Biased DNA Integration in *Colletotrichum gloeosporioides* f. sp. *aeschynomene* Transformants with Benomyl Resistance

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ABSTRACT

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A procedure is presented for transforming *Colletotrichum gloeosporioides* f. sp. *aeschynomene* to benomyl resistance by using a mutant β-tubulin gene from *Neurospora crassa*. Hybridization between the *N. crassa* β-tubulin gene and transformant DNAs digested with *Sty*I indicated that the integration site in all transformants was in a specific region of the genome. Transformants tolerated up to 300 μg of benomyl per milliliter but differed in pigmentation, growth rate, and pathogenicity. All transformed strains remained benomyl resistant after repeated subculture on medium lacking benomyl. We speculate that the bias in the site of integration was due to selection against transformants with other configurations between the *N. crassa* β-tubulin gene and *C. g. aeschynomene* genome, which were unstable, lethal, or unsuitable for expression of the benomyl phenotype.

Colletotrichum gloeosporioides (Penz.) Succ. f. sp. *aeschynomene* causes anthracnose of northern jointvetch (*Aeschynomene virginica* (L.) B.S.P.). Because of its relatively narrow host range, the fungus was approved by the U.S. Environmental Protection Agency for agricultural application to control the weed in rice and soybean fields (2) . In view of its use in the environment, there are several reasons why availability of stable, recombinant, fungicide-resistant *C. g. aeschynomene* strains would be valuable. For example, strains with the fungicide resistance phenotype could be used in population studies in the field to distinguish the introduced organisms from indigenous soil fungi and wild-type strains. Also, fungicide-resistant *C. g. aeschynomene* strains could be sprayed in mixtures containing agrofungicides; the *C. g. aeschynomene* would be used to control northern jointvetch, whereas the fungicide(s) would control naturally occurring phytopathogenic fungi.

Many fungi have been transformed to fungicide resistance with the mutant β-tubulin gene from *Neurospora crassa*, which confers resistance to benomyl (Ben^r; methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate) (4,7,12,13), or with the hygromycin B (*hyg*) resistance gene from *Escherichia coli* (1,5,6,8,9,12). It was evident in some of these studies that transformants were frequently unstable and reverted to fungicide sensitivity by aborting the foreign sequence (1,5,8). Certainly, transformants that tend to lose the foreign gene(s) would not be useful for field application. However, there are other events whose effects on long-term gene stability and function are not as obvious. For example, although the Hyg^r phenotype of several *Glomerella* transformants was retained after subculture on a nonselective medium, the hygromycin B resistance gene often rearranged itself among *Hin*dIII restriction fragments (13). Similarly, the hygromycin B and benomyl resistance genes in *Fusarium* transformants often were deleted and rearranged in isolates recovered 3 wk after the organism was inoculated on corn plants (3).

The apparent frequency of gene instability in recombinant fungi has led to investigations of the genetic factors associated with persistence and function of transforming DNA. Keller et al (7) studied mitotic stability in *Cochliobolus heterostrophus* transformed with a hygromycin B resistance gene that had a promoter from the fungus. They noted that stability depended on whether the foreign DNA was present as a single copy or as tandemly repeated copies in homologous or heterologous sites. Also, Leslie and Dickman (9) looked at the fate of foreign DNA during meiosis in *Gibberella* transformed with the hygromycin B resistance gene; single copies of the gene segregated according to Mendelian inheritance, whereas transformants with multiple copies produced proportionately fewer Hyg^r progeny.

Our objective was to obtain stable, biologically vigorous, virulent, Ben^r strains of *C. g. aeschynomene* that can be used in the environment.

MATERIALS AND METHODS

Fungal strain and culture conditions. The wild-type *C. g. aeschynomene* strain 3.1.3 (provided by D. TeBeest, University of Arkansas, Fayetteville) was cultured at 30 C for 2–3 days on torula yeast agar (TYA; 15 g of torula yeast, 15 g of starch, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 15 g of agar, and 1 L of H_2O) or in Emerson's YpSs broth (4) amended with streptomycin (25 μg/ml) and chloramphenicol (25 μg/ml). Benomyl (systemic fungicide, 50% active ingredient; concentrations represent quantity of active ingredient) at 10 μg/ml were added to media for selection and routine maintenance of Ben^r transformants.

Preparation of protoplasts. After 2–3 days at 30 C, spores on TYA plates were suspended in sterile water by gentle scraping of the surface with a pipet and centrifuged three to five times for 5 min at 1,000 *g*. Protoplasts were obtained using a modification of the procedure of Kistler and Benny (8). Each batch of spores from a single plate was suspended in 10 ml of 1.0 M $MgSO_4 \cdot 7H_2O$ in 10 mM Tris-HCl (pH 5.8) containing Novozym 234 (17 mg/ml; Novolabs, Danbury, CT) and incubated for 5 h at 32 C. After adding 10 volumes of 1.2 M sorbitol, the protoplasts were centrifuged for 10 min at 2,000 *g*. To remove nuclease contaminants in the Novozym, the protoplasts were washed five times by centrifugation for 3 min at 2,000 *g* in 1.2 M sorbitol buffered with 10 mM Tris-HCl (pH 7.0). Protoplasts were resuspended in the sorbitol solution for DNA transformation.

DNA transformation. Plasmid pBT (Fig. 1) consisted of the 3.2-kb phagemid pBS(+/−) vector (Stratagene, La Jolla, CA) and a 2.6-kb *Sal*I insert encoding the mutant β-tubulin gene from *N. crassa* (15). From 10 to 20 μg of pBT was added to each milliliter of the protoplast suspension containing $1–2 \times 10^6$ protoplasts per milliliter as determined with a hemacytometer. After adding an equal volume of 30% polyethylene glycol 4000 (PEG) in 10 mM $CaCl_2$, the suspension was incubated 30 min at 20–25 C, followed by centrifugation for 3 min at 3,000 *g* to remove the PEG. The pellet of protoplasts was resuspended in 1.2 M sorbitol in 10 mM Tris-HCl (pH 7.0), and 0.1-ml volumes were spread immediately on agar containing 0.8 M sucrose. After 24 h, protoplasts were overlayed with 5 ml of 1% YpSs agar containing 10 μg/ml of benomyl and incubated at 30 C. To avoid the benomyl-tolerant, nonrecombinant clones that appeared as background colonies after 5–7 days, Ben^r colonies were picked on day 5 and streaked on TYA containing 10 μg/ml of benomyl. After 2 days at 30 C, isolated colonies were repeatedly picked and streaked on the same medium three separate times before further experiments.

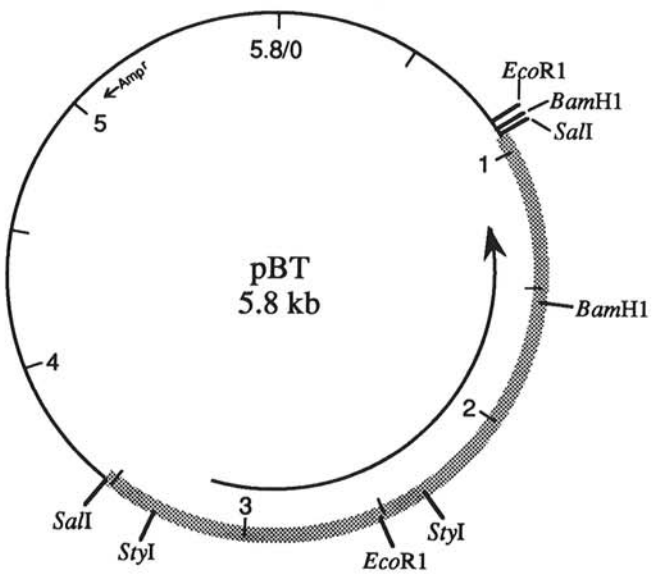

Fig. 1. Restriction map of pBT. The solid perimeter is pBS(+/−) DNA; the shaded perimeter is *Neurospora crassa* DNA containing the β-tubulin gene. The arrow shows the extent of β-tubulin gene and direction of transcription. Numbers refer to the number of kilobases.

Tests for mitotic stability, score viability, and growth rate. Mitotic stability of the transformed DNA was tested by subculturing transformants in 100 ml of YpSs broth lacking benomyl. After growth for 2–3 days at 30 C, 0.1 ml was transferred to a second volume of YpSs broth. The subculturing was done five times before each culture was diluted and plated in duplicate on TYA in the presence or absence of benomyl. Colonies were counted after 3 days at 30 C.

The efficiency of germination of transformant spores was determined by spreading aqueous suspensions of spores on TYA and TYA amended with 10 μg/ml of benomyl and comparing the colony numbers. These results were compared with direct counts (using a hemacytometer) of spores in the original suspensions to measure spore viability.

For comparisons of growth rates, 0.5 × 0.5 cm plugs were removed from media covered with hyphal growth of parent or transformant strains and placed on TYA with or without 100 μg/ml of benomyl. The radius of hyphal growth was measured after 7 days at 30 C.

DNA extraction. Fungi were cultured in YpSs broth containing 10 μg/ml of benomyl, collected by filtration, lyophilized, ground with a mortar and pestle, and the DNA was extracted by the CTAB method (10). The powder was suspended in 1% hexadecyltrimethylammonium bromide (CTAB) (containing, 0.7 M NaCl, 10 mM EDTA, 1% 2-mercaptoethanol [v/v], and 50 mM Tris-HCl [pH 8.0]). This was heated 1 h at 55 C, mixed with an equal volume of chloroform/isoamyl alcohol (24:1, v/v), and centrifuged 10 min at 10,000 *g*. The supernatant liquid was mixed with one-tenth volume of 10% CTAB in 0.7% NaCl. This was extracted again with chloroform/isoamyl alcohol. An equal volume of 1% CTAB in 10 mM EDTA and 50 mM Tris-HCl (pH 8.0) was added. After 30 min at 20–25 C, the suspension was centrifuged at 4,000 *g* for 5 min. The pellet was dissolved in CsCl solution (density, 1.62; 0.5% sodium *n*-lauroylsarcosine, 10 mM EDTA, 100 μg/ml of ethidium bromide, and 50 mM Tris-HCl [pH 8.0]), and the DNA was purified by centrifugation at 436,000 *g* for at least 8 h in an ultracentrifuge (Beckman TLV-100) (14).

DNA hybridization. About 0.75–1.5 μg of *C. g. aeschynomene* DNA per reaction was digested with either *Sty*I (Stratagene) or a combination of *Sal*I and *Eco*RI (Bethesda Research Laboratory, Bethesda, MD) according to manufacturer's instructions. DNA fragments were separated by electrophoresis in 0.7% agarose with Tris-acetate buffer by standard methods (14). The DNAs were transferred to a Nytran membrane by the "rapid transfer method" in alkaline buffer (directions from Schleicher & Schuell, Keene, NH) and baked for 1 h at 80 C. Membranes were saturated with 6× saline sodium citrate solution (SSC; 1× SSC contained 0.15 M NaCl and 0.05 M Na citrate [pH 7.0]) (15) and incubated for 4 h at 65 C in 25 ml of blocking solution (5× SSC, 0.5% blocking agent [1096 176, Boehringer-Mannheim, Indianapolis, IN], 0.1% sodium *n*-lauroylsarcosine, 0.1% sodium dodecyl sulfate [SDS], 100 μg/ml of salmon sperm DNA [Sigma, St. Louis, MO],

and 50 mM $NaPO_4$ [pH 6.5]). All but 3.5 ml of blocking solution was removed before adding probe DNA. The *Sal*I fragment encoding the *N. crassa* β-tubulin gene (15) was purified by electrophoresis and Geneclean (Bio 101 Inc., La Jolla, CA), labeled with ^{32}P using a random primers extension kit (according to manufacturer's instructions; Bethesda Research Labs), and purified through a Nu-Clean D50 spun column (VWR Scientific). Hybridization was carried out for 16 h at 65 C. This was followed by four washes in 2× SSC containing 0.1% SDS (twice at room temperature, 5 min; once at 55 C, 15 min; once at 65 C, 15 min). Hybrids were detected by autoradiography with Kodak X-OMAT film (14).

Pathogenicity testing. Northern jointvetch (*A. virginica*) seeds were washed in 10% NaOCl, scarified with sandpaper, germinated on moistened filter paper for 24 h at 28 C, and planted in vermiculite in 8-cm pots. Seedlings were raised at 28 C in a Conviron E7 growth chamber with a 16-h light period and watered by continuous flow. Spores were harvested with water from *C. g. aeschynomene* cultured on TYA. The cotyledonary node of each seedling was inoculated with 20 μl of water or water containing 2×10^6 spores/ml. The plants were incubated in a dew chamber for 24 h at 28 C and returned to the growth chamber. After 7 days, the extent of disease was rated qualitatively on a scale of 0 to 5: 0 = no anthracnose; 1 = nonexpanding, 1–2 mm diameter lesions on <25% of the plant; 2 = expanding, 2–4 mm diameter lesions on 25–50% of the plant; 3 = lesions on 50–75% of the plant; 4 = lesions on >75% of the plant; 5 = dead plant. The test was performed twice.

RESULTS

Transformation of *C. g. aeschynomene* with *N. crassa* β-tubulin gene. Transformation of *C. g. aeschynomene* to Ben^r using the *N. crassa* β-tubulin gene in pBT occurred at a relatively low frequency. One stable Ben^r clone was obtained from each of three independent transformation experiments. These transformants were identified as II-4, IV-2, and III-5. In these experiments, the frequencies of transformants per 10^8 protoplasts were 50, 2, and 17, respectively. The corresponding calculations for transformants per 100 μg of DNA were 2, 3, and 3. No transformants were observed during five additional experiments involving a total of 570 μg of DNA and 1.9×10^8 protoplasts. The overall frequency of stable transformants for all eight experiments was 12 transformants per 10^9 protoplasts or four transformants per milligram of DNA. Variations in the amount of added DNA between 1 and 35 μg of DNA per 10^6 protoplasts did not increase the appearance of transformants.

A variety of tests were performed to compare the biological properties of the three transformants. Isolates II-4, IV-2, and III-5 tolerated up to 300 μg/ml of benomyl, and the resistance phenotype proved to be mitotically stable after five subculturings in YpSs lacking benomyl. Comparisons between colony numbers on unamended TYA and visual spore counts revealed differences among the percentages of viable spores: wild type, 61%; II-4, 45%; IV-2, 14%; III-5, 0.37%. Spores of transformants were also cultured on TYA and on TYA amended with 10 μg/ml of benomyl. For all strains, colony numbers on TYA plus benomyl were about 90% (II-4, 93%; IV-2, 87%; III-5, 89%) of the numbers on unamended media. Only isolate II-4 exhibited the orange pigment of the parent strain; isolate IV-2 was dark brown, and III-5 was brown-black. On TYA containing 100 μg/ml of benomyl, isolate II-4 grew slower than the parent strain on TYA but faster than IV-2 and III-5 on TYA plus benomyl. The radius of growth of the parent strain around an agar plug was 1.6 cm after 7 days, whereas the radii for strains II-4, IV-2, and III-5 were 0.9, 0.5, and 0.6 cm. The virulence of transformant II-4 on northern jointvetch was comparable to the parent strain; strain IV-2 had reduced virulence, and III-5 was avirulent (Table 1).

Characterization of *C. g. aeschynomene* transformants by DNA hybridization. The unusual pigmentation, low spore viability, slow growth, and reduced virulence of IV-2 and III-5 suggested they might be contaminants. This was discounted by observing the similarity between the *Sty*I fragment patterns of the wild-type strain and transformants (Fig. 2A). To compare the insertion sites of the *N. crassa* β-tubulin gene in the transformants, the *Sty*I fragments shown in Figure 2A were transferred to a Nytran membrane and probed with the β-tubulin gene (Fig. 2B). Relatively high stringency conditions (final wash: 2× SSC containing 0.1% SDS, 65 C, 15 min) were used in the hybridization to favor base pairing between sequences having extensive homology with the *N. crassa* DNA. Under these conditions, no hybridization occurred between the *N. crassa* DNA probe and pBS(+/−) DNA (data not shown). However, the probe hybridized strongly with a 3.7-kb *Sty*I fragment (lane 1) and weakly to several other *Sty*I fragments that were common to the parent and the three transformants. Three additional strongly hybridizing fragments were unique to the transformants. The smallest was equal in size to the 0.98-kb fragment between the two *Sty*I sites in the *N. crassa* DNA of pBT (Fig. 1). The other fragments were 5.2- and 6.3-kb long and presumably were the remainder of a single copy of the integrated pBT and *C. g. aeschynomene* sequences flanking the transforming DNA. Hybridization of transformant DNAs digested with both *Eco*RI and *Sal*I indicated that the entire length of *N. crassa* DNA in pBT was present in the transformants; each

TABLE 1. Pathogenicity of parent and Ben^r transformant strains of *Colletotrichum gloesporioides* f. sp. *aeschynomene* on northern jointvetch plants

Treatment[a]	Disease ratings on individual plants[b]												Disease rating average
	1	2	3	4	5	6	7	8	9	10	11	12	
Experiment 1													
Untreated	0	0	0	0	0	0	0	0	...	...	...	...	0
Water	0	0	0	0	0	0	0	0	...	...	...	...	0
Parent strain	0	5	5	5	0	0	5	5	...	...	...	...	3.1
Transformant II-4	5	5	5	5	5	5	5	5	...	...	...	...	5
Transformant IV-2	3	1	3	3	3	3	1	3	...	...	...	...	2.5
Transformant III-5	0	0	0	0	0	0	0	0	...	...	...	...	0
Experiment 2													
Untreated	0	0	0	0	0	0	0	0	0	...	...	...	0
Water	0	0	0	0	0	0	0	0	0	0	...	...	0
Parent strain	5	5	5	5	5	5	5	5	5	5	...	...	5
Transformant II-4	5	5	5	5	5	5	5	5	5	5	5	5	5
Transformant IV-2	5	3	2	5	3	2	2	4	2	2	2	...	3
Transformant III-5	0	0	0	0	0	0	0	0	0	0	...	...	0

[a] Cotyledonary nodes of 1-day-old seedlings of *Aeschynomene virginica* uninoculated or inoculated with 20 μl of water or water containing 2×10^6 spores/ml.

[b] Anthracnose was assessed after 7 days at 28 C; 0 = no anthracnose; 1 = nonexpanding, 1–2 mm diameter lesions on <25% of the plant; 2 = expanding, 2–4 mm diameter lesions on 25–50% of the plant; 3 = lesions on 50–75% of the plant; 4 = lesions on >75% of the plant; 5 = dead plant.

transformant contained the expected 1.0- and 1.6-kb fragments (Fig. 1). The presence of some pBS(+/−) DNA in the transformants was confirmed by probing their DNAs with pBS(+/−) (data not shown).

DISCUSSION

Although we successfully transformed *C. g. aeschynomene* to Ben^r with a mutant β-tubulin gene from *N. crassa*, transformants were rare. The frequency of stable transformants was 12 per 10^9 protoplasts, which was equivalent to four per milligram of transforming DNA. This was considerably lower than reported results for the occurrence of transformants, e.g., 200–1,000/mg of DNA for Ben^r *Fusarium* (3), 13,000/mg of DNA for Ben^r *Colletotrichum graminicola* (11), and 60,000/mg of DNA for Hyg^r *Leptosphaeria maculans* (5). We propose that the scarcity of *C. g. aeschynomene* transformants may have been due to a relatively unique recombination event that was necessary for formation of stable, viable transformants with the Ben^r phenotype.

Although the three stable transformants we studied were obtained from three independent experiments, in all three the gene was inserted in a 12.5-kb *Sty*I region (the sum of the 0.98-, 5.2-, and 6.3-kb *Sty*I fragments), which included 2.6-kb of *N. crassa* DNA, the *C. g. aeschynomene* genomic sequences, and pBS(+/−) DNA. Therefore, the size of the insert was between 2.6 and 5.8 kb, depending on the amount of pBS(+/−) DNA flanking the *N. crassa* DNA. This means that the transforming DNA inserted itself near the middle of a 7–10 kb *Sty*I region of the *C. g. aeschynomene* genome. There were no measurable differences in the lengths of the *Sty*I fragments from the three isolates. Therefore, the "hot spot" region targeted for DNA integration was no larger than about 100 base pairs (i.e., the approximate limit of resolution for determining fragment lengths by comparisons with size standards). However, despite the similarity of the integration sites in the three transformants, the clones differed in pigmentation, spore viability, growth rate, and virulence on northern jointvetch (Table 1). Transformant II-4 was like the wild type with respect to pigmentation and virulence, whereas strain IV-2 had reduced virulence, and III-5 was avirulent. Strains IV-2 and III-5 also had altered pigmentation and grew considerably slower than the wild type. Clearly, these differences were not due to differences in the amount of integrated *N. crassa* DNA, because the entire *N. crassa Sal*I insert of pBT was present in each transformant (Fig. 2B). More likely, the differences reflected insertion of *N. crassa* DNA into dissimilar sites within the "hot spot" region, and/or presence of pBS(+/−) vector DNA in one or more sites that affect pigmentation, spore viability, growth rate, and virulence.

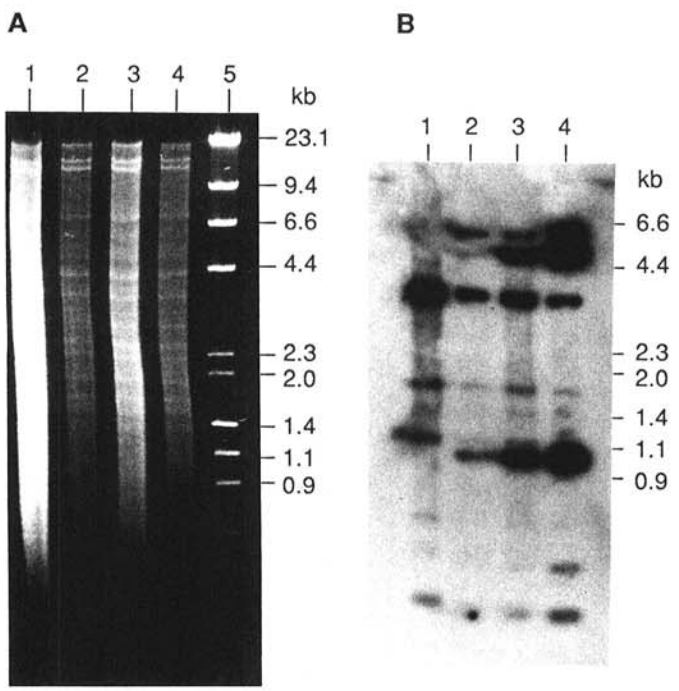

Fig. 2. Comparison of *Sty*I digests of DNA from *Colletotrichum gloeosporioides* f. sp. *aeschynomene* parent and transformant strains. **A,** Ethidium bromide-stained gel over ultraviolet light: lane 1, parent strain; lanes 2–4, transformants II-4, IV-2, and III-5; lane 5, marker DNAs (mixture of lambda DNA digested with *Hin*dIII and X174 digested with *Hae*III). **B,** autoradiogram of DNAs digested with *Sty*I and hybridized with a ^{32}P-labeled *Neurospora crassa* β-tubulin gene probe: lane 1, parent strain; lanes 2–4, transformants II-4, IV-2, and III-5. Numbers on the right indicate sizes of marker DNAs.

Even when DNA hybridization was conducted under highly stringent reaction conditions, probing with the *N. crassa* β-tubulin gene identified a single 3.7-kb *Sty*I fragment in the nontransformed Ben^s *C. g. aeschynomene* parent strain. The high degree of homology between these DNAs suggested that at least one genomic β-tubulin gene is located within this fragment. However, the 3.7-kb *Sty*I fragment also appeared in *Sty*I digests of transformant DNAs. Thus, despite the homology between the 3.7-kb segment and the *N. crassa* β-tubulin gene, transformation occurred in a region that lacked sufficient homology to hybridize with the *N. crassa* β-tubulin under our hybridization reaction conditions. It is unknown whether the integration site was in a genomic β-tubulin gene. The possible presence of two divergent *C. g. aeschynomene* β-tubulin genes that vary in relatedness with the *N. crassa* β-tubulin gene is analogous to *C. graminicola* (11), which has two genomic sequences that hybridize with the *N. crassa* β-tubulin gene; one hybridizes when the stringency of hybridization reaction is strict, whereas both hybridize under more relaxed conditions.

In some cases, DNA transformation in fungi involves integration at "random" sites (7,11,12) and typically yields transformants with the foreign gene inserted into a variety of positions. This may also be the case for *C. g. aeschynomene* Ben^r transformants. But, the unique property shared by the transformants we studied points to a distinct arrangement between the *C. g. aeschynomene* genome and the *N. crassa* gene in viable transformants. We speculate that other arrangements occurred, but they were unstable, lethal, or unsuitable for expression of the Ben^r phenotype. This selection would have culled most clones containing other arrangements of the *N. crassa* gene.

From a practical standpoint, a Ben^r strain of *C. g. aeschynomene* could be useful in the field. It could be sprayed in conjunction with benomyl to control northern jointvetch as well as Ben^s fungal pathogens. However, to be effective, a strain like this would have to retain its drug resistance property and compete with indigenous microorganisms. Pure culture studies with isolate II-4 indicated it was a good candidate for field use because the resistance phenotype was stable, and it grew rapidly in the presence of benomyl.

For recombinant fungi to have practical value, more information is needed on how to transform the host without disabling it, control the site of integration, stabilize an integrant once it is in the genome, and produce constructs that enable continued "normal" expression of the foreign gene. To clarify these factors in *C. g. aeschynomene*, we also propose sequencing the insertion sites in the Ben^r transformants.

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