Polymerase Chain Reaction-Based Assays for Species-Specific Detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*

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This research was supported by the Bundesminsterium für Forschung und Technologie, Germany (project BEO 0318897B).
We thank M. E. Takács for skillful technical assistance and L. W. Burgess, University of Sydney, Australia, J. A. G. van Leur, ICARDA, Addis Ababa, Ethiopia, Th. Miedaner, State Plant Breeding Institute, Stuttgart, Germany, H. Nirenberg, Biologische Bundesanstalt, Berlin, A. von Thiedemann, University of Göttingen, Germany, and W. Winter, ETH, Zurich, for generously providing fungal isolates. We thank W. Beyer and G. Gang for supplying infected samples of rye and B. Koopmann, University of Göttingen, Germany, for providing genomic DNA of *Rhizoctonia solani*.
Accepted for publication 25 January 1996.

ABSTRACT

Schilling, A. G., Möller, E. M., and Geiger, H. H. 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86:515-522.

Differential detection assays employing polymerase chain reaction (PCR) amplification of sequence-characterized amplified regions were developed for *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Unique fragments from randomly amplified polymorphic DNA profiles that differentiated *F. culmorum* and *F. graminearum* were cloned and sequenced. Based on the sequences, pairs of 20-mer oligonucleotide primers were designed to yield distinguishable amplicons of different molecular weight. Single fragments were amplified with each of the primer pairs that were specific for *F. culmorum* and *F. graminearum*. Screening a broad range of isolates of *Fusarium* spp., other cereal pathogens, and potential host plants revealed no significant cross-reactions for any assay. The assays were capable of detecting less than 10^{-12} g of fungal DNA and enabled the detection of individual *Fusarium* spp. directly in extracts of infected stem tissue and grains of rye and wheat showing disease symptoms. Additionally, internal transcribed spacer regions (ITS) of nuclear ribosomal DNA were amplified with universal primers and analyzed for sequence variation among the species. The ITS sequences of *F. culmorum* and *F. graminearum* were not polymorphic enough to design species-specific primers. ITS-1 and -2 of both species were compared to those of *F. avenaceum* and revealed sufficient sequence variation, especially in ITS-2, to derive primers for specific amplification of *F. avenaceum*. These specific, sensitive PCR assays represent valuable, versatile new tools for diagnosis, epidemiology, screening of breeding material for *Fusarium* resistance, and fungal population genetics.

Additional keywords: cereals, diagnostic kit, foot rot disease, *Gibberella* spp., head blight, molecular identification.

Complex communities of fungal pathogens are involved in foot rot and head blight diseases of small grain cereals and grasses as well as ear and stalk rot of corn. In temperate regions, *Fusarium* species, including *F. culmorum* (W.G. Sm.) Sacc. (teleomorph unknown), *F. graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch), and *F. avenaceum* (Fr.) Sacc. (teleomorph *G. avenacea* R.J. Cooke), are the most abundant and aggressive pathogens (12). Infections of seedlings and basal stems of plants are initiated by seed- or soilborne inoculum, and major yield losses result from damaged seedlings, preharvest lodging, and impaired grain filling.

Mixed infections of several *Fusarium* species and often with other foot and root rot pathogens, such as *Pseudocercosporella herpotrichoides* and *Microdochium nivale*, occur frequently. As a consequence, visual diagnosis is often hampered. Diagnostic methods for identifying *Fusarium* species are based on morphological characteristics observed on selective media (3,13). Considerable expertise is required to differentiate and identify the closely related *F. culmorum* and *F. graminearum*, because their traits exhibit variation on a continuous scale that may overlap between species. This situation is further complicated by the species *F. crookwellense* L.W. Burgess, P.E. Nelson, & T.A.Toussoun (2), which shares characteristics of *F. culmorum* and *F. graminearum* but has unclear pathogenic capabilities (3).

An enzyme-linked immunosorbent assay (ELISA)-based test for quantitative assessment of *F. culmorum*, *F. graminearum*, and *F. avenaceum* is available (1). The assay is reliable across various physiological stages of the host, but it is not capable of differentiating among the species. The utilization of molecular markers for species-specific detection assays recently has become very popular (reviewed in 6). Based on polymerase chain reaction (PCR), highly sensitive diagnostic assays have been implemented successfully for important plant pathogens, such as *Verticillium dahliae* and *V. albo-atrum* (11) and *Leptosphaeria maculans* (30). Most of these tests were developed by exploiting sequence polymorphisms within internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA). Alternative strategies for developing species- or strain-specific markers have employed unique sequences of mitochondrial DNA (18) or cloned restriction fragments of genomic DNA (8). Recently, Wiglesworth et al. (27) isolated a distinct fragment of randomly amplified polymorphic DNA (RAPD) of *Peronospora tabacina* representing a repetitive sequence. Using specific primers, the amplification of this sequence enabled the detection of minute amounts of fungal DNA contained in one or a few sporangiospores (27). The approach of sequence-characterizing RAPD fragments (SCARs) was first applied by Paran and Michelmore (16) to mark downy mildew resistance genes in lettuce.

The objective of our investigation was to develop reliable and sensitive diagnostic assays for selective detection of pathogenic

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Publication no. P-1996-0226-01R

Fusarium species on cereals. Previously, genetic diversity among isolates of a worldwide collection of *F. culmorum* and *F. graminearum* was assessed by RAPD fingerprinting (19,20). In this study, we explored the use of SCARs (16) to generate specific PCR markers from informative RAPDs. Additionally, we evaluated the utility of sequence variation in the ITS regions of selected isolates of *F. avenaceum*, *F. culmorum*, and *F. graminearum* to distinguish among the three species.

MATERIALS AND METHODS

Fungal cultures. Isolates of *Fusarium* spp. and other genera (Table 1) obtained from international culture collections (Centraalbureau voor Schimmelcultures, Baarn, Netherlands, and German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and collaborating researchers were single-spored from all original cultures.

For isolating *Fusarium* spp. from infected host tissue, grain samples of wheat and rye were surface-sterilized (3). Kernels were immersed in 1% sodium hyperchlorite and 0.1% Tween 20 (Merck, Darmstadt, Germany) for 10 min and rinsed three times in sterile distilled water. The material was placed on sterile filter paper in a laminar flow hood for a few minutes to dry excess moisture. Kernels were cut in half with a sterile scalpel. One half of each kernel was placed on synthetic nutrient-poor agar (SNA) medium (14) and incubated at 24°C for 1 day in the dark. The other half kernels were pooled to five pieces each and homogenized to a fine powder with a mixer-mill MM2 (Retsch, Haan, Germany).

DNA extraction. Total genomic DNA was isolated from mycelium (19) by a microextraction protocol according to Möller et al. (9) including treatment with RNaseA. Genomic DNA of infected plant material (stem tissue or grains) was extracted in the same manner, except that the extraction buffer was supplemented with a dash of sodium bisulfite. The DNA was quantified by UV spectrophotometry at 260 nm and by comparison to DNA standards by agarose gel electrophoresis. All stated DNA template amounts are based on gel estimates.

For a quick preparation of fungal DNA from freshly grown cultures, mycelium was scraped off the plate and placed in 1 ml of distilled water. The sample was boiled for 10 min, immediately cooled on ice for 5 min, and centrifuged at 14,000 × *g* for 5 min to pellet cell debris. An aliquot of 2 or 5 µl was subjected to PCR in various reaction mixes containing SCAR primers (described below).

TABLE 1. Isolates of *Fusarium* spp. and other cereal pathogens tested with polymerase chain reaction-based assays specific for *F. avenaceum*, *F. culmorum*, and *F. graminearum*

Isolate	No. of tested isolates
Fusarium sp.	
F. acuminatum	2
F. avenaceum	9
F. compactum	1
F. crookwellense	3
F. culmorum	69
F. equiseti	2
F. graminearum group 1	5
F. graminearum group 2	34
F. oxysporum	1
F. poae	2
Other genera	
Alternaria alternata	1
Bipolaris sorokiniana	1
Cladosporium herbarum	1
Epicoccum purpurascens	1
Gibberella fujikuroi	13
Microdochium nivale	6
Pseudocercosporella herpotrichoides	2
Rhizoctonia solani	1
Stagonospora nodorum	1

PCR conditions for RAPD. Decamer primer kit 100/1 and kit T were obtained from the University of British Columbia, Vancouver, and Operon Technologies, Alameda, CA, respectively. Based on the RAPD protocol of Williams et al. (29), PCR conditions were adjusted for *Fusarium* spp. genomic DNA as described elsewhere (19).

Restriction enzyme digestion. Total genomic DNA from isolates of *F. avenaceum*, *F. graminearum* groups 1 and 2, *F. crookwellense*, and *F. culmorum* (1 to 3 µg) was digested, each with 10 to 30 units of restriction enzyme for 3 h following the manufacturer's instructions. Restriction fragments were separated overnight on 0.8% agarose gels in 1× TAE buffer (0.04 M Tris-HCl and 0.002 EDTA adjusted to pH 8.0 with 98% acetic acid) with 0.5 V cm^{-1}. Prior to Southern blotting, gels were stained in ethidium bromide solution (5 µg ml^{-1}) for 3 min and destained in distilled water for a minimum of 30 min. Gel images were recorded with Polaroid film 667 (Polaroid Corp., Cambridge, MA) or a CCD video camera system (Cybertech, Berlin) connected to an image-processing workstation.

Southern hybridization. Gel-fractionated PCR products or restriction enzyme-digested genomic DNA were transferred onto nylon membranes (Hybond N, Amersham-Buchler, Braunschweig, Germany) by vacuum blotting following the manufacturer's protocol. The filters were prehybridized in a solution containing 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0), 1× Denhardt's solution, 5% sodium dodecyl sulfate (SDS), and a final concentration of 0.5 mg of denatured herring sperm DNA per ml for 1 h at 65°C and were hybridized overnight after adding 100 ng of denatured biotinylated probes per ml. Probes were prepared by PCR amplification of cloned RAPD fragments with M13 sequencing primers and biotin-16-dUTP (Boehringer, Mannheim, Germany) according to standard protocols (7). Filters were washed twice at room temperature at 2× and 0.2× SSC plus 0.1% SDS for 5 min each with two final high-stringency washes at 65°C and 0.16× SSC plus 0.1% SDS for 15 min each.

Membranes were equilibrated briefly in TBS-SDS buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% SDS) at room temperature and blocked with TBS-SDS plus 3% bovine serum albumin (Fraction V) for 2 h at 62°C. For signal detection, filters were carefully shaken in 0.07 µl per cm^2 of streptavidine-alkaline phosphatase conjugate (Life Technologies, Eggenstein, Germany), diluted to 10^{-3} in TBS-SDS at room temperature for 10 min. Membranes were transferred to TBS-SDS buffer to remove excess conjugate. The washes were repeated four times at room temperature, and the membranes were preincubated twice in substrate buffer (100 mM diethanolamine, 0.01% NaN_3, 1 mM $MgCl_2$, pH 10) for 10 min each. The chemiluminescent substrate CSPD (Tropix, Bedford, MA) was diluted in substrate buffer to 2×10^{-3}, and the filters were bathed in 0.05 ml per cm^2 for 10 min. Signals were detected by exposing the filters to Fuji X-ray film (Fuji Photofilm Europe GmbH, Düsseldorf, Germany) for 3 h.

PCR of ITS regions. The ITS-1 and -2 and the intervening 5.8S coding rDNA region were amplified from total genomic DNA of one isolate each of *F. avenaceum*, *F. culmorum*, and *F. graminearum* with primers I5 and I4 (26), which were deduced from the conserved flanking regions of 18S and 25S coding rDNA of ascomycetous consensus sequences. Amplifications were performed in 50-µl reactions, composed as described below for the species-specific PCR assays by the programmable thermal cyclers PREMIII (LEP Scientific Ltd., Andover, U.K.) or MJ Research PTC100-60 (Watertown, MA). Cycling parameters were the same as those described below, except for the annealing temperature of 56°C.

Cloning and sequencing of RAPD and ITS fragments. Bands were excised from the gel, and the DNA was purified with a Geneclean kit (Bio101, La Jolla, CA). Following gel quantification of isolated DNAs, the fragments were blunt-end ligated

into the *Sma*I cloning site of pUC18 with a SureClone ligation kit (Pharmacia Biotech, Freiburg, Germany). Ligated plasmids were used to transform competent cells of *Escherichia coli* strain DH5α according to established procedures (5). Positive clones were selected by PCR amplification of inserts with M13 sequencing primers on plasmids that had been extracted by a miniplasmid preparation protocol (28). Additionally, inserts of the cloned RAPD fragments were amplified with the corresponding arbitrary primers. Clones were tested as probes by hybridizing them against blotted RAPD profiles. Double-stranded DNA sequencing was performed by the dideoxy-chain termination method with a T7 sequencing kit (Pharmacia Biotech, Germany).

Primer design, PCR, and electrophoresis. Based on the full-length sequence, two specific primers were designed for each selected clone with the aid of the computer program Primer-Find 3.0 (written by K. Ellinger, Erlangen, Germany). The oligonucleotide primers (20 bases in length) included complete or part of the original arbitrary primer sequences and had a GC content of 50 to 60%. Specific primers were custom synthesized by Pharmacia Biotech, Roosendaal, Netherlands.

PCR amplifications were conducted in 50-µl reactions containing 1× *Taq* polymerase buffer (50 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$) and 1 unit of *Taq* polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each dNTP (Ultrapure; Pharmacia, Germany), and 25 pmol of each forward and reverse primer. Amplification of species-specific fragments was performed by routinely adding 1 to 5 ng of total genomic DNA or as described in the captions of Figures 7 and 8. Each PCR experiment included a positive control of a known amount of template DNA and a negative control without DNA. Cycling profiles consisted of 30 cycles of 1 min at 94°C and 1 min at 59, 55, or 61°C for annealing the respective primers specific for *F. avenaceum*, *F. culmorum*, and *F. graminearum*. An extension step of 2 min at 72°C was added. At the beginning of the cycling profile, reactions were held for 2 min at 94°C to denature the genomic DNA templates, and the final cycle was extended to 5 min at 72°C.

After completion of PCR, samples were cooled immediately to 15°C and stored at 4 or –20°C until gel separation. A gel-loading solution (10 µl) was added, and 10 µl of the total product volume (60 µl) was resolved on 1.5% agarose (Loewe Biochemica, Munich) in 1× TAE buffer for 2 h at 3.5 V cm^{-1} aside with a 100-bp ladder (Pharmacia, Germany) as the size standard. Gels were stained in ethidium bromide, and images were recorded as described above.

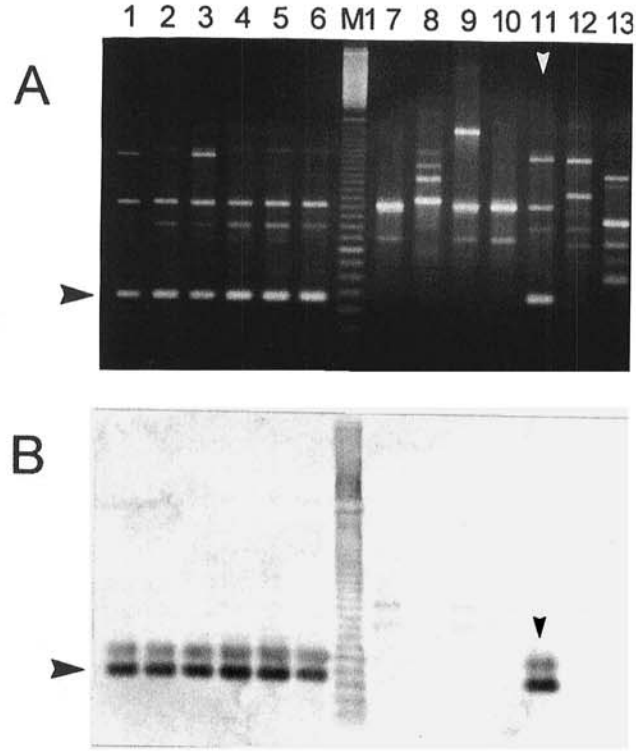

Fig. 1. A, Agarose gel showing randomly amplified polymorphic DNA (RAPD) profiles of *Fusarium culmorum*, *F. graminearum*, *F. crookwellense*, and *F. avenaceum* amplified with arbitrary primer OPT18 and **B,** corresponding Southern blot hybridized with a cloned RAPD product. **A,** The black arrow points to a unique fragment of approximately 470 bp present in all profiles of *F. culmorum* isolates (lanes 1 to 6) but not in *F. graminearum* (lanes 7 to 10), *F. crookwellense* (lane 12), or *F. avenaceum* (lane 13). The RAPD pattern in lane 11 marked by a white arrow identifies a falsely classified *F. graminearum* isolate as belonging to *F. culmorum* (19). **B,** Southern blot of the RAPD gel hybridized with the *F. culmorum*-specific fragment. The molecular weight standard (lane M1) is a 100-bp ladder (Pharmacia Biotech, Germany) with the 800-bp fragment marked by double intensity.

RESULTS

Species-specific SCARs derived from RAPDs. Different isolates of *F. culmorum* and *F. graminearum* were analyzed for RAPD profiles with arbitrary primers OPT18 (5′-GATGCCAGAC-3′) and UBC85 (5′-GTGCTCGTGC-3′). Primer OPT18 amplified a fragment of about 470 bp that was unique to all *F. culmorum* isolates (Fig. 1A). Primer UBC85 amplified a distinct fragment of 410 bp that was unique to *F. graminearum* group 2 (Fig. 2A). The two

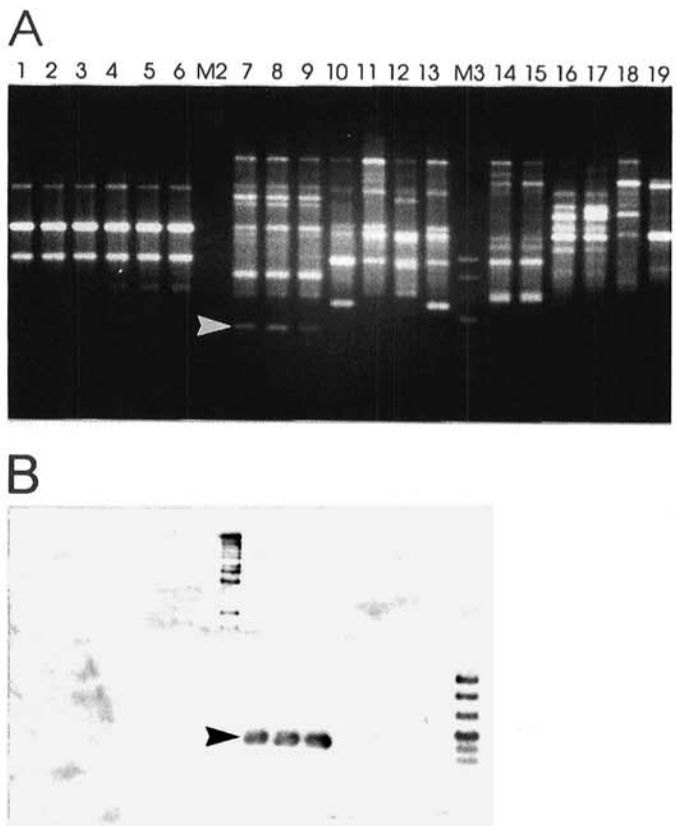

Fig. 2. A, Agarose gel showing randomly amplified polymorphic DNA (RAPD) profiles of *Fusarium culmorum*, *F. graminearum*, *F. crookwellense*, *F. avenaceum*, *Microdochium nivale*, and *Pseudocercosporella herpotrichoides* amplified with arbitrary primer UBC85 and **B,** Southern blot hybridized with a cloned RAPD product. **A,** The white arrow points to a unique fragment of approximately 410 bp present only in profiles of *F. graminearum* group 2 isolates (lanes 7 to 9) but not in profiles of *F. culmorum* (lanes 1 to 6), *F. graminearum* group 1 (lanes 10 to 13), *F. crookwellense* (lanes 14 and 15), *F. avenaceum* (lanes 16 and 17), *M. nivale* (lane 18), or *P. herpotrichoides* (lane 19). **B,** Southern blot of the RAPD gel hybridized with the *F. graminearum* group 2-specific fragment. A black arrow points to a single, strong hybridization signal. The molecular weight standards (lanes M2 and M3) are *Bst*EII-digested lambda DNA and STANVIII (Boehringer Gmbh, Mannheim, Germany), respectively.

TABLE 2. Sequences of specific primers for detecting *Fusarium avenaceum*, *F. culmorum*, and *F. graminearum*

Target species	Specific primer[a]	Sequence[b]	Amplicon	
			Size (bp)	Designation
F. avenaceum	FA-ITSF	CCAGAGGACCCAAACTCTAA	272	SCAR2-14
	FA-ITSR	ACCGCAGAAGCAGAGCCAAT		
F. culmorum	$OPT18F_{470}$	GATGCCAGACCAAGACGAAG	472	SCAR2-35
	$OPT18R_{470}$	GATGCCAGACGCACTAAGAT		
F. graminearum group 2	$UBC85F_{410}$	GCAGGGTTTGAATCCGAGAC	332	SCAR85
	$UBC85R_{410}$	AGAATGGAGCTACCAACGGC		

[a] Primer nomenclature is according to Paran and Michelmore (16), except for the primers derived from internal transcribed spacer sequences.
[b] Underlined nucleotides indicate sequences of the corresponding arbitrary primers.

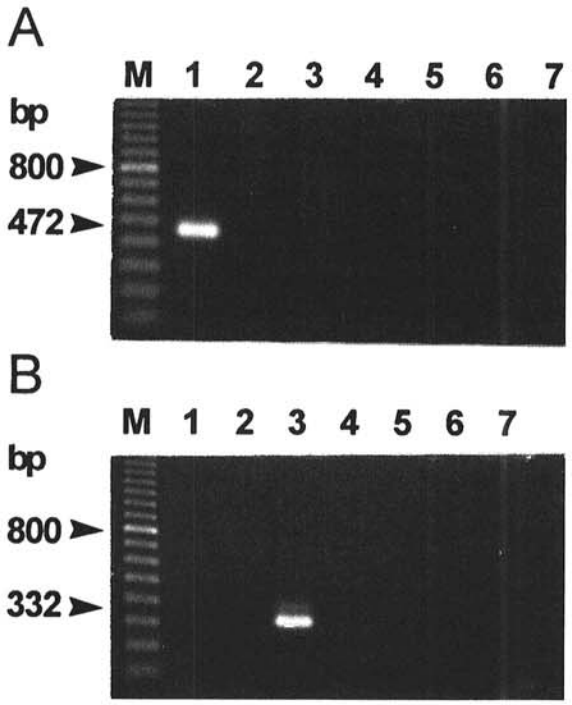

Fig. 3. Specific detection of *Fusarium culmorum* and *F. graminearum* by SCAR2-35 and SCAR85 amplified from total genomic DNA of various *Fusarium* spp. and other fungal species. **A,** SCAR2-35: a single fragment of 472 bp is specifically amplified from templates of *F. culmorum* (lane 1). No amplicons are observed in reactions of representative isolates of *F. crookwellense*, *F. graminearum* groups 2 and 1, *F. avenaceum*, *Microdochium nivale*, or *Pseudocercosporella herpotrichoides* (lanes 2 to 7, respectively). **B,** SCAR85: a single fragment of 332 bp is specifically amplified from templates of *F. graminearum* group 2. Lanes 1 to 7, the same isolates as in **A**. The molecular weight standard (lane M) is a 100-bp ladder (Pharmacia Biotech, Germany) with the 800-bp fragment marked by double intensity.

RAPD fragments were purified from the gel and cloned into the *Sma*I site of pUC18. To determine possible homologies among the amplicons derived from each arbitrary primer, the resulting clones, pLAP2-35 and pLAP85, were labeled with biotin and hybridized to filters displaying the respective RAPD patterns. Each of the two probes produced a single, strong hybridization signal that was common to all isolates of *F. culmorum* and *F. graminearum* group 2, respectively (Figs. 1B and 2B). These corresponded in size to the progenitor RAPD fragments.

The probe prepared from pLAP2-35 produced additional, but less intense, signals on RAPD fragments of *F. culmorum* amplified with primer OPT18 that were approximately 100 bp larger than the targeted fragment. Very faint hybridization signals of different fragment sizes also were noticed on RAPD patterns of some *F. graminearum* isolates. The probe prepared from pLAP85 also hybridized to RAPD fragments of *F. culmorum*, *F. crookwellense*, and *F. graminearum* group 1, but again, signals were very weak and of different sizes. These may represent related or partially homologous amplicons.

The complete sequences of clones pLAP2-35 and pLAP85 were 472 and 409 bp long, respectively, and the overall GC content was 47%. On the basis of these sequences, specific primers were designed (Table 2). Ten bases at the 5′ ends of the specific primer pair for *F. culmorum* were identical to those of the progenitor arbitrary primer. Therefore, the original fragment of 472 bp was amplified and designated SCAR2-35 (Fig. 3A). Due to matching bases at both 5′ ends of the insert sequence of pLAP85, specific primers for *F. graminearum* had to be chosen from internal sequences. The resulting amplicon was 332 bp long and designated SCAR85 (Fig. 3B).

PCR conditions were optimized for both assays to amplify single fragments of total genomic DNA of *F. culmorum* or *F. graminearum*. Both assays were tested with a large collection of *Fusarium* species and isolates of other fungal pathogens commonly associated with foot and ear rot-diseased plants (Table 1). No cross-reactions or additional fragments were observed for any other tested species. Examples are presented in Figure 3A and B. Furthermore, no amplifications were achieved with plant genomic DNA of rye or maize. SCAR2-35 was efficiently amplified from genomic DNA of 65 of 69 *F. culmorum* isolates tested that were obtained from different countries and continents. The four *F. culmorum* isolates that did not amplify the expected SCAR fragment were confirmed to belong to *F. culmorum*. Employing arbitrary primer UBC85 for RAPD profiling of these four isolates, typical and monomorphic fragments were amplified as shown for the *F. culmorum* samples in Figure 2A.

Similarly, SCAR85 was amplified selectively from genomic DNA of 34 *F. graminearum* group 2 isolates but not from isolates of *F. graminearum* group 1. Occasionally, a very faint 520-bp band was observed with genomic DNA of *F. culmorum*. However, lowering the annealing temperature by 5°C (from 61 to 56°C) resulted in multiple fragments that were polymorphic among *F. crookwellense*, *F. culmorum*, and *F. graminearum* (Fig. 4). Compared to the distinct, strong amplification of the SCAR85 fragment of *F. graminearum* group 2 isolates, all additionally amplified fragments of *F. culmorum* and *F. crookwellense* targets were less intense, except for a 870-bp fragment of *F. crookwellense*. In contrast, SCAR2-35 primers did not yield extra fragments from various *Fusarium* spp. targets when the annealing temperature was decreased from 55 to 50°C.

Southern blot hybridization. To investigate the capabilities of SCAR2-35 and SCAR85 for differentiating *Fusarium* species by restriction fragment length polymorphism (RFLP) analysis, Southern blots were hybridized with each SCAR fragment prepared as probe. On blots of *Eco*RV-digested genomic DNA of several isolates of *F. avenaceum*, *F. culmorum*, *F. crookwellense*, and *F. graminearum*, SCAR2-35 hybridized to a single 2.8-kbp band for *F. culmorum* (Fig. 5A). Interestingly, on one *F. culmorum* isolate two hybridization signals of approximately 7 and 1.4 kbp were obtained (Fig. 5A, lane 1). This was one of the four isolates that did not amplify SCAR2-35 with the *F. culmorum*-specific primers.

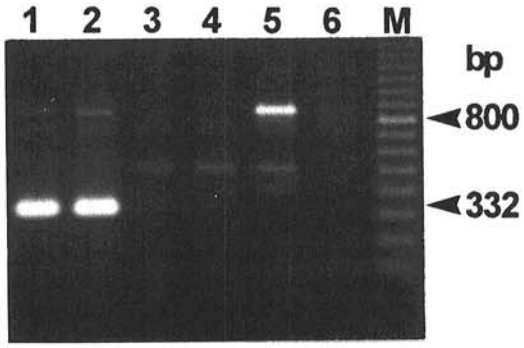

Fig. 4. Amplicons generated with SCAR85 primers under less stringent annealing conditions (the stringent temperature of 61°C was decreased by 5°C). Lanes 1 and 2, two *Fusarium graminearum* group 2 isolates with the species-specific 332-bp amplicon. Additional faint fragments occur with *F. culmorum* isolates (lanes 3 and 4), and a more intense band occurs with *F. crookwellense* (lane 5). No fragments are amplified from *F. avenaceum* (lane 6). The molecular weight standard (lane M) is a 100-bp ladder (Pharmacia Biotech, Germany) with the 800-bp fragment marked by double intensity.

Signals of homologous sequences also were identified for *F. graminearum*, with one or two bands of 2.5 or 2.5 and 5.8 kbp, and for *F. crookwellense,* with two bands of 1.6 and 3.8 kbp, but no hybridization was observed for *F. avenaceum* (Fig. 5A). Notably, within the SCAR2-35 sequence no *Eco*RV cleavage site existed.

Using SCAR85 as a probe on blots of genomic DNA of one isolate each of *F. avenaceum*, *F. culmorum*, *F. crookwellense*, and *F. graminearum* group 1 and two isolates of *F. graminearum* group 2 revealed only single bands on digests of any of the five restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, and *Pst*I. Within the sequence of SCAR85, no cleavage sites were found for these enzymes. Among *F. culmorum*, *F. crookwellense*, and *F. graminearum* and also between groups 1 and 2 of *F. graminearum*, all obtained signals were polymorphic, except for those obtained from *Pst*I fragments. No hybridizations signals were observed on *F. aveneaceum* genomic DNA digested with any of the five restriction enzymes. Polymorphic bands of *Eco*RV-digested DNA of *Fusarium* species are presented in Figure 5B.

Species-specific SCARs derived from ITS. To examine the suitability of ITS regions for distinguishing *Fusarium* spp., we initially analyzed only one isolate each of *F. avenaceum*, *F. culmorum*, and *F. graminearum*. Primers I4 and I5 generated unique PCR products of approximately 570 bp from templates of isolates of *F. culmorum* (CBS251.52) and *F. graminearum* (CBS389.62) and of about 600 bp from *F. avenaceum*. The fragments' identity to ITS regions, including the 5.8S coding sequences, was confirmed by separate digestions with the restriction enzymes *Cla*I, *Eco*RI, and *Sph*I, respectively (data not shown). According to previous studies on ascomycetes (10,15), each of these enzymes cleaves the 5.8S region once at conserved sites. Additionally, positive signals were obtained from blotted fragments hybridized with a heterologous probe prepared from a cloned 5.8S coding region of *Saccharomyces carlsbergensis* (obtained from G. Bahnweg, Munich).

The resulting subfragments were cloned, and both strands were sequenced. To detect variation among the species, sequences were aligned and compared to published ITS and 5.8S sequences of *F. sambucinum* (15). For *F. culmorum* and *F. graminearum*, the ITS-1 region was 146 bp long, and for *F. avenaceum*, it was 148 bp. The ITS-2 region exhibited variable lengths of 153, 149, and 160 bp for *F. culmorum*, *F. graminearum*, and *F. avenaceum*, respectively. Within ITS-1, no base differences were detected between the sequences of *F. culmorum* and *F. graminearum*. However, within the ITS-2 sequences, minor polymorphisms were confined to two deletions/insertions of a single base and to an array of three bases. Despite efforts to design several primers by exploiting the few sequence differences, no differential amplification was obtained. Moreover, these primers amplified similar size fragments from other *Fusarium* species, hampering their usefulness for species-specific detection assays.

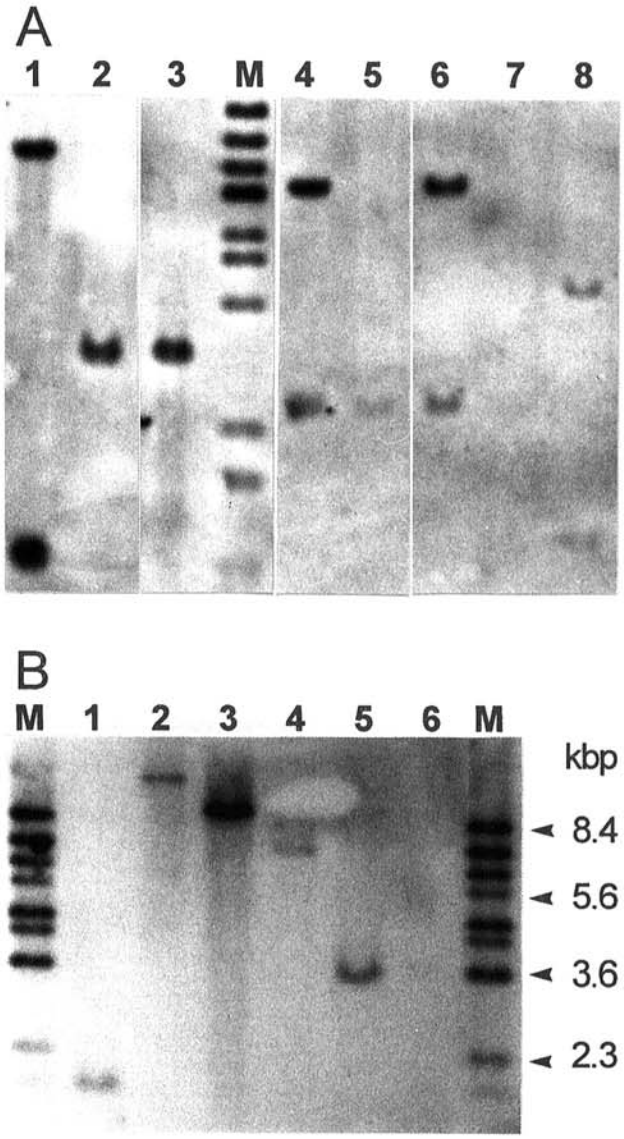

Fig. 5. Southern blots of *Eco*RV-digested genomic DNA of representative isolates of *Fusarium avenaceum*, *F. crookwellense*, *F. culmorum*, and *F. graminearum* groups 1 and 2 hybridized with SCAR2-35 and SCAR85 fragments as probes. **A,** SCAR2-35: a single and monomorphic fragment of 2.8 kbp is detected for isolates of *F. culmorum* (lanes 2 and 3). One *F. culmorum* isolate (lane 1) shows a deviating restriction fragment length polymorphism pattern with hybridizing fragments of 1.4 and approximately 7 kbp. Lanes 4 to 6, three *F. graminearum* isolates with one or two signals of 2.5 or 2.5 and 5.8 kbp. Lane 8, a *F. crookwellense* isolate with two signals of 1.6 and 3.8 kbp. No hybridizing fragments are detected for *F. avenaceum* (lane 7). **B,** SCAR85: single and polymorphic fragments are detected among *Fusarium* spp. Lanes 1 and 4 to 6, one isolate each of *F. culmorum*, *F. graminearum* group 1, *F. crookwellense*, and *F. avenaceum*, respectively. Lanes 2 and 3, two isolates of *F. graminearum* group 2. Similar to SCAR2-35 (**A**), no hybridizing fragments are detected for *F. avenaceum*. The molecular weight standards (lanes M) are *Bst*EII-digested lambda DNA.

Comparing ITS-1 and -2 of both *F. culmorum* and *F. graminearum* to that of *F. avenaceum*, several sites of base substitutions and deletions/insertions were apparent. ITS-2 showed extensive intervals of sequence differences. Polymorphic regions within ITS-1 and -2 were used to design primers specific for *F. avenaceum* (Table 2). PCR conditions were adjusted to exclusively amplify a 272-bp fragment of the *F. avenaceum* ITS, designated SCAR2-14

(Fig. 6). The *F. avenaceum*-specific primers were tested with the same collection of isolates (Table 1) as in the *F. culmorum* and *F. graminearum* selective PCR assays. SCAR2-14 was amplified from genomic DNA of nine *F. avenaceum* isolates examined from different habitats. Overall, no cross-reactions were observed among the three assays nor with any of the fungal and plant species tested.

Sensitivity of species-specific detection assays. To determine the minimum amount of fungal DNA that can be detected by the established PCR assays, reactions were set up with variable quantities of genomic DNA ranging from 50 ng to 5 fg. In addition, all reactions were spiked with a constant amount of about 10 ng of rye or 30 ng of maize genomic DNA. As little as 50 pg of total genomic DNA of *F. culmorum* was sufficient for reliable amplification of the specific SCAR2-35 fragment. By subjecting total sample volumes of the PCR to an agarose gel, the SCAR fragment was unambiguously visible after ethidium bromide staining (Fig. 7). The *F. graminearum*-specific fragment SCAR85 was amplified from even smaller amounts of total genomic DNA. As little as 5 pg was sufficient to yield PCR products that could be visualized (Fig. 8A). The sensitivity of detection of fungal targets was further increased by blotting the gel in Figure 8A and probing it with the labeled SCAR85 fragment. The lowest detectable amount of fungal target DNA approached as little as 500 fg, thereby achieving a 10-fold higher sensitivity compared to gel analysis (Fig. 8B).

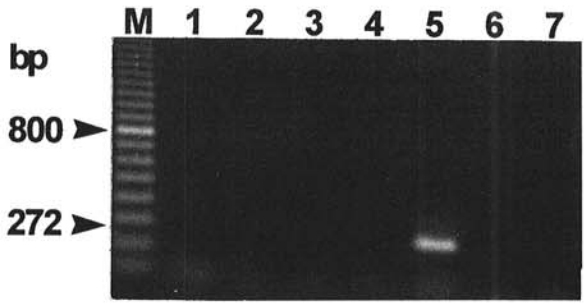

Fig. 6. Amplicons of internal transcribed spacer regions of *Fusarium avenaceum* utilizing species-specific primers. A 272-bp fragment is exclusively amplified from *F. avenaceum* (lane 5). No amplifications are detected for *F. culmorum*, *F. crookwellense*, or *F. graminearum* groups 2 and 1 (lanes 1 to 4, respectively). Lanes 6 and 7, *Microdochium nivale* and *Pseudocercosporella herpotrichoides*. The molecular weight standard (lane M) is a 100-bp ladder (Pharmacia Biotech, Germany) with the 800-bp fragment marked by double intensity.

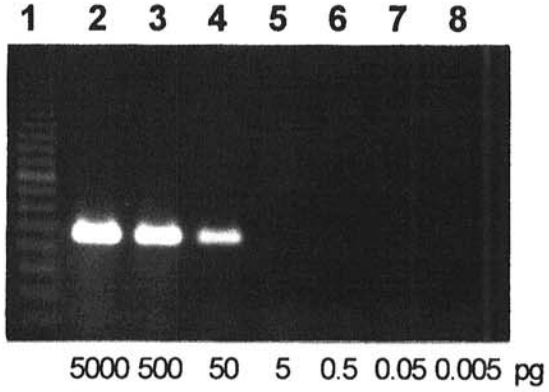

Fig. 7. Sensitivity of the *Fusarium culmorum*-specific polymerase chain reaction assay. SCAR2-35 is amplified from decreasing amounts of *F. culmorum* genomic DNA ranging from 5,000 to 0.005 pg (lanes 2 to 8). All reactions are spiked with 10 ng of rye genomic DNA each. Total sample volumes are subjected to gel electrophoresis. Lane 1, the molecular weight standard is a 100-bp ladder (Pharmacia Biotech, Germany).

Analysis of host tissues. To investigate conditions for diagnostic applications of the assays, extracts of stem bases of rye artificially infected with either *F. culmorum* or *F. graminearum* were analyzed. The samples were first scored visually for typical symptoms, and disease ratings were assigned (W. Beyer, *personal communication*). In all instances, amplification of SCAR2-35 and SCAR85 specifically revealed the presence of *F. culmorum* and *F. graminearum* in the plant-tissue extracts (Fig. 9). No plant compounds were recognized that may have inhibited the PCR of fungal target DNA. These samples were analyzed previously by an immunological test that enables the quantification of *Fusarium* spp. in stems of cereals (1). The ELISA and PCR assays were generally in good accordance. However, to evaluate the superiority of the available assays, further experiments are required that compare the two techniques in detail. With the presented PCR assays, no further quantification was pursued.

To test the PCR assays for their ability to detect infection of *F. culmorum* and *F. graminearum* in seeds of cereals, samples of rye and wheat grains were analyzed. Rye heads were taken from a field-inoculation experiment with *F. culmorum*. Wheat heads were obtained from a commercial field in southern Germany that was naturally infected with *F. graminearum*. Both grain samples showed only minor symptoms of *Fusarium* disease. The two PCR assays were capable of detecting the specific fungal contaminants in extracts of kernels with low infection (Fig. 10A and B). Also, solutions of boiled mycelia from obviously mixed cultures growing from surface-sterilized and incubated grain pieces were sufficient to amplify SCAR2-35 and SCAR85. The distinct fragments were amplified to amounts that were easily detected on ethidium bromide-stained gels (Fig. 10A and B).

DISCUSSION

We have successfully applied RAPDs for differentiation of *F. culmorum*, *F. graminearum*, and *F. avenaceum* (19,20). Species-

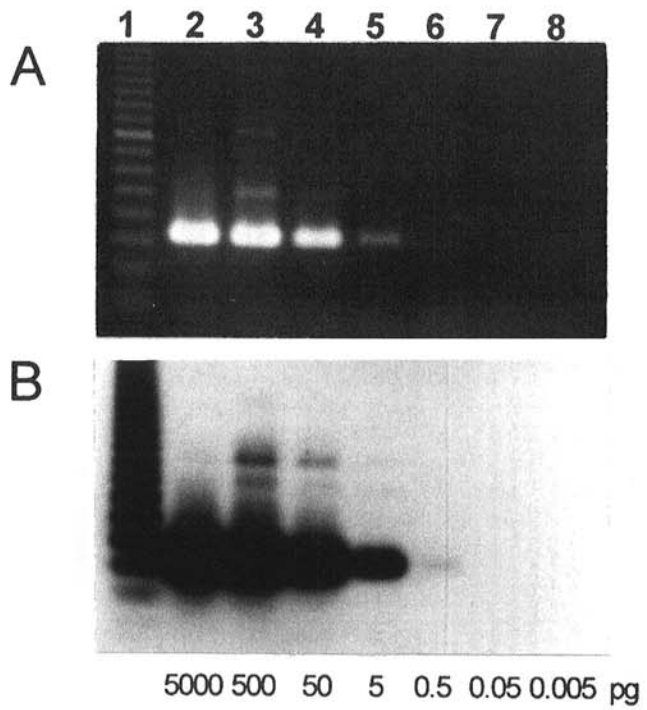

Fig. 8. Sensitivity of the *Fusarium graminearum* group 2-specific polymerase chain reaction assay. **A,** SCAR85 is amplified from decreasing amounts of *F. graminearum* group 2 genomic DNA ranging from 5,000 to 0.005 pg (lanes 2 to 8). All reactions are spiked with 10 ng of rye genomic DNA each. Total sample volumes are subjected to gel electrophoresis. **B,** Corresponding Southern blot hybridized with SCAR85 as probe. Lane 1, the molecular weight standard is a 100-bp ladder (Pharmacia Biotech, Germany).

specific fragments were easily identified and used to develop primers for selective amplification of *F. culmorum* and *F. graminearum* DNA. The two PCR assays presented were very reliable in identifying the fungal species directly in extracts of infected plant tissue and at various degrees of disease severity.

Of the many fungal isolates tested with both PCR assays, SCAR2-35 was not amplified from four isolates of *F. culmorum*. However, these isolates were confirmed by morphological reidentification of *Fusarium* specialists and by molecular data from our lab as *F. culmorum*. Possible explanations for this result could be that the four isolates possess genomic alterations in the primer annealing sites. Also, the genomic region tagged by the SCAR primers might have been rearranged by insertion/deletion events. As a result, the SCAR primers could not anneal specifically, or the two primer annealing sites would have been separated too far from each other, preventing the amplification of a distinct fragment. For one of the four identified *F. culmorum* isolates that failed to amplify SCAR2-35, Southern analyses of digested total DNA provided evidence that genomic alterations most likely affected the SCAR2-35 region. More data is required to obtain a better understanding of the particular alterations involved. The other three *F. culmorum* isolates that failed to amplify SCAR2-35 will need to be investigated by Southern hybridization to compare their RFLP patterns.

SCAR2-35 and SCAR85 most likely correspond to low or single-copy sequences as indicated by Southern analyses of digested genomic DNA of *Fusarium* spp. isolates. Single hybridization signals were observed on Southern blots of digested genomic DNA of *F. culmorum* or *F. graminearum* when probing with the specific SCAR fragments of the respective species. Except for the specific primers, neither of the sequences of both SCARs was unique to each of the two species. Instead, Southern analyses of digested genomic DNA and hybridizations to the RAPD profiles revealed homologies among the species *F. culmorum*, *F. graminearum* groups 1 and 2, and *F. crookwellense*. Utilizing these DNA homologies, the SCAR fragments could be used to differentiate and identify strains of *Fusarium* spp. by RFLP analyses. Employing SCAR85 as a probe provided a well-suited RFLP marker for differentiating the two groups within *F. graminearum*. Using morphological characters, isolates of groups 1 and 2 could not be distinguished. These two groups are regarded as differently adapted populations because they differ in the abundance of perithecia formation, origin of inocula, disease symptoms caused on stem bases, climatic requirements for infection, and geographic distribution (3).

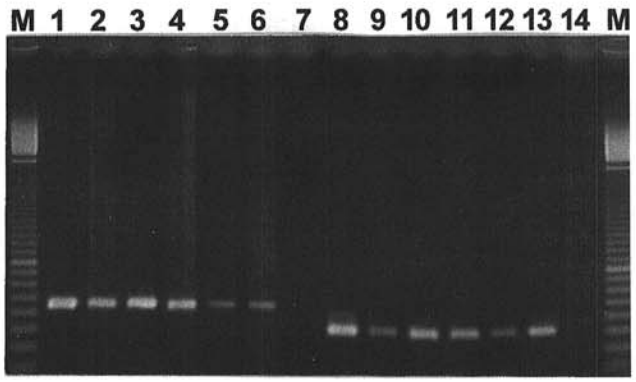

Fig. 9. Examples of in vivo detection of *Fusarium culmorum* and *F. graminearum* in infected stem-base tissues of rye. SCAR2-35 (lanes 1 to 7) and SCAR85 (lanes 8 to 14) are amplified from tissue extracts of stem bases. Lanes 1 and 8, positive controls showing amplicons obtained from 5 ng of purified genomic DNA of *F. culmorum* and *F. graminearum*, respectively. Lanes 2 to 6 and 9 to 13, tissue samples infected by *F. culmorum* and *F. graminearum*, respectively. Lanes 7 and 14, negative controls from noninfected rye tissue. Lanes M, molecular weight standard is a 100-bp ladder (Parmacia Biotech, Germany.

No signal was obtained for *F. avenaceum* in any of the hybridization and PCR experiments with SCAR2-35 and SCAR85 or the respective primers. This suggests that *F. culmorum*, *F. crookwellense*, and *F. graminearum* are more closely related to each other than any of them is related to *F. avenaceum*. Comprehensive RAPD and RFLP data from our own studies (19,20, and E. M. Möller, *unpublished data*) support a close relationship among *F. culmorum*, *F. crookwellense*, and *F. graminearum* and the more distant relation of these species to *F. avenaceum*. Hence, our data corroborate the generally accepted taxonomic relations of these *Fusarium* species (12).

The suitability of the ITS region as a multiple-copy sequence and its use as a diagnostic tool is known for several plant-pathogenic fungi (6). Based on ITS sequences, species-specific primers could be designed for *F. avenaceum*. In contrast, we could not utilize the few base deletions/insertions that exist between *F. culmorum* and *F. graminearum* to construct species-specific primers. Nazar et al. (11) found only 1% sequence variation in ITS-1 and -2 of *V. albo-atrum* and *V. dahliae* but were able to construct distinct primers that differentiated between the two species. In their case, polymorphisms were due mainly to clustered base changes rather than to deletions/insertions as in our study. Sequence align-

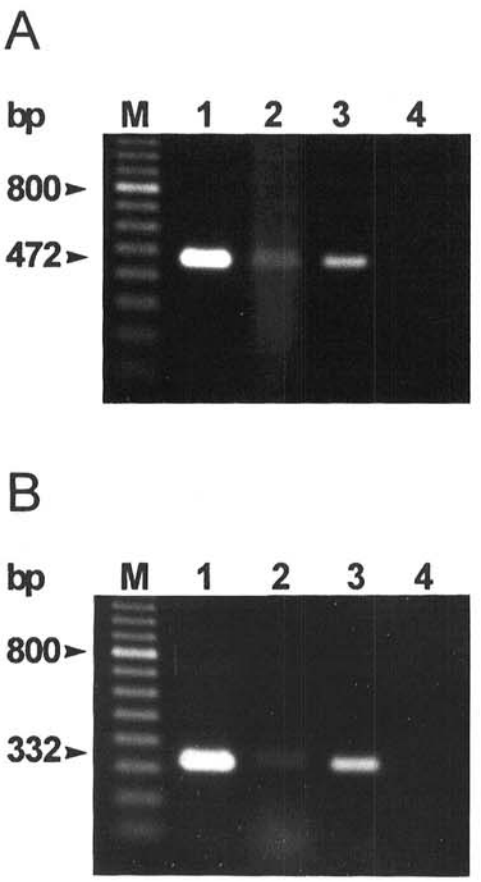

Fig. 10. Examples of in vivo detection of **A,** *Fusarium culmorum* and **B,** *F. graminearum* in infected grains of rye and wheat, respectively. **A,** SCAR2-35: lane 1, positive control showing SCAR2-35 amplified from 5 ng of purified genomic DNA of *F. culmorum*. Lane 2, amplicon from a DNA aliquot extracted from five half kernels of rye. The total sample volume was subjected to gel electrophoresis. Lane 3, amplicon from a solution of boiled mycelium growing on the other half kernels on an agar plate after 1 day. **B,** SCAR85: lane 1, positive control showing SCAR85 amplified from 5 ng of purified genomic DNA of *F. graminearum*. Lane 2, amplicon from an extract of infected wheat kernels as in **A**. Lane 3, amplicon from a boiled mycelium solution growing on wheat kernels processed as in **A**. Lane 4 (**A and B**), negative control with no sample DNA in the polymerase chain reaction mixes. Molecular weight standard (lane M) is a 100-bp ladder (Pharmacia Biotech, Germany) with the 800-bp fragment marked by double intensity.

ment of our data to *F. sambucinum* (15) indicated that *F. avenaceum* exhibited more sequence homology to *F. sambucinum* than to either *F. culmorum* or *F. graminearum*. However, more sequence work is required to estimate the ITS homology among and within the species, as well as to confirm the detected sequence polymorphisms.

Recently, Wiglesworth et al. (27) successfully used a distinct RAPD fragment for establishing a diagnostic PCR assay for *Peronospora tabacina* based on an anonymous repetitive sequence. The assay was capable of detecting the fungus in planta with high specificity and sensitivity. In our study, the presented SCARs allowed the detection of the closely related fungal pathogens *F. culmorum* and *F. graminearum* separately and directly in extracts of infected plant tissue at various degrees of disease severity. A minimum amount of 5 pg of *F. graminearum* total genomic DNA was sufficient for specific and reliable amplification of SCAR85 that was visible in the gel. The detection limit was further improved by applying a hybridization assay to the amplified product that provided readable signals from 0.5 pg of initial template DNA. Hence, a minimum of 20 to 40 nuclei, based on size estimates for the genomes of *F. graminearum* and *F. avenaceum* of 24 to 27 Mbp, respectively (4,23), would constitute the minimum amount of genomic DNA required for detecting *Fusarium* spp.

Current methods of testing *Fusarium* spp. contamination of stem tissue or cereal grains are based on morphological characters (13, 14). Other methods focus on assaying secondary metabolites or utilize immunological and enzymatic tests (17,21,22,25). The great majority of these techniques are labor-intensive, expensive, delicate, and some are neither species nor *Fusarium* specific. The PCR assays we developed are simple, robust, and fast. They are primarily qualitative tests for species-specific detection of *F. culmorum*, *F. graminearum*, and *F. avenaceum*. The assays are capable of identifying the particular species in vivo, i.e., in weakly infected stem tissues and at early stages of disease with barely visible symptoms as well as in infected cereal grains. In contrast, the reliability and precision of enzymatic and immunological test systems can be affected by samples having a high protein content. Furthermore, the presented PCR assays are highly selective and sensitive in detecting particular *Fusarium* species, even from tissue with multiple infections and at low incidence. Similar to our PCR assays, a simple and highly selective PCR test was developed by Taylor (24) to detect virulent strains of *L. maculans* in rape seeds. To consider routine usage of the PCR assays for in vivo detection of *Fusarium* spp. in grains, our tests will have to be further optimized. However, the PCR assays could become valuable diagnostic tools in epidemiological studies, especially for analyzing competition between different pathogen species during seedling and stem-base invasion. The assays also could be applied to monitor the growth of pathogens over various developmental stages of the host plant. In addition, they could be valuable when testing advanced breeding materials for stem rot and head blight resistance and for basic research in fungal population genetics.

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