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Expression of the *Escherichia coli* β-Glucuronidase Gene in *Pseudocercosporella herpotrichoides*

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The plant-pathogenic fungus *Pseudocercosporella herpotrichoides* has been successfully transformed by using two different positive selection systems in combination with the *Escherichia coli gusA* gene. The selectable markers used in this study were the hygromycin B phosphotransferase gene (*hph*) from *E. coli* and the gene (*bml*) for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa*. A lower transformation rate was obtained with the *bml* system than with the *hph* system. Conversely, cotransformation frequencies, as determined with medium plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, were higher with *bml* than with *hph* as the selectable marker. The hygromycin-resistant transformants were mitotically stable, and both the selectable gene and *gusA* were maintained through conidiation. The vector DNA was integrated into the genome, and the number and sites of insertion varied among transformants. Enzyme assays of mycelial extracts showed that β-glucuronidase activity was highest in transformants with a high *gusA* copy number. Expression of *gusA* during growth of the fungus on plants was easily detectable and did not affect pathogenicity. These results form the basis for construction of a versatile and sensitive reporter gene system for *P. herpotrichoides*.

Pseudocercosporella herpotrichoides (Fron) Deighton is the causative agent of eyespot disease, an economically important disease of wheat, rye, and barley (4). The teleomorph, *Tapesia yallundae*, has only recently been identified (14, 17), and the use of classical genetics in the study of this fungus is still in the early stages. Hence, the application of molecular genetic techniques would facilitate the study of several aspects of the biology of this fungus.

Genetic transformation, a powerful molecular genetic tool, has already been done with *P. herpotrichoides* (1). The use of gene fusions has proven to be a valuable tool for the analysis of gene structure and function in many organisms. In particular, the gene fusion system based on the *Escherichia coli gusA* gene has been developed as an effective reporter gene system for bacteria, animals, plants, and, most recently, fungi (6, 7, 12, 15). The numerous assays of the β-glucuronidase (GUS) system that allow rapid and sensitive activity measurements by fluorimetry, by spectrometry, in medium plate tests, and in tissue sections make it an excellent reporter system for gene fusion analysis or for monitoring growth and development of an organism.

The objective of this study was to develop "tagged" fungal strains, by using the GUS gene fusion system, for use in genetic analysis of this fungus and in studies directed toward understanding pathogenesis. Here we report the successful transformation of *P. herpotrichoides* with the *gusA* gene and show that GUS expression does not have deleterious effects on the fungus or its pathogenicity.

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MATERIALS AND METHODS

Strains and media. *P. herpotrichoides* was from the Monsanto culture collection and was originally collected in Washington state. The fungus was maintained on MYG medium (5.0 g of malt extract, 2.5 g of yeast extract, 10.0 g of glucose, and 15.0 g of agar per liter) (1). Protoplasts were plated on protoplast regeneration medium (PRM) (Sigma Chemical Co., St. Louis, Mo.), which contains the following (in grams per liter): sucrose, 10.0; $NaNO_3$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; L-asparagine, 2.0; thiamine, 0.002; sorbitol, 219.0; and agar, 10.0. For selective media, hygromycin B (Calbiochem Corp., San Diego, Calif.) was included at 25 μg/ml and benomyl (Du Pont Co., Wilmington, Del.) was included at 1 μg/ml (from a stock solution of 1 mg/ml in ethanol) in the media. 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) and 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sigma) were included in the agar media at 25 μg/ml. For sporulation, mycelial plugs were placed on water agar plates (1.0% agar) and the plates were held at 11°C with 10 h of near-UV light per day. Conidia developed in 4 to 8 days.

Vectors. Plasmid pMP10 contains the *Cla*I-to-*Sph*I fragment from pDH25 (2) cloned into the *Sma*I site of pUC118. The plasmid carries the hygromycin B phosphotransferase gene (*hph*) from *E. coli* with the *trpC* terminator fragment from *Aspergillus nidulans* downstream. There is no fungal regulatory sequence upstream of *hph* in this construct (10). Plasmid pBT3 carries the gene (*bml*) for β-tubulin, along with its flanking 5′ and 3′ regions, from a benomyl-resistant mutant of *Neurospora crassa* (8). Plasmid pNOM102 carries the GUS gene (*gusA*) from *E. coli* flanked by the *gpd* promoter fragment from *A. nidulans* upstream and the *trpC* terminator fragment downstream (12).

Protoplast formation and transformation. Protoplasts were prepared as described by Stanway and Buck (16) with some modifications. Approximately 3×10^5 conidia from a 7-day-old spore plate culture were added to 250 ml of half-strength potato dextrose broth (Difco Laboratories, Detroit, Mich.). The culture was grown at 23°C with shaking (100 rpm) for 60 to 72 h. The mycelium was harvested by filtering through miracloth (Calbiochem) with suction and rinsed with stabilizing buffer (0.2 M NaH_2PO_4 [pH 5.8], 0.6 M KCl). The mycelium (ca. 5 g) was resuspended in 50 ml of stabilizing

buffer containing 5 mg of lysing enzymes (Sigma, catalog no. L2265) and 5 mg of cellulase (Sigma, catalog no. C0901) per ml and incubated at 22 to 24°C for 4 h with gentle shaking. Protoplasts were collected by filtration through a glass wool plug to remove mycelial fragments and pelleted by centrifugation (5 min at 1,500 × *g*). The protoplasts were washed twice with stabilizing buffer and finally resuspended in 2 to 3 ml of transformation buffer (25 mM $CaCl_2$, 25 mM Tris [pH 7.6], 1 M sorbitol).

The transformation procedure of Wang et al. (18) was used with the additional modifications described by Pilgeram and Henson (9). The protoplast concentration was adjusted to 5 × 10^8 protoplasts per ml. Plasmid vector DNA (10 μg each of pNOM102 and either pMP10 or pBT3) was added to 200 μl of protoplasts. The protoplasts and plasmids were mixed, and a polyethylene glycol-$CaCl_2$ solution was added as described by Wang et al. (18). After protoplasts were collected by centrifugation, they were immediately resuspended in 3 ml of PRM broth with no selection and incubated with gentle shaking overnight at 20°C. Treated protoplasts were pelleted by spinning at 1,000 × *g* for 5 min and then resuspended in 1 ml of PRM broth, and 200-μl aliquots were added to 4 ml of molten PRM top agar (0.8% agar) containing the appropriate selective antibiotic. The top agar was spread onto PRM plates containing selective antibiotics. The plates were incubated at 19°C, and transformants could be detected within 10 days.

DNA isolation and Southern hybridization. DNA was isolated from mycelium grown by inoculating approximately 10^5 conidia into 75 ml of half-strength PDB and incubating with shaking (100 rpm) at 22 to 24°C for 3 to 4 days. The mycelium was collected by filtration, and total DNA was extracted by the protocol of Dellaporta et al. (3). DNA samples were digested with restriction enzymes (Promega Corp., Madison, Wis.) according to the manufacturers' directions. Electrophoresis, blotting, and hybridization were done according to standard procedures (13). Each lane of agarose gels contained 1 to 2 μg of fungal genomic DNA. The DNA was blotted to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.). Radioactive probes were labeled by using the hexamer labeling kit from Pharmacia (Piscataway, N.J.), and hybridization was in 50% formamide at 42°C.

Protein extraction and GUS activity analysis. Protein was extracted from mycelium, grown as described above, by the extraction protocols of Jefferson (5). Mycelium was frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. The powder was transferred to a centrifuge tube, and GUS extraction buffer was added (1 ml of buffer per g [fresh weight] of mycelium). The buffer and mycelium were thawed and mixed thoroughly, and the tubes were spun at 12,000 × *g* for 10 min. The supernatant was filtered through a glass wool plug into a sterile 50-ml tube, and extracts were stored at −80°C. GUS activity was assayed quantitatively by fluorimetry with MUG as a substrate according to the procedure of Jefferson (5). One unit of GUS activity was defined as the amount of enzyme producing one nanomole of 4-methylumbelliferrone (MU) min^{-1} mg of $protein^{-1}$ at 37°C. Protein was estimated with the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.). GUS was measured qualitatively either by fluorescence with transilluminator or by the presence of blue on medium plates containing X-Gluc.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 10 to 20% polyacrylamide gradient gels (Integrated Separation Systems, Hyde Park, Mass.). The proteins were blotted onto Immobilon-P polyvinylidene difluoride paper (Millipore Corp., Bedford, Mass.) by using a dry transfer system as described by Sambrook et al. (13). Detection of GUS protein on the solid support was performed by using rabbit anti-GUS serum as the primary antibody and goat anti-rabbit alkaline phosphatase conjugate as the secondary antibody. Both antibodies were purchased from CLONTECH Laboratories (Palo Alto, Calif.), and detection of GUS protein on polyvinylidene difluoride paper was done according to the manufacturers' directions.

Pathogenicity assays. Transformants were tested for pathogenicity on 10-day-old wheat (cv. Hart) seedlings. Inoculations were made by spraying the base of the stems of 90 seedlings with 5 ml of spore suspensions (200,000 conidia per ml) of the transformants. After being sprayed, the stems were covered with moist vermiculite and the plants were maintained at 14 to 15°C and 85% humidity with a 12-h exposure to light. Ten days after inoculation, six plants from each treatment were harvested for GUS expression studies. GUS expression was measured qualitatively by incubating inoculated wheat stem sections in GUS extraction buffer containing 1 mM MUG overnight at 37°C. Fluorescence was visualized by placing the tubes containing the stems on a UV transilluminator. The remaining plants were rated for disease after 5 weeks.

RESULTS

The wild-type *P. herpotrichoides* strain was inhibited by hygromycin B (25 μg/ml) and benomyl (1 μg/ml). *P. herpotrichoides* was cotransformed with pMP10 and pNOM102 to obtain hygromycin B-resistant (Hyg^r) strains and with pBT3 and pNOM102 to obtain benomyl-resistant (Ben^r) strains. Transformation frequencies ranged from 2 to 20 transformants per μg of vector DNA with the Hyg^r selection system and from 1 to 3 transformants per μg of vector DNA with the Ben^r selection system. Both systems gave few if any background colonies on control plates. The cotransformation frequency, as measured by GUS expression on X-Gluc medium plates (GUS^+), was 38 to 51% for pMP10-derived transformants and 80 to 92% for pBT3-derived transformants. The pMP10-derived transformants are assumed to result from the insertion of the promoterless *hph* gene near a regulatory element, with expression resulting from this position effect. Other investigators routinely select for Hyg^r transformants by using hygromycin B levels of 100 to 300 μg/ml (10); the low selection pressure used in this study allowed for selection of transformants that would have weak expression attributable to a position effect.

Initial colonies growing on PRM selection plates were transferred to MYG medium plates containing selective antibiotics. Approximately 10% of these colonies failed to grow upon subsequent transfer. The transformants that grew after initial transfer were stable and maintained hygromycin or benomyl resistance through several transfers on nonselective medium. The pBT3-derived transformants exhibited a very slow growth rate and altered morphology on both selective and nonselective media as compared with the wild-type strain and were not further characterized.

Total genomic DNA was isolated from wild-type *P. herpotrichoides*, the P84-control strain (derived from protoplasts treated with polyethylene glycol but no vector DNA), and four Hyg^r transformants. Three of these transformants (P84-7, P84-8, and P84-18) exhibited a GUS^+ phenotype on MYG–X-Gluc plates (data not shown). Uncut DNA was probed separately with a DNA fragment containing the *hph*

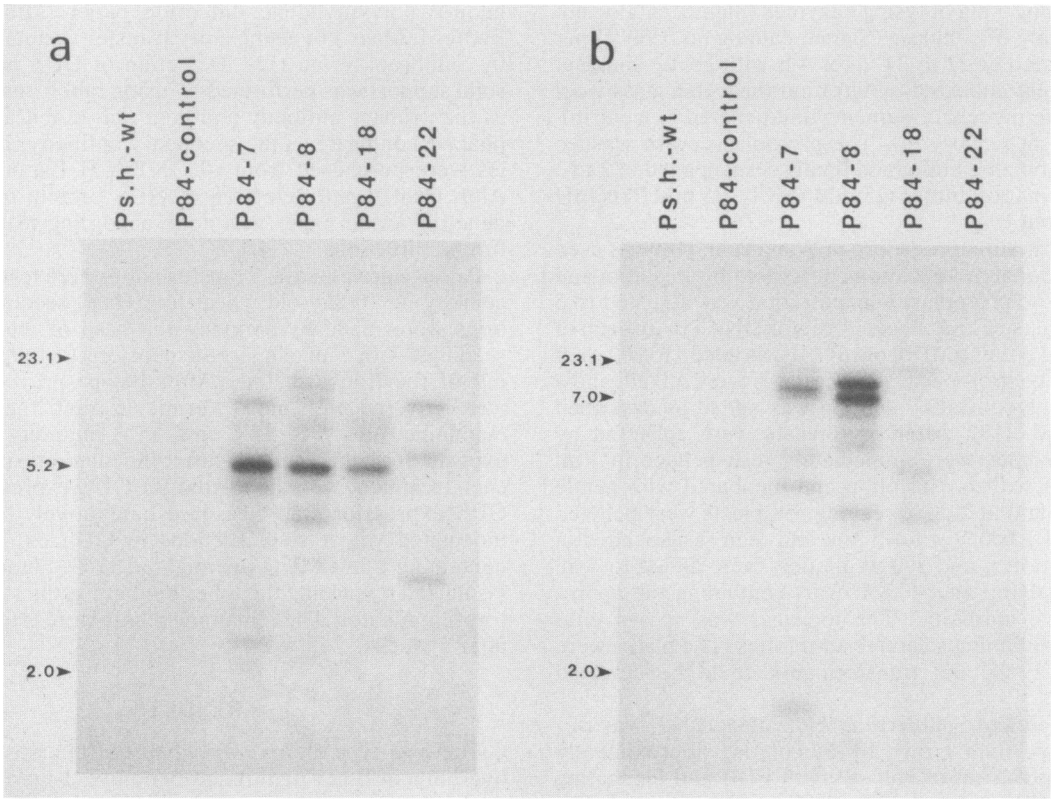

FIG. 1. Southern blot of DNA from wild-type *P. herpotrichoides* (Ps.h.-wt), the control strain, and Hyg^r transformants. The DNA was digested with *Hin*dIII, an enzyme that cuts once within pMP10 and pNOM102 but does not cut within either *hph* or *gusA*. (a) Blot hybridized with the labeled *hph* gene fragment from pMP10; (b) blot hybridized with the labeled *gusA* gene fragment from pNOM102. Numbers at left indicate sizes in kilobases.

gene from pMP10 or the *gusA* gene from pNOM102. Homology with the *hph* gene was present only in the DNA from the four Hyg^r transformants, and homology with *gusA* was present only in the DNA from transformants exhibiting the GUS$^+$ phenotype (data not shown). This homology was restricted to high-molecular-weight DNA, suggesting that the vector integrated into the genome of each transformant and was not replicating autonomously.

To determine the number of sites of integrated pMP10 and pNOM102 in the transformants, genomic DNA was digested with *Hin*dIII, a restriction enzyme that linearizes each plasmid, and hybridized separately with the ^{32}P-labeled *hph* fragment from pMP10 and the ^{32}P-labeled *gusA* fragment from pNOM102. All the transformants showed multiple insertions of the hph gene (Fig. 1a). The sites of integration varied among the transformants as evidenced by the different sizes of homologous fragments. The three GUS$^+$ transformants also showed multiple insertions of the *gusA* gene (Fig. 1b).

Expression of the *gusA* gene at the translational level in transformants and in untransformed *P. herpotrichoides* and the P84-control strain was characterized by immunodetection techniques and by fluorometric measurements of enzyme activity. When protein preparations were fractionated by SDS-PAGE, specific antibodies raised against GUS detected enzyme only in the transformants expressing the GUS$^+$ phenotype (Fig. 2). The protein detected had the same molecular weight as the purified bacterial GUS (purchased from Sigma), indicating that a full-length, stable

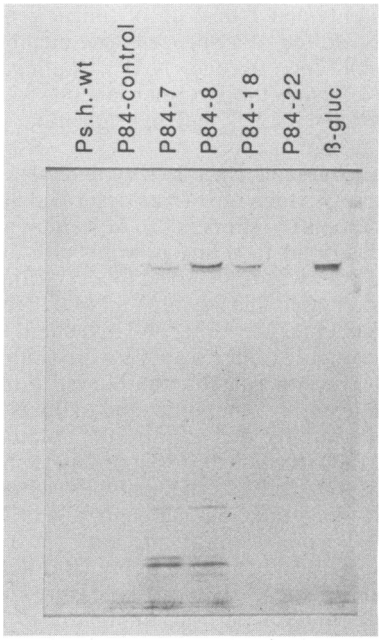

FIG. 2. Western blot of total protein from mycelial extracts of control *P. herpotrichoides*, wild-type *P. herpotrichoides* (Ps.h.-wt), and transformants. Purified bacterial GUS (β-gluc) (Sigma) was used as a control marker. The blot was probed with antibodies raised against GUS.

TABLE 1. GUS activity in extracts of untransformed *P. herpotrichoides* and transformants

Isolate	Phenotype	GUS activity[a]
Wild type	Hyg^s GUS^-	<0.005
P84-control	Hyg^s GUS^-	<0.005
P84-7	Hyg^r GUS^+	1,821
P84-8	Hyg^r GUS^+	7,066
P84-18	Hyg^r GUS^+	393
P84-22	Hyg^r GUS^-	<0.005

[a] Expressed as nanomoles of MU per minute per milligram of protein. Values are averages of four replicates.

protein is made in these transformants. In the fluorometric assay, there was no significant GUS activity detected in the wild-type strain, the P84-control strain, or P84-22, a Hyg^r GUS^- transformant (Table 1). The Hyg^r GUS^+ transformants exhibited high GUS activity levels (10^2 to 10^3-fold higher than the minimal detectable limits) and showed complete correlation between enzyme activity and the qualitative MYG–X-Gluc plate assays.

Transformants expressing GUS on MYG–X-Gluc plates have a normal growth rate and morphology as compared with the untransformed wild-type strain. In pathogenicity assays all four transformants gave symptoms on wheat that were indistinguishable from those of the wild-type control (data not shown). When infected wheat stems were incubated in GUS extraction buffer containing 1 mM MUG and assayed for MU fluorescence with a UV transilluminator, the Hyg^r GUS^+ transformants still expressed GUS during growth in plant tissues (Fig. 3). Only weak background fluorescence was seen in the uninoculated controls and in plants inoculated with the wild-type strain or with P84-22, the Hyg^r GUS^- transformant. The amount of infection or fungal growth on the wheat stems was not standardized, so the amount of fluorescence seen in vivo is not directly comparable to the quantitative in vitro enzyme activity data.

DISCUSSION

Cotransformation, or transformation with genes not selected for directly but added as additional plasmid DNA along with plasmid DNA carrying a selectable gene (i.e., antibiotic resistance), is well established for filamentous fungi (9, 11, 12). The results presented here indicate that it is also possible to cotransform *P. herpotrichoides*. The high cotransformation frequencies reported here are similar to those found for other filamentous fungi. This suggests that cotransformation of a wide variety of plasmids may be possible with *P. herpotrichoides*.

The *hph* gene proved to be an effective selectable marker, and the same inconsistent, aberrant growth of the Ben^r transformants as previously reported by Blakemore et al. (1) was observed. The *gusA* gene was integrated into the genome of the fungal transformants and was mitotically stable, being maintained through conidiation. Multiple copies of both the selectable *hph* gene and the nonselected *gusA* gene were inserted in all the cotransformants tested. The variation in integration sites seen among the transformants indicates that both of these genes inserted at random sites. The Southern blot probed with *hph* DNA (Fig. 1a) showed an intense hybridizing band of approximately 5.2 kb, and the blot probed with *gusA* DNA (Fig. 1b) had an intense hybridizing band at 7.4 kb, the same sizes as the respective vectors, pMP10 and pNOM102, in the DNA of some of the transformants. These bands may result from several insertions occurring in tandem, which may be due to homologous recombination of the circular vector DNA before integration into the genome.

The cotransformants all showed high levels of GUS activity, and the levels are similar to those expressed by transgenic *Aspergillus* spp. transformed with the same vector (12). Activity in *P. herpotrichoides* transformants may be correlated with the copy number of the *gusA* gene. Cotransformant P84-8 had the *gusA* highest copy number as shown by Southern blot analysis (Fig. 1b). P84-8 also had the highest level of GUS activity in the fluorometric assay (Table 1) and gave the most intensely staining band on the Western blot (immunoblot) (Fig. 2).

Cotransformants expressing GUS on MYG–X-Gluc plates exhibited normal growth and morphology as compared with the wild type. Expression of GUS also had no discernible effect on pathogenicity; all the cotransformants tested were as pathogenic as the wild type in the assay used. The presence of the GUS-producing transformants on the wheat stems could be detected by a simple fluorescence assay, as shown in Fig. 3, well before symptoms developed. Since *P. herpotrichoides* is a relatively slowly growing fungus, GUS^+ transformants may be useful in studies when rapid detection is essential.

The GUS gene fusion system has been developed as a useful tool in molecular biology. The utility of this system has been shown for *P. herpotrichoides*, and its potential use as a reporter gene has many applications in plant pathology. Tagged fungi can be used to study plant-pathogen interactions, in epidemiological studies, and for rapid screening of fungal resistance in plant breeding efforts.

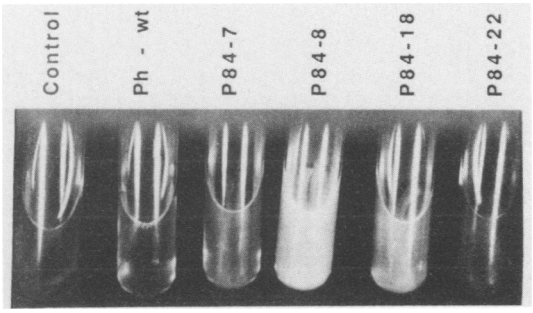

FIG. 3. MU fluorescence from wheat stems 10 days after inoculation with *P. herpotrichoides* and transformants. Fluorescence was visualized with a UV transilluminator.

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