A PCR-Based Technique for Detection of *Neotyphodium* Endophytes in Diverse Accessions of Tall Fescue

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ABSTRACT

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A previously described polymerase chain reaction (PCR)-based method used for detection of *Neotyphodium coenophialum* in tall fescue detected *Neotyphodium* endophytes in some, but not all, infected plants from a geographically diverse sample. In the study reported here, a different set of primers, based on intervening sequences of the tubulin 2 gene, were prepared and used for PCR. PCR with these primers yielded the expected 444 base pair amplification product with DNA from 104 of the 106 infected accessions tested. In addition, one accession originally scored as endophyte-free on the basis of a tissue culture test was correctly rated as endophyte-infected using the PCR procedure. Results suggest that primers based on intervening sequences of the tubulin 2 gene can be used for PCR-based detection of *Neotyphodium* endophytes in tall fescue accessions of diverse origin.

Additional keywords: *Acremonium*, *Festuca arundinacea*

Tall fescue, *Festuca arundinacea*, an important forage, pasture, and turf grass (3), is sometimes infected with endophytic fungi that grow asymptotically within the grass foliage (1,22). These fungi, which were formerly placed in the genus *Acremonium*, have recently been assigned to the new genus *Neotyphodium* Glenn, Bacon and Hanlin gen. nov. (11), based on DNA sequence analysis. Molecular analysis has demonstrated that they are descended from fungi of the genus *Epichloë* (Ascomycotina, Clavicipitaceae; 16,20,21).

Grasses infected with endophytes can benefit from the association in several ways, including enhancement of vigor, greater drought tolerance, and increased resistance to attack by insects and other herbivores (20,21). Insect resistance is conferred by the presence of toxic alkaloids produced by the fungus that are present in the grass foliage (20,21). Other fungal-derived alkaloids, however, can be

harmful to livestock, reducing the forage value of endophyte-infected grass (20,21). Hence, from an agronomic perspective, whether or not a tall fescue genotype is infected with endophyte is an important determinant of end use.

Endophytes can be detected in planta using histochemical (2,5,17,25), immunological (13,14), and tissue culture (8) techniques (10). In addition, we recently described a method for detection of *Neotyphodium coenophialum* in tall fescue (9) that employed the polymerase chain reaction (PCR; 18). When we attempted to use the PCR-based technique to detect *Neotyphodium* endophytes in a geographically diverse sample of tall fescue, the diagnostic 1,000 base pair (bp) amplification product was not detected in 3 out of 8 endophyte-infected (E+) plants. The oligonucleotide primers used with this PCR method were based on the nucleotide sequence of a fragment of genomic DNA present in *N. coenophialum* that had been isolated from "Kentucky 31," a tall fescue cultivar widely grown in the United States. The *Neotyphodium* endophyte (i.e., *N. coenophialum*) present in this cultivar, and in the other forage-type tall fescues derived from it, is genetically uniform (4,15,21); whereas, as a group, the *Neotyphodium* endophytes infecting tall fescue are quite diverse (24). Thus, our inability to detect endophytes in some infected plants suggested that the primers used in the original PCR-based detection procedure did not hybridize efficiently with fungal DNA

from *Neotyphodium* endophytes present in some tall fescue accessions of diverse origin. Indeed, many of the Mediterranean accessions examined in this study harbored *Neotyphodium* isolates that could not be accommodated by the taxon *N. coenophialum* (6) as presently defined (26). The objective of this study was to develop a PCR-based method that would detect symbiotic *Neotyphodium* endophytes in a diverse sample of tall fescue accessions.

MATERIALS AND METHODS

Plant materials. The tall fescue accessions used were from the germplasm collection at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Western Regional Plant Introduction Station, Pullman, Washington. Seed of these accessions was collected during a 1994 plant exploration trip to North Africa and Italy (Sardinia; 6). Fresh tillers of the plants were shipped from Pullman to Corvallis, Oregon on ice. Samples were coded so that personnel carrying out the PCR-based tests were unaware of the endophyte infection status, which had been determined using a tissue culture assay (6).

DNA extraction. DNA was extracted from small cylinders of the basal tissue of grass tillers using the method of Steward and Via (23), with four tillers per extraction (25 to 50 mg). Small plastic pestles fitted to microcentrifuge tubes (Kontes, Vineland, NJ) were used for homogenization. DNA was taken up in either 400 or 100 µl of water.

PCR and detection of amplification product. PCR was carried out using the 11-1 and 11-2 primers described previously (9), or two primers (IS-1 and IS-3) based on the nucleotide sequence of introns 1 and 3 of the tubulin 2-4 gene (Tub 2 primers; Table 1). The tubulin 2-4 gene was present in all of the tall fescue endophyte isolates examined by Tsai et al. (24). The PCR reaction volume was 25 µl, and the composition was as follows: 10 mM TRIS-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; 200 µM each deoxynucleotide triphosphate (Promega, Madison, WI); 1.0 µM each oligonucleotide primer, 0.625 units Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA), 1 µl DNA extract.

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Reactions were formulated on ice and then held at 94°C for 1 min, followed by 35 cycles with a 94°C melting temperature for 15 s and an annealing/reaction temperature of 60°C for 1 min (Coy Thermocycler II, Grass Lake, MI). After cycling, reactions were held at 72°C for 10 min to complete polymerizations. Amplification products were then separated by agarose gel electrophoresis using a 2% agarose gel (cat no. A 9539, Sigma Chemical Co., St. Louis) with a TRIS-phosphate buffer system, and detected by staining the gel with ethidium bromide (19).

RESULTS AND DISCUSSION

When a sample of 12 tall fescue plants comprising 2 Kentucky 31 derivatives and 10 accessions from North Africa and Italy (Sardinia) was subjected to the PCR-based detection procedure described previously (9), the diagnostic 1 kilobase (kb) amplification product was not detected with 3 of the 8 E+ plants (Fig. 1). Histological examination (25) confirmed the results from tissue culture, and demonstrated that the PCR-based technique had failed to detect endophytes in some E+ plants (Table 1).

Table 1. *Neotyphodium* endophyte infection status of 12 tall fescue accessions determined using several diagnostic methods

Accessions	Tissue culture	Histological	PCR (11-1/11-2 primers) ^a	PCR (Tub-2 primers) ^b
Q1 ^c	+	+	+	+
1-16067T	+	+	+	+
1-16075T	+	+	+	+
4-16003T	+	+	—	+
Q2 ^c	—	—	—	—
2-16094T	—	—	—	—
2-160036T	—	—	—	—
5-16144T	—	—	—	—
1-15778M	+	+	—	+
4-16144S	+	+	+	+
42T	+	+	—	+
43T	+	+	+	+

^a PCR = polymerase chain reaction. Primer 11-1, 5'-TCA TAC CGG CTA ACC GGC ATT-3'; primer 11-2, 5'-TGT TAC AGG ATT GGT AGA GGC-3'.

^b Tub-2 primers: Primer IS-1, 5'-GGT GTT GAG CCC CCC TGA TTT-3'; primer IS-3, 5'-GTC TCA TCT CCG GGG CGG TAT-3'.

^c Q1 and Q2 are Kentucky 31 accessions.

With some endophyte-free (E-) plants, an amplification product with a molecular size slightly greater than the diagnostic 1,000-bp fragment was obtained when an annealing/reaction temperature of 60°C was used with the 11-1 and 11-2 primers (Fig. 1). This product, presumably derived as the result of nonspecific priming, disappeared with an annealing temperature of 68°C.

The PCR technique we employed was developed using primers based on the nucleotide sequence of a fragment of genomic DNA from an endophyte (*N. coenophialum*) isolated from a Kentucky 31 tall fescue derivative. The failure to detect endophyte in some E+ plants with this method suggested that the primers used did not hybridize with DNA from all of the *Neotyphodium* endophytes in tall fescue. Accordingly, new primers, based on the intervening sequence of a B-tubulin gene (tub 2-4) that was present in all of the isolates of *Neotyphodium* spp. from tall fescue examined by Tsai et al (24), were prepared and used for PCR. With these primers, all eight of the endophyte-infected tall fescue accessions in the test sample yielded the expected 444 bp amplification product (Fig. 1, Table 1).

Subsequently, we examined a tall fescue germplasm collection originating from seed collected in the wild from Italy (Sardinia) and North Africa. Of 160 accessions in this collection, 106 were E+, and the PCR method confirmed the endophyte

Fig. 1. Ethidium bromide-stained agarose gel showing 1,000 base pair (bp) amplification product obtained using 11-1 and 11-2 primers (upper lanes) and 444 bp product obtained with Tub-2 primers (lower lanes). Tall fescue accession designations are indicated above lanes. Q1 and Q2 are Kentucky 31 derivatives. Molecular weight standards are in rightmost lanes. Note that in some cases with the 11-1 and 11-2 primers and an annealing temperature of 60°C, nonspecific priming gave rise to amplification products of nearly the same size as the diagnostic product. Such products, which are seen here as weak bands, could be eliminated by increasing the annealing temperature to 68°C. Tub-2 primers yield more amplification product with the lower annealing temperature than with the higher temperature.

infection status in all but 3 cases. With one of these (an accession from Sardinia), formerly scored as E-, a diagnostic amplification product was observed, suggesting that the tissue culture procedure had yielded incorrect results. Histological examination of this accession confirmed the presence of endophyte, as did a subsequent tissue culture test. With two other infected accessions, diagnostic amplification products were not detected with several assays, even though histological examination confirmed the presence of endophyte. With DNA from several barley accessions infected with *Neotyphodium* endophytes (7), a diagnostic amplification product was not formed with either the Tub 2 or the 11-1 and 11-2 primers. In addition, a Kentucky 31 derivative originally used in our tests as an E- control was found, through histological examination, to possess an endophytic fungus other than a *Neotyphodium* sp. DNA from this accession also gave a negative response with both pairs of primers. These results suggested that the PCR-based assay using the Tub-2 primers was specific for *Neotyphodium* endophytes of tall fescue.

The PCR-based technique has some advantages over other methods used for endophyte detection. It is rapid, requiring only a day to screen 60 or more plants. It is also sensitive and requires only a few milligrams of tissue. Unlike the methods that require skills in endophyte identification, the PCR-based method is easy to learn and can provide reliable results even if persons carrying out the test have no previous experience with endophytes. Finally, this technique should be useful for detecting *Neotyphodium* spp. in tall fescue accessions that harbor genetically diverse endophytes.

The PCR-based technique does have some pitfalls. False positives are not observed if the usual care is taken to avoid DNA contamination. However, we have occasionally encountered false negatives, which presumably arise, in at least some cases, because the fungus is not uniformly present in the sampled tissue. For this reason, it is important to repeat DNA extraction and amplification with any plant scored as E- in the first round of testing, and to sample tissue from several grass tillers. Alternatively, testing could be carried out on duplicate or triplicate samples of all plants. It is also important, as a check on the DNA extraction procedure, to include in each set of samples grass tissue known to be either endophyte-free or endophyte-infected. In addition, an extract of DNA that can act as a template for synthesis of the diagnostic amplification product should be included in each set of amplification reactions.

Groppe and Boller (12) recently described a PCR assay for *Epichlœ*-type endophytes that used primers based on the

nucleotide sequence of a variable microsatellite region in the genomic DNA from *Epichlœ* spp. Although in planta detection of *Neotyphodium* endophytes in tall fescue was not demonstrated with these primers, amplification products were obtained when DNA isolated from several *Neotyphodium* spp. was used as a template, and it is likely that these microsatellite-derived primers could also be used for detection of *Neotyphodium* endophytes in tall fescue tissue. However, the anomalous response to changes in annealing temperature observed when such primers were used with several *Neotyphodium* isolates suggests that optimization of PCR conditions could be difficult.

In general, PCR-based methods for detection of fungal endophytes of grasses provide a convenient means of ascertaining infection status. The Tub-2 primers described in this report were superior to the 11-1 and 11-2 primers described earlier (9) in detecting *Neotyphodium* endophytes in tall fescue accessions from a diverse geographic range.

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