS sequences within the species of *F. sambucinum*. We used ITS primers 1 and 4 to amplify the entire 5.8S rDNA gene, both ITS regions I and II, and a portion of the 18S nuclear small-subunit rDNA gene. The amplified DNA was sequenced with the M13 forward and reverse sequencing primers to develop a genus-specific PCR assay for the rapid identification of *Fusarium* genus isolated from Egyptian cotton.

As the primary factors in developing a PCR detection protocol are specificity and sensitivity, it is essential that the parameters used in the PCR are optimal, resulting in a maximal outcome. Despite the abundance of literature on the dynamics and stoichiometry of PCR reagents and conditions, the need for these parameters to be empirically optimized for a specific set of conditions is generally regarded as crucial (Arnheim and Erlick, 1992). In the current research the annealing temperature, $MgCl_2$ concentration and primer concentration were optimized

prior to verifying the specificity and determining the sensitivity of the PCR amplifications. In testing for specificity, it is impractical and unrealistic to test for cross reactivity with every extent organism. Nevertheless, the current approach of testing known related species is justified primarily by the use of rDNA as the basis for specificity. The nucleotide sequence analysis of rDNA region has been widely accepted to have phylogenetic significance, and is therefore useful in taxonomy and the study of phylogenetic relationships (Hibbett, 1992). This approach, designing primers from the rDNA region has far superior reliability compared to the use of random non-defined probes or primers.

Primer specificity was tested using the primers ITS-Fu-f and ITS-Fu- r, which specifically amplified a 398 bp fragment from all *Fusarium* species isolates. No cross-reaction was detected with genomic DNA from any isolates of other fungi that are occasionally associated with cotton, such as *R. solani* and *M. phasolina*, all of which were tested for non-specific amplification. According to the results of the dilution assay, even 100 fg of DNA is sufficient for amplification using these specific primers. Schilling (1996) has previously reported that 50 pg of *F. culmorum* and 5 pg of *F. graminearium* DNA were sufficient to yield PCR products with species-specific primers. Also, Nicholson et al. (1998) showed that the primer pairs developed for *F. culmorum* and *F. graminearium* were found to be highly sensitive, although when used in multiplex or competitive PCR, the detection limit was reduced to approximately 1 pg of genomic DNA for both species. For DNA extracted from *F. oxysporum* f. sp. *vasinfectum* mycelium, 50 fg to 100 ng was found to be suitable for PCR (Moricca et al., 1998). The presented PCR assays are highly selective and sensitive in detecting the *Fusarium* genus. To consider routine usage of the PCR assays for *in vivo* detection of *Fusarium* species in infected host tissue, our tests will have to be further evaluated and optimized.

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