Transient Expression of the β-Glucuronidase Gene Introduced into *Uromyces appendiculatus* Uredospores by Particle Bombardment

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ABSTRACT

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Plasmids carrying the β-glucuronidase gene (GUS) under the control of the promoter from the previously cloned gene specific for infection structure, INF24, were constructed by inserting the INF24 promoter in front of the GUS gene carried in the Bluescript vector. These plasmid DNAs were then introduced into uredospores of the bean rust fungus, *Uromyces appendiculatus*; the biolistic particle delivery system was used. With germination and differentiation on collodion membranes, GUS activity assayed histochemically with X-gluc was evident only in those germlings that were bombarded with the plasmid containing the entire promoter region; it was not evident in those that were mock-bombarded or bombarded with the plasmid lacking the promoter.

Additional keywords: biolistic transformation, gene-gun.

Uredospore germlings of the obligately parasitic rust fungus, *Uromyces appendiculatus* (Pers.:Pers.) Unger, develop infection structures on the bean plant, *Phaseolus vulgaris* L., to infect its host. The appressorium forms when the germ tube contacts the lip of the stomatal guard cell (14) or a simple 0.5-μm ridge on a plastic surface (4). Differentiation of the infection structures also is induced during germination on oil-collodion membranes (9). To decipher the molecular steps of the signal transduction pathway, we wish to identify protein factors that bind to specific sequences in the promoter region of the gene in regulating gene expression.

Traditional methods for identifying *cis*-acting elements in fungal genes have employed protoplast-based transformation techniques (5,12). Here, we introduced DNA into uredospores by using the high-velocity microprojectile technique (1,7,8). We report, for the first time, the use of this procedure for introducing promoter-GUS constructs into uredospores of *U. appendiculatus*. The GUS gene was expressed in germ tubes, appressoria, and vesicles.

MATERIALS AND METHODS

Spores. Uredospores of *U. appendiculatus* were used in all the experiments described in this paper. Their collection, germination, and differentiation were previously described (3).

Plasmid constructions. The plasmid pBI121 containing the GUS gene from *Escherichia coli* was obtained from CLONTECH Laboratories, Inc., Palo Alto, CA. The 2.0-kb *Bam*HI-*Eco*RI fragment making up the coding region of the GUS gene was subcloned into the *Bam*HI-*Eco*RI region of INF24, thus replacing the coding region and the 3′ noncoding region of INF24 (3). The resulting plasmid, pL48A1, is shown in Figure 1.

A second plasmid, pLB12, was constructed by deleting the *Bam*HI-*Spe*I region of pL48A1 (Fig. 1). This was accomplished by digesting pL48A1 with *Bam*HI and *Spe*I and by intramolecular ligation of the free ends after a filling-in reaction with Klenow enzyme in the presence of all four deoxynucleotide triphosphates.

Particle bombardment. The original design of Du Pont's biolistic particle delivery system (PDS1000) has been described (8). The current model of the gun that we used in our studies (PDS1000/He) is essentially the same except that helium at high pressure is used in place of gun powder as a propellant (1,10,11). The M10 tungsten particles (Sylvania, GTE Products Corp., Towanda, PA) had a mean diameter of 1.1 μm and were coated with DNA essentially as described (8). Briefly, 3.0 mg of M10 tungsten particles suspended in 100 μl of water were mixed with 10 μg of plasmid DNA (2.5 μg/ml) on ice. After the addition of 100 μl of cold 2.5 M $CaCl_2$, the mixture was vortexed, 40 μl of cold 0.1 M spermidine was added, and the particle-DNA mixture was vortexed again at 4 C for 15 min. The particles were sedimented at room temperature in a microcentrifuge for

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5 s, washed once with water, once with 70% ethanol, and once with absolute ethanol. Finally, the particles were suspended in 75 μl of absolute ethanol and sonicated in a sonicator water-bath (Branson 100) for 5 s. Six microliters of coated particles was spread on a Kapton flying-disc (10) and used for bombardment. The particles were propelled towards the uredospores by release of helium at pressures of 31,050 and 62,100 kdyn/cm^2 (450 and 900 psi).

Before DNA was introduced into uredospores, spores treated for 20 min with β-ionone vapors to reduce self-inhibition (2) were dusted onto oil-collodion membranes prepared in 10-cm glass petri dishes and stored in a moist chamber at 4 C until bombardment (about 90 min). By this time, the spores had hydrated but not germinated. For transformation, a petri dish was placed into the chamber of the particle delivery system at a distance of 14.0 cm from the launch site. The spores were bombarded once or twice with M10 tungsten particles coated with one of the plasmid DNAs. After bombardment, spores were allowed to germinate and differentiate, and appressoria and vesicles formed. Development was stopped by pipetting about 5 ml of GUS staining solution (see below) onto the membranes.

Histochemical staining. To prepare the chromogenic GUS staining solution, a 1-ml aliquot of a 50-mg/ml stock solution of X-gluc (Jersey Lab Supply, Irvington, NJ) dissolved in N,N′-dimethylformamide or dimethyl sulfoxide was added to 100 ml of buffer (0.05 M sodium phosphate buffer, pH 7.4, which contained 1 mM EDTA, 0.1% Triton X-100, and 0.1 mM $K_3Fe[CN]_6$). The solution was pipetted onto the membrane, the petri dishes were incubated at 37 C for 1–2 h, then returned to room temperature for 2–3 days in a moist chamber to minimize evaporation. We observed germlings for staining by using a light microscope, and we photographed them by using Kodacolor Gold 100 film with a blue filter.

RESULTS AND DISCUSSION

As part of our long-term goal to stably transform the rust fungus, we undertook studies to determine if the high velocity microprojectile technique could be used to introduce foreign DNA into uredospores of the bean rust fungus. To visualize reception of DNA by the spores and to assess the ability of rust uredospores to express the foreign DNA, we employed the GUS transient expression assay. In this assay, the spores were allowed to germinate for up to 10 h after introduction of the DNA. During this time, the DNA is transcribed into mRNA, which in turn is translated to yield the enzyme product for assay by histochemical staining. The foreign DNA is probably not stably integrated into the chromosome of the rust fungus, but no attempts were made to recover spores that might have been stably transformed. Hence, we use the terms "transient expression" or "transient transformation" throughout this study.

Plasmid constructs pL48A1 and pLB12 (Fig. 1) were used to transform uredospores of *U. appendiculatus*. High velocity microprojectiles coated with one of the plasmid DNAs were used to bombard nongerminated uredospores that had been dusted onto oil-collodion membranes. After bombardment, spores were allowed to germinate and differentiate; subsequently, the germlings were stained for GUS activity as described above. The GUS activity was present in appressoria (Fig. 2A), vesicles (Fig. 2B–D), and germlings (Fig. 2F,G). Only those bombardments that employed particles coated with pL48A1 produced GUS-stained germlings; control bombardments with uncoated particles or particles coated with the plasmid pLB12 that lacked the promoter did not give rise to germlings that expressed the GUS gene (Fig. 2E).

Because INF24 is expressed at low levels in undifferentiated germlings (2,3), it was not surprising to find GUS activity in germ tubes and spores. Why some spores stained more intensely than others was not determined. It is possible that such spores received more copies of the GUS gene because more than one DNA-coated particle entered the spore. Multiple entry is a fairly frequent event in experiments of particle bombardment (1,7).

In general, most of the GUS-stained spores or germlings were concentrated around the center of the plate, and the numbers decreased radially. Conceptually this is expected because the maximum thrust of the pressurized helium gas is at the center and falls off towards the edge of the plate. Although the photographs (Fig. 2F,G) were chosen to show the number of stained and unstained spores, we observed as few as a single spore, germ tube, or appressorium among the many unstained spores. In contrast to the particle gun that employed gun powder to dispel the particles, the helium-based particle gun yielded predictably heavier distributions of stained spores in the center (S. M. Bhairi and R. C. Staples, *unpublished data*).

We examined the effect of multiple bombardments on the transformation efficiency, because increasing the probability of collisions between particles and spores should improve the chances of a spore receiving DNA. Although multiple bombardments did appear to improve the efficiency of transient expression at helium pressures of both 31,050 and 62,100 kdyn/cm^2, the data (not shown) were quite variable. Because a number of parameters, including the ratio of DNA to the number of particles, particle diameter, and the distance between the launch site and sample, affect the efficiency of transformation (1,10,11), there probably is room for optimizing our system to obtain consistently better efficiency of transient expression. M5 tungsten particles (mean

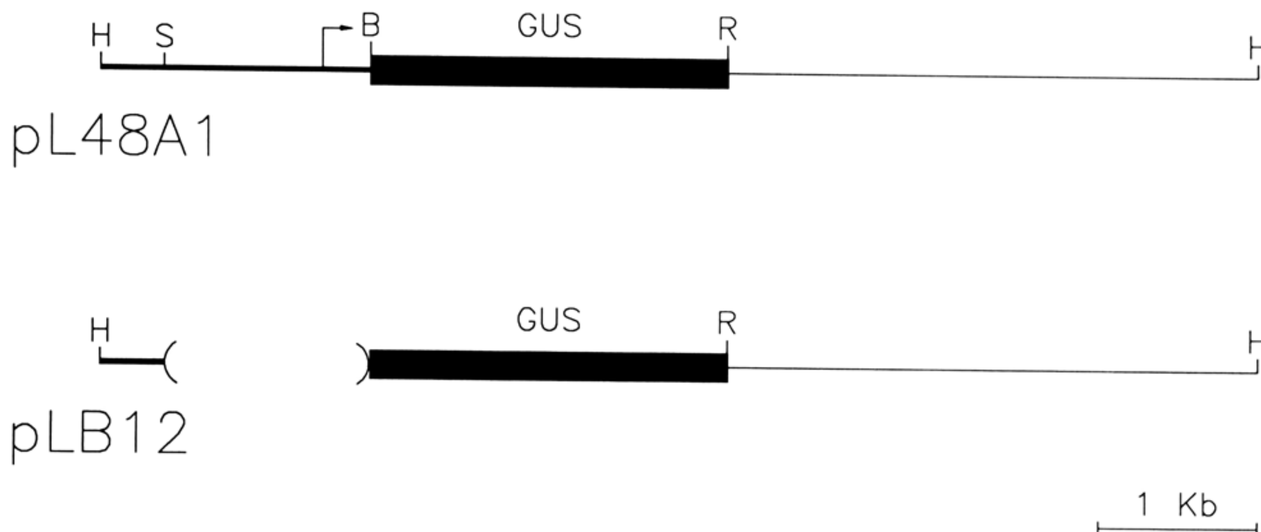

Fig. 1. Restriction maps of the plasmids used in the study. Thick line, the promoter region of INF24 (3); thin line, Bluescribe vector; closed box, the coding region of the *Escherichia coli* β-glucuronidase (GUS) gene. Restriction sites are: *Hind*III (H), *Eco*RI (R), *Spe*I (S). In pLB12, the region within the parentheses was deleted from the promoter region. The arrow in pL48A1 shows the position(s) of the transcription start site(s).

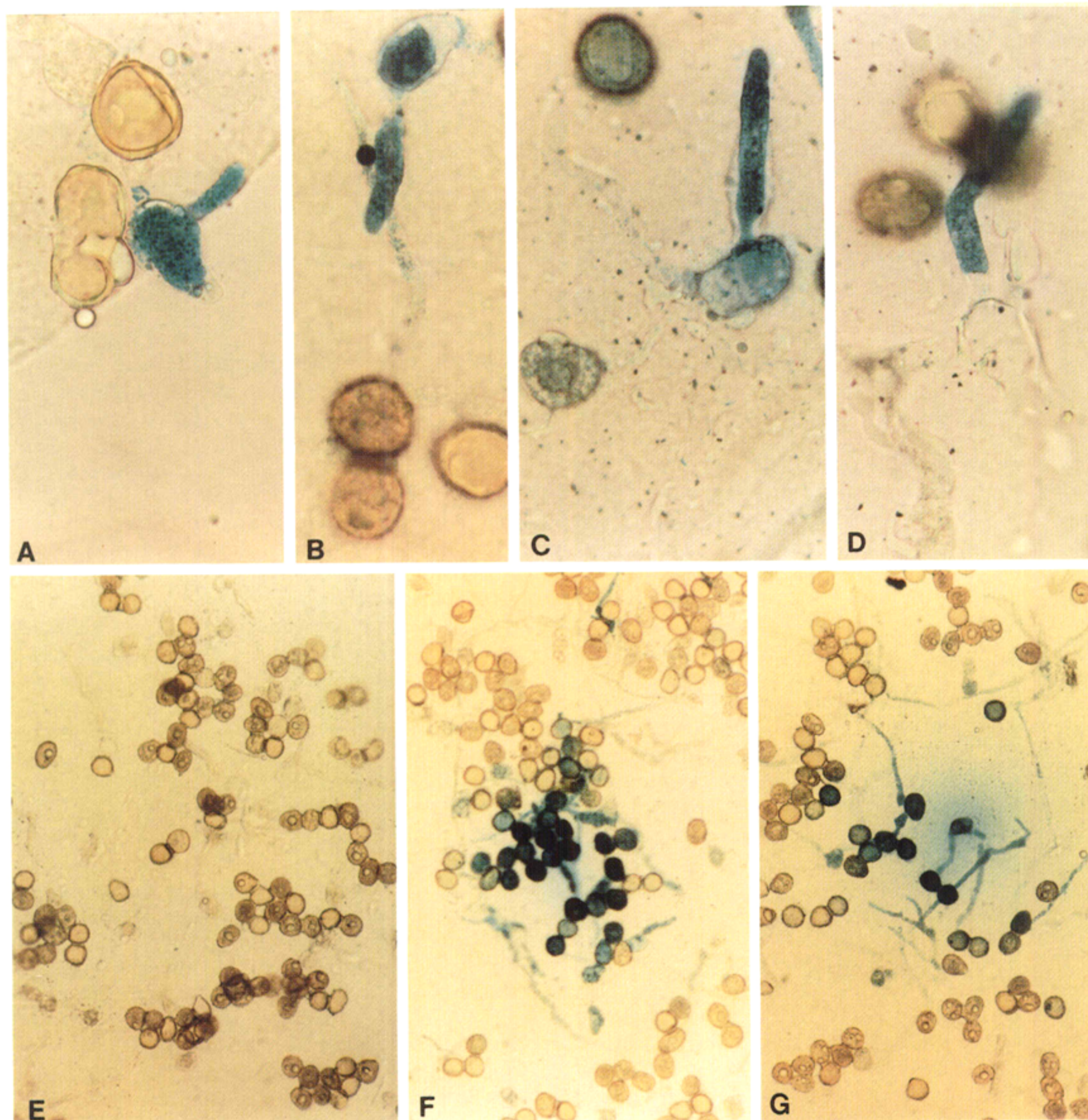

Fig. 2. Transient expression of β-glucuronidase (GUS) in uredospore germlings of *Uromyces appendiculatus*. Nongerminated uredospores dusted on oil-collodion membranes were bombarded with tungsten particles coated either with the DNAs (pL48A or pLB12) or mock-coated. Subsequently, spores were allowed to germinate and differentiate, and they produced appressoria and vesicles. After differentiation, the germlings were stained for GUS activity. **A,** Appressorium expressing the GUS gene; **B** and **C,** the cytoplasm containing GUS activity has partly moved into the vesicle; **D,** all the cytoplasm has moved into the vesicle; **E,** typical results with uredospores that were bombarded with mock-coated particles or particles coated with pLB12. **F** and **G,** Several germlings and spores that stained positive for GUS.

diameter 0.771 μm) appeared less efficient; however, the effect of size was not thoroughly investigated. Tungsten rather than gold was chosen for the particles for reasons of economy.

Our results have several potential applications. First, employing a quantitative assay that uses the fluorescent substrate, methyl umbelliferylglucuronide (MUG, Sigma Chemical Co., St. Louis, MO), should make it possible to examine the effects of deleting various amounts of DNA in the promoter region on GUS expression. In our initial feasibility studies, we bombarded 36 plates containing nongerminated uredospores on membranes, germinated the spores after bombardment, and prepared total cell extracts by grinding the germlings along with the membranes in liquid nitrogen as we did before (2). The powdered spores and membranes were added to a GUS buffer (6), and GUS activity was measured with MUG substrate. Extracts from spores on bombarded plates contained four times as much activity as did extracts of spores from plates that had not been bombarded; however, the data are not shown because the ability to quantitate GUS activity appeared to depend on the efficiency of the bombardment protocol. Hence, before optimizing the quantitative protocols, we need to study various parameters that affect the efficiency of GUS expression in uredospores by histochemical staining. An alternative possibility is to use polymerase chain reaction techniques for quantitating the levels of GUS mRNA relative to the levels of endogenous INF24 mRNA, as described in published procedures (13). It should then be possible to identify nucleotide sequences in the promoter region of INF24 that are required for thigmotropic induction of gene expression.

The results also indicate that attempts can now be made to stably transform the rust fungus by infecting plants with spores subjected to the bombardment procedures described above.

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