APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1994, p. 4155–4159
0099-2240/94/$04.00+0

Vol. 60, No. 11

Isolation, Characterization, and Expression of a Second β-Tubulin-Encoding Gene from *Colletotrichum gloeosporioides* f. sp. *aeschynomene*†

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Received 10 June 1994/Accepted 8 September 1994

***Colletotrichum gloeosporioides* f. sp. *aeschynomene* is a fungal plant pathogen of *Aeschynomene virginica*. A β-tubulin-encoding gene (*TUB2*) from this pathogen was cloned and sequenced. The deduced amino acid sequence of *TUB2* had a high degree of homology to other fungal β-tubulins. A portion of *TUB2* from a benomyl-resistant *C. gloeosporioides* f. sp. *aeschynomene* mutant was also cloned and sequenced. A point mutation resulting in a glutamic acid-to-lysine substitution at amino acid 198 likely confers benomyl resistance. The mutation is relevant for use as a selectable marker in developing a gene transfer system in *C. gloeosporioides* f. sp. *aeschynomene*. Northern (RNA) hybridizations with *C. gloeosporioides* f. sp. *aeschynomene* *TUB2* and another *C. gloeosporioides* f. sp. *aeschynomene* β-tubulin-encoding gene (*TUB1*) as probes showed differential expression of these genes in different cell types.**

Colletotrichum gloeosporioides (Penz.) Sacc. f. sp. *aeschynomene* incites anthracnose on *Aeschynomene virgininica*, which is commonly called northern jointvetch (4). Northern jointvetch is a leguminous weed in rice and soybean fields in some southern states. Contaminating northern jointvetch seeds greatly reduce the market value of rice (20). *C. gloeosporioides* f. sp. *aeschynomene* has been commercially marketed as a mycoherbicide (COLLEGO) to decrease populations of northern jointvetch in rice and soybean fields.

Development of a transformation system would be extremely useful for investigating the molecular biology of *C. gloeosporioides* f. sp. *aeschynomene*. Attempts to transform *C. gloeosporioides* f. sp. *aeschynomene* by using heterologous molecular markers have met with little success (1a). Thus, cloning of an endogenous molecular marker may facilitate development of an efficient transformation system.

Benomyl (BEN) is an effective systemic fungicide (active ingredient, methyl-2-benzimidazole carbamate). BEN binds to the microtubule subunit β-tubulin (βTub) to inhibit the growth of many fungi (14). Point mutations in a highly conserved gene for βTub, designated *TUB2*, confer BEN resistance in *Neurospora crassa* (13) and *Aspergillus nidulans* (8). *C. graminicola* contains two genes encoding βTub (15). A divergent βTub gene (*TUB1*) from a BEN-resistant *C. graminicola* strain successfully transformed wild-type *C. graminicola* to BEN resistance (16).

C. gloeosporioides f. sp. *aeschynomene* also contains two βTub-encoding genes, including a βTub gene with a high degree of amino acid homology to *C. graminicola TUB1* (2). Here we report the nucleotide sequence of a second gene for βTub (*TUB2*) in *C. gloeosporioides* f. sp. *aeschynomene* and identify a molecular lesion which likely confers BEN resistance. Interestingly, the β-tubulin genes are differentially expressed in conidia, conidiating mycelia, and vegetative mycelia and the *TUB1* transcript is considerably larger than expected.

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† Journal series no. 10745, Agricultural Research Division, University of Nebraska.

MATERIALS AND METHODS

Strains and plasmids. Wild-type *C. gloeosporioides* f. sp. *aeschynomene* 3.1.3, used in this work, was isolated from northern jointvetch. B-21 is a pathogenic, ethylmethylsulfonate-induced, BEN-resistant mutant derived from strain 3.1.3. Both strains were provided by Dave TeBeest, University of Arkansas. Clone pCGTUB2 (15) was obtained from R. M. Hanau, Purdue University. The *Escherichia coli* XL-1 and plasmid vectors pBluescript KS+ and KS− were used for subcloning. Helper phage VCS-M13 was used for single-stranded DNA generation.

Media and culture conditions. YpSs (21) agar plates were inoculated with spore suspensions of each *C. gloeosporioides* f. sp. *aeschynomene* strain stored at −70°C. B-21 was grown on medium with 1 μg of BEN per ml. Cultures were grown for several days at 24°C with a 12-h photoperiod under white fluorescent light. For DNA isolation, 250 ml of YpSs liquid medium (amended with 1 μg of BEN per ml for B-21) was inoculated with approximately 0.5-cm^2 plugs of mycelia from YpSs agar plates. Cultures were grown for several days at room temperature on a rotary platform at 100 rpm. For RNA isolation, 100 to 1,000 ml of YpSs liquid medium was inoculated with a sterile loop of *C. gloeosporioides* f. sp. *aeschynomene* conidia from a YpSs agar plate. To obtain vegetative mycelia, cultures were grown on a bench without agitation for approximately 2 weeks. Conidia and conidiating mycelia were obtained from cultures grown for several days at room temperature on a rotary platform at 100 rpm. Conidia were filtered through sterile cheesecloth to separate conidia from conidiating mycelia. All fungal samples were examined by light microscopy to distinguish whether samples contained mycelia, conidia, or both mycelia and conidia. Conidiating mycelium samples contained some conidia but conidial samples contained only conidia. *E. coli* cultures were grown on Luria-Bertani agar or 2xYT liquid medium (18). Selective medium contained 100 μg of carbenicillin per ml and 10 μg of tetracycline per ml as necessary.

Nucleic acid isolation. Fungal genomic DNA was isolated as previously described (16), except that total DNA was purified in a single cesium chloride gradient. Total RNA was purified in accordance with previously published procedures (3). Plasmids were isolated as previously described (18).

Library construction, isolation of clones, and sequencing. A genomic library of wild-type *C. gloeosporioides* f. sp. *aeschynomene* was constructed in the vector EMBL3 (6). Genomic DNA was partially digested with *Sau*3A and size fractionated on sucrose gradients. Fragments of 15 to 20 kb were ligated with *Bam*HI-digested EMBL3 arms, and the ligation products were packaged in vitro by incubation with bacteriophage lambda packaging extracts. The resulting library was transfected into *E. coli* P2392. Plaque hybridization was performed by the method of Benton and Davis as described by Sambrook et al. (18), by using nick-translated (17) pCGTUB2 as the probe. Subcloning involved standard procedures (18). Deletions were constructed with restriction endonucleases, ligation, and transformation. DNA was sequenced by using the dideoxy-chain termination method (19) as modified by Nelson et al. (12). Oligonucleotides were synthesized on an Applied Biosystems nucleic acid synthesizer to sequence any DNA not sequenced from deletions. All enzymes were purchased and used in accordance with the manufacturer's specifications.

PCR. A portion of the *TUB2* allele from genomic B-21 DNA was amplified by PCR. Reactions were prepared in accordance with the protocol of Perkin Elmer Cetus, by using 50 ng of heat-denatured, *Apa*I-digested genomic B-21 template DNA and 140 ng each of two primers (TB201 [5′-CTCCATCTCG TCATACC] and TB203 [5′-TTATCCGCCTTGCCCCT-3′]). Reactions were incubated as follows: a denaturing cycle of 94°C for 5 min; three cycles of 94°C for 1.5 min, 40°C for 2 min, and 72°C for 3 min, four cycles of 94°C for 1.5 min, 45°C for 2 min, and 72°C for 3 min; 18 cycles of 94°C for 1.5 min, 50°C for 2 min, and 72°C for 3 min; and a 7-min extension at 72°C. The resulting 1.65-kb fragments were digested with *Bam*HI-*Xho*I to produce 1,028-nucleotide fragments. These were ligated into pBluescript KS− and transformed into *E. coli* XL-1.

Northern (RNA) hybridizations. RNA (20 μg) was mixed with 0.72 μg of ethidium bromide, 1.5 μl of 10× loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll, 10 mM EDTA), and 3 μl of 5× TBE buffer (18) in a 15-μl volume. Samples were heated at 75°C for 5 min prior to loading of 1% agarose–1× TBE gels. Gels were run at 3 V/cm for 3.5 h in 1× TBE buffer. Gels were soaked in 7% formaldehyde for 10 min, rinsed twice with water, and then soaked in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]) for 10 min. RNA was blotted onto nylon filters with 20× SSPE for 16 to 20 h and subsequently fixed via UV irradiation. Blots were hybridized in 5× SSPE–5× Denhardt's solution–0.5% sodium dodecyl sulfate–20 μg of single-stranded salmon sperm DNA per ml. Probes were labeled with [α-^{32}P]dCTP by using the random priming method of Feinberg and Vogelstein (5). Hybridizations included 25 ng of the probe in 10 ml of solution at 60°C for 20 h. Filters were washed under high stringency including two washes in 0.2× SSPE at 60°C for 20 min each. Filters were exposed to Kodak X-Omat film with intensifying screens for about 7 days.

Nucleotide sequence accession number. The GenBank accession number for *C. gloeosporioides* f. sp. *aeschynomene TUB2* is U14138.

RESULTS

Cloning of *C. gloeosporioides* f. sp. *aeschynomene TUB2*. A genomic phage library of wild-type *C. gloeosporioides* f. sp. *aeschynomene* was screened by using pCGTUB2 (16) as a probe. Several strongly hybridizing plaques were isolated. DNA purified from individual phage clones was digested with several restriction enzymes. Subsequent Southern hybridizations revealed a single hybridizing fragment of 2.65 kb after digestion with *Apa*I-*Pst*I. This fragment was cloned into *Apa*I-*Pst*I-digested pBluescript KS+ and KS−. Restriction endonuclease digestion produced the map in Fig. 1.

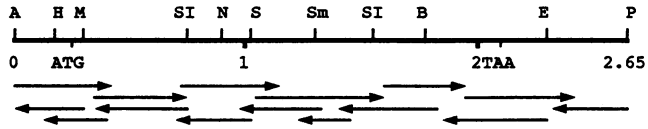

FIG. 1. Restriction map of *C. gloeosporioides* f. sp. *aeschynomene TUB2* and flanking regions. Map coordinates are in kilobases. The relative positions of the ATG and TAA codons are indicated below the map. The pCGAWTTUB2 clone contains the entire *Apa*I-*Pst*I insert. The arrows indicate the directions and extents of the *TUB2* regions sequenced. Restriction sites are designated as follows: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Nco*I; P, *Pst*I; SI, *Sac*I; S, *Sal*I; Sm, *Sma*I.

Sequence analysis. Both strands of the 2.65-kb *Apa*I-*Pst*I fragment were completely sequenced (Fig. 1) by using dideoxy-chain termination sequencing (19). Sequence alignment with *C. graminicola TUB2* showed that this fragment contained a complete *TUB2* gene from *C. gloeosporioides* f. sp. *aeschynomene*. Alignment of the *C. gloeosporioides* f. sp. *aeschynomene TUB2* coding sequence (Fig. 2) showed 93.6 and 91.2% nucleotide sequence identity with *C. graminicola TUB2* (15) and *N. crassa tub-2* (13), respectively. Sequence alignment with *C. gloeosporioides* f. sp. *aeschynomene TUB1* (2) showed 69.1% nucleotide sequence identity.

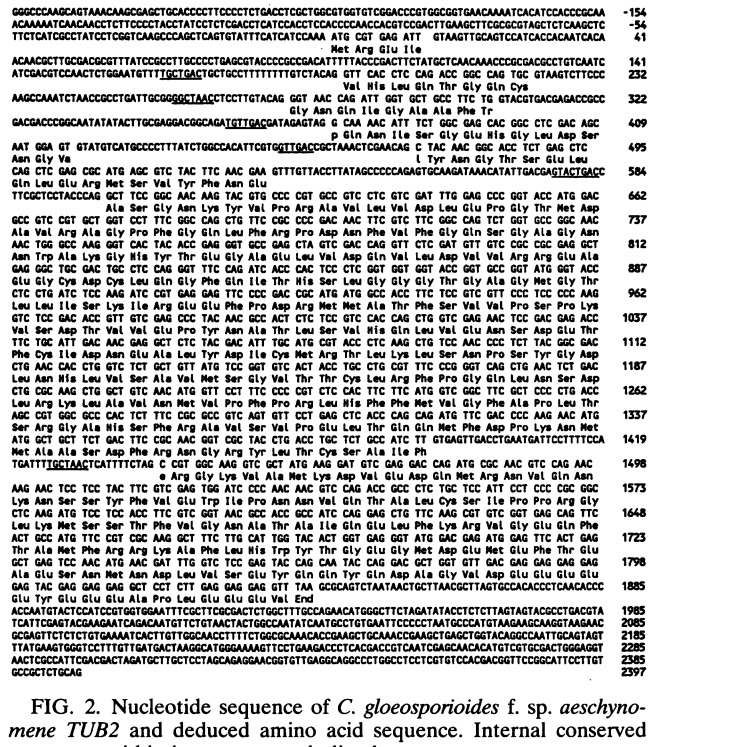

FIG. 2. Nucleotide sequence of *C. gloeosporioides* f. sp. *aeschynomene TUB2* and deduced amino acid sequence. Internal conserved sequences within introns are underlined.

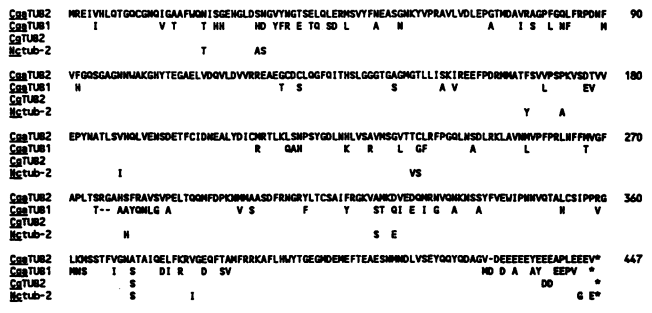

FIG. 3. Comparison of deduced amino acid sequences of *C. gloeosporioides* f. sp. *aeschynomene* (*Cga*) TUB2 and TUB1 (2), *C. graminicola* (*Cg*) TUB2 (15), and *N. crassa* (*Nc*) tub-2 (13) βTubs. Amino acids identical to those in the *C. gloeosporioides* f. sp. *aeschynomene* TUB2 sequence were omitted, and dashes were introduced to maintain maximum sequence homology.

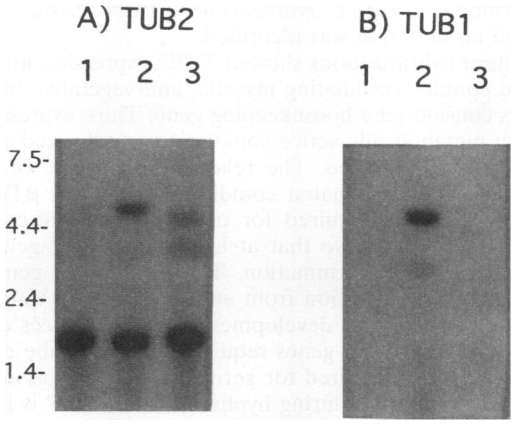

FIG. 4. Northern hybridizations of RNAs isolated from *C. gloeosporioides* f. sp. *aeschynomene* conidia (lane 1), conidiating mycelia (lane 2), and vegetative mycelia (lane 3) probed with pCGAWTTUB2 (panel A) and pNPWTTUB1 (2) (panel B). The numbers to the left of panel A are molecular sizes in kilobases.

Sequence alignment of *C. gloeosporioides* f. sp. *aeschynomene TUB2* revealed six introns in positions identical to those in *C. graminicola TUB2* and *N. crassa tub-2*. All intron-exon borders contain sequences conserved among other reported filamentous fungal introns (1). Furthermore, all introns in *C. gloeosporioides* f. sp. *aeschynomene TUB2* contain internal consensus sequences (Fig. 2) found in other fungal introns (13). Splicing of the intervening sequences produced a single 1,341-nucleotide (447-amino-acid) open reading frame.

Alignment of the deduced amino acid sequence of the *C. gloeosporioides* f. sp. *aeschynomene* TUB2 protein (Fig. 3) revealed 78.7% amino acid identity to the *C. gloeosporioides* f. sp. *aeschynomene* TUB1 protein (2), 99.3% amino acid identity to the *C. graminicola* TUB2 protein (15), and 96.6% amino acid identity to the *N. crassa* tub-2 protein (13).

The coding region of *C. gloeosporioides* f. sp. *aeschynomene TUB2* has a G+C content of 59.2% and shows biased codon usage. Only 42 of 61 sense codons are used in *C. gloeosporioides* f. sp. *aeschynomene TUB2*, with 78.3% of the codons ending in G or C. Codon bias in *C. gloeosporioides* f. sp. *aeschynomene TUB2* is similar to that of *C. graminicola TUB2* (41 codons) and *N. crassa tub-2* (42 codons) but is much more pronounced than the codon bias of *C. gloeosporioides* f. sp. *aeschynomene TUB1* (57 codons).

Detection of a BEN-resistant mutation. PCR was employed to generate 1.65-kb fragments (nucleotides 60 to 1714 of *C. gloeosporioides* f. sp. *aeschynomene TUB2* [Fig. 2]) by using DNA from pathogenic, BEN-resistant strain B-21 as the template. This region of the gene was amplified because every identified mutation which confers BEN resistance in a plant pathogen is found within this region of β-tubulin (9, 22). Fragments were digested with *Bam*HI-*Xho*I and cloned. Four fragments from two separate reactions were cloned. A sequence was obtained for each of the four clones for nucleotides 777 to 1077 (amino acids 113 to 212). Each clone contained a single mutation, a G-to-A transition at nucleotide 1032, resulting in a lysine substitution for glutamic acid at amino acid 198.

Northern analysis. RNA was isolated from *C. gloeosporioides* f. sp. *aeschynomene* conidia, conidiating mycelia, and vegetative mycelia. RNA was separated on agarose gels, transferred to nylon, and probed with radiolabeled pCGAWTTUB2 (Fig. 1) or pNPWTTUB1 (2), which contains the complete *C. gloeosporioides* f. sp. *aeschynomene TUB1* gene (Fig. 4). *TUB2* was expressed in all three cell types. Autoradiographs exposed for shorter lengths of time indicated that *TUB2* was expressed at the highest levels in vegetative mycelia and at the lowest levels in conidia, although the difference in expression among cell types was not significant (data not shown). *TUB1* appeared to be expressed only in conidiating mycelia (Fig. 4B, lane 2). The *TUB2* transcript was approximately 1.8 kb long (Fig. 4A), while the *TUB1* transcript was approximately 4.8 kb long (Fig. 4B). Running RNA on denaturing formaldehyde gels and then subjecting it to Northern hybridization did not resolve the extra faint signals and revealed transcript sizes identical to those shown in Fig. 4 (data not shown).

DISCUSSION

A second βTub-encoding gene from *C. gloeosporioides* f. sp. *aeschynomene* was cloned. This gene shows a high degree of identity to conserved βTub genes characterized in other filamentous fungi, including *N. crassa tub-2* (13) and *C. graminicola TUB2* (15). Genomic Southern hybridizations using the *C. gloeosporioides* f. sp. *aeschynomene TUB2* gene as the probe (data not shown) were identical to those using *C. graminicola TUB2* as the probe (2).

Pathogenic, BEN-resistant strain B-21 possesses an altered *TUB2* allele which codes for lysine instead of glutamic acid at amino acid 198. Mutations that confer BEN resistance have been identified in *TUB2* homologs from several fungi. *TUB2* mutations that produce an amino acid (lysine, alanine, or valine) substitution for glutamic acid at amino acid 198 have been identified in highly BEN-resistant isolates of *Venturia inaequalis*, *Monilinia fructicola*, and *Penicillium expansum* (9). Lysine and alanine substitutions for glutamic acid at amino acid 198 were reported for two *TUB2* alleles from BEN-resistant field isolates of *Botrytis cinerea* (22). Substitutions for glutamic acid at amino acid 198 have been identified in three *TUB2* alleles from BEN-resistant isolates of *A. nidulans* (8). Furthermore, a *TUB2* allele with a glycine substitution for glutamic acid at amino acid 198 has been shown to confer BEN resistance in *N. crassa* (7). Thus, the molecular lesion identified in the *TUB2* allele from a BEN-resistant *C. gloeosporioides* f. sp. *aeschynomene* isolate likely confers BEN resistance. Identification of a mutation which likely confers BEN resistance is relevant for developing a gene transfer system in *C. gloeosporioides* f. sp. *aeschynomene*. Interestingly, a divergent βTub gene, *TUB1*, from the closely related fungus *C. graminicola*

transformed wild-type *C. graminicola* to BEN resistance (16), although no mutation was identified.

Northern hybridizations showed *TUB2* expression in ungerminated conidia, conidiating mycelia, and vegetative mycelia. *TUB2* is considered a housekeeping gene. Thus, expression of *TUB2* in metabolically active conidiating mycelia and vegetative mycelia is expected. The relatively high level of *TUB2* transcripts in ungerminated conidia suggests that βTub encoded by *TUB2* is required for or immediately after spore germination. We believe that at least two sets of genes are required for spore germination. The first set of genes are required for the transition from an ungerminated spore to a germinated spore. This developmental switch induces expression of a second set of genes required for germ tube elongation. Many genes required for germ tube elongation are also presumably expressed during hyphal growth. *TUB2* is likely a housekeeping gene that is expressed in vegetative mycelia, and such hyphae, especially hyphal tips, are similar, if not physiologically identical, to germ tubes. Thus, *TUB2* transcripts in ungerminated conidia are likely present for germ tube elongation.

Production of RNA transcripts is metabolically expensive. Fungal spores contain a finite amount of energy and catabolic sources for basic building molecules such as ribonucleotides. Transcripts in conidia are likely generated at the expense of conidiating mycelia during conidial development. Furthermore, conidial germination often occurs in the absence of exogenous nutrients, especially for many foliar plant pathogens which germinate on leaf surfaces. Thus, conidia containing transcripts would be more efficacious than conidia produced without transcripts.

Transcripts of the *cot-1* gene from *N. crassa* are also present in ungerminated conidia of *N. crassa* (23). Because *cot-1* encodes a kinase required for hyphal tip elongation, it is unlikely that the *cot-1*-encoded protein needs to be present to maintain viability of ungerminated conidia. These data strongly suggest that ungerminated conidia contain transcripts which encode proteins requiring rapid de novo synthesis during spore germination. This phenomenon is apparently not unique to *C. gloeosporioides* f. sp. *aeschynomene TUB2* or *N. crassa cot-1*. For example, transcripts of the *HTS-1* gene encoding HC toxin synthetase are present in ungerminated conidia of *Cochliobolus carbonum* (4a). Furthermore, transcripts of three *C. trifolii* genes, i.e., those that encode calmodulin and two kinases, are present in ungerminated conidia of *C. trifolii* (2a). The temporal expression of these genes may facilitate establishment of a fungus in its environmental niche. The temporal expression of specific genes is likely very important for establishment of pathogens on hosts.

Transcripts of the divergent βTub-encoding gene, *TUB1*, from *C. gloeosporioides* f. sp. *aeschynomene* were detected in conidiating mycelia but not ungerminated conidia or vegetative mycelia. Thus, the *TUB1*-encoded protein appears to be involved in conidial development. The divergent βTub-encoding gene, *tubC*, from *Aspergillus nidulans* also appears to participate in conidial development in *A. nidulans* (10). However, the divergent βTub gene, *TUB1*, from *C. graminicola* showed similar levels of expression in conidiating and nonconidiating cultures of *C. graminicola* (15). Thus, the *C. gloeosporioides* f. sp. *aeschynomene TUB1* gene shows novel expression relative to *C. graminicola TUB1* because it is expressed only in conidiating mycelia.

The *C. gloeosporioides* f. sp. *aeschynomene TUB2* transcripts were approximately 1.8 kb long, which correlates well to the 1,341-nucleotide coding sequence of *TUB2*. However, the estimated transcript size of 4.8 kb for *C. gloeosporioides* f. sp. *aeschynomene TUB1* is much greater than the 1,338-nucleotide coding sequence for this gene. It is possible that *TUB1* transcripts contain a secondary structure which alters their migration through agarose gels. However, use of denaturing formaldehyde gels followed by Northern hybridization did not alter the apparent transcript size of *TUB1*. Furthermore, *TUB1* shows nearly 70% identity to *TUB2*, which migrated as expected through agarose gels. Thus, the large size of *TUB1* transcripts is difficult to explain. This may reflect unique sequences flanking the *TUB1* coding sequence. Alternatively, *TUB1* may be transcribed as part of a polycistronic precursor mRNA as shown for α-Tub and β-Tub genes from trypanosomes (11). Importantly, *C. gloeosporioides* f. sp. *aeschynomene TUB1* is expressed as a large transcript in conidiating mycelia of *C. gloeosporioides* f. sp. *aeschynomene*, whereas *C. graminicola TUB1* is expressed as a normally sized transcript in conidiating and vegetative mycelia of *C. graminicola*. These data represent fundamental differences between the tubulin genes in these related fungal plant pathogens.

ACKNOWLEDGMENTS

We thank Cindy Stryker for technical assistance.

This research was funded in part by the Leva B. and Elda R. Walker Fund, the U. S. Harkson Fund, and the DOE/NSF/USDA Joint Program in Collaborative Research in Plant Biology (grant 92-37310-7821).

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