Colletotrichum graminicola Transformed with Homologous and Heterologous Benomyl-Resistance Genes Retains Expected Pathogenicity to Corn

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Colletotrichum graminicola was efficiently transformed to benomyl resistance by β-tubulin genes cloned from *C. graminicola* and *Neurospora crassa* benomyl-resistant mutants. Southern blot hybridizations showed that the β-tubulin genes used as selectable markers share homology with distinct regions of the *C. graminicola* genome, suggesting the presence of two divergent β-tubulin genes in the fungus. Plasmids containing either selectable marker integrated at a variety of loci and were often present in more than one copy per genome. Plasmid integration was not detected in genomic sequences with homology to the selectable markers. The benomyl-resistant phenotype of transformants was highly stable during *in vitro* culture in the absence of selection. In addition, the benomyl-resistant phenotype persisted in isolates recovered from susceptible corn seedlings that had been inoculated with transformants. When transformants obtained with either selectable marker were used to inoculate susceptible and resistant corn seedlings, each produced symptoms typical of those caused by the untransformed, parental strain.

Additional keywords: anthracnose, divergent genes, filamentous fungi, gene transfer, plasmid integration, β-tubulin

Colletotrichum graminicola (Ces.) Wils. is the causal agent of anthracnose of corn, sorghum, and a variety of other grasses. The incidence and severity of anthracnose on corn in the United States has risen dramatically during the last 25 yr, and much of this increase has been attributed to the emergence of new pathotypes of *C. graminicola* on corn. Whether these pathotypes represent specific races of the fungus remains a controversy (Wheeler *et al.* 1974; Nicholson and Warren 1976; Forgey *et al.* 1978; Schall *et al.* 1980; Nicholson and Warren 1981; White *et al.* 1987). The suggestion that races exist is further complicated by studies that show that the response to infection is significantly influenced by the maturity of the corn leaf (Leonard and Thompson 1976; Nicholson *et al.* 1985; Jamil and Nicholson 1987). Related studies have suggested the presence of races within geographically diverse populations of *C. graminicola* pathogenic to sorghum but not to corn (Ali and Warren 1987). Gene-transfer techniques such as DNA-mediated transformation could be extremely useful in experiments designed to characterize the molecular basis for physiologic specialization exhibited by *C. graminicola*.

Systems for DNA-mediated transformation of fungi have been the subject of considerable research since they were first described for *Saccharomyces cerevisiae* (Hinnen *et al.* 1978) and *Neurospora crassa* (Mishra 1979). Until recently, however, there have been no reports describing the development of transformation systems for phytopathogenic species. One hindrance has been the lack of strains with well-defined, auxotrophic mutations that can be complemented by recombinant clones that contain the corresponding wild-type allele. To overcome this, genes that

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can be directly selected have been employed. For example, the *amdS* gene of *Aspergillus nidulans*, which permits the utilization of acetamide, has been used as a selectable marker in transformations of *Cochliobolus heterostrophus* (Turgeon *et al.* 1985) and *Glomerella cingulata* (Rodriguez and Yoder 1987). A chimeric marker, consisting of a *Cochliobolus* promoter fused to the *hygB* gene of *Escherichia coli*, has also been utilized to select for resistance to the antibiotic hygromycin B in transformations of these fungi (Turgeon *et al.* 1987; Rodriguez and Yoder 1987). This chimeric construction has also been reported to transform a variety of phytopathogenic fungi that included *C. graminicola*, *Leptosphaeria maculans*, *Nectria haematococca*, and *Fusarium sporotrichioides* (Turgeon *et al.* 1987). Work on transformation in other laboratories has resulted in the development of systems for *Ustilago maydis* (Wang *et al.* 1988), *Fulvia fulva* (Oliver *et al.* 1987), and *Fusarium oxysporum* (Powell *et al.* 1987) that also use the *hygB* gene as a selectable marker. A notable exception has been work by Parsons *et al.* (1987), in which transformation of a *Magnaporthe grisea arg3* mutant was achieved by using an *ArgB*⁺ gene from *A. nidulans* as a selectable marker.

Another antibiotic for which resistance genes have been cloned is the antimicrotubule compound, benomyl. Benomyl resistance in several Ascomycetes has been attributed to mutations in genes encoding β-tubulin (Sheir-Neiss *et al.* 1978; Thomas *et al.* 1985; Orbach *et al.* 1986). It has been demonstrated that a mutant allele of the *N. crassa tub-2* gene, which encodes a β-tubulin that confers resistance to benomyl, serves as an efficient selectable marker in transformation of *N. crassa* (Orbach *et al.* 1986; Vollmer and Yanofsky 1986). In this paper we report the cloning of a β-tubulin gene from a benomyl-resistant mutant of *C. graminicola* and its subsequent use as a selectable marker in transformation of the fungus. We also demonstrate that the mutant *tub-2* allele from *N. crassa* is

equally effective as a selectable marker in transformation of *C. graminicola*. Because reports of transformation in other fungi have indicated that the integration of plasmid DNA and the frequency of homologous recombination can vary greatly, it was of interest to compare the integration of a plasmid containing a *C. graminicola* β-tubulin gene with that of a plasmid containing the *tub-2* allele from *N. crassa*.

MATERIALS AND METHODS

Fungal strains and culture. An isolate of *C. graminicola* from maize, designated CgM2, was used as the wild-type strain and as the recipient in transformations. Strain CgM2BmlR3 is a UV-induced, benomyl-resistant mutant of strain CgM2 and served as the source of DNA from which the gene encoding the benomyl-resistant β-tubulin was cloned. Isolates of the fungus, including transformants, produce uninucleate conidia when cultured on solid medium (Politis and Wheeler 1973; Panaccione and Hanau, unpublished); prior to analyses of transformants, each was grown from a single conidium produced on solid medium to resolve any heterokaryons that may have formed during the transformation procedure.

To generate mycelia and conidia for preparation of DNA and protoplasts, 1 L of liquid-complete medium (Leach *et al.* 1982) was inoculated with conidia from two potato dextrose agar (PDA) cultures that had been incubated for 3 wk at 24° C under white fluorescent light. The liquid cultures were incubated at 30° C for 18–36 hr on a gyratory shaker (200 rpm). The mycelium was harvested by filtering the cultures through two layers of sterile cheesecloth. Conidia were collected from the filtrate by centrifugation at 10,000 × *g* for 15 min.

Preparation of DNA. Fungal DNA was prepared by grinding conidia and mycelia under liquid nitrogen with a mortar and pestle. The resulting powder from each gram (fresh weight) of tissue was suspended in 3 ml of LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-Cl, pH 7.5, 0.5% sodium dodecyl sulfate [SDS]; O. C. Yoder, personal communication) and stirred for 15 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was then added, and the mixture was stirred for an additional 15 min. After centrifugation at 12,000 × *g* for 15 min, the aqueous phase was recovered and mixed with an equal volume of 5 M ammonium acetate. The sample was chilled on ice for several hours and the high molecular weight RNA was pelleted by centrifugation at 10,000 × *g* for 15 min. The remaining nucleic acids were precipitated from the supernatant with an equal volume of 2-propanol and recovered by centrifugation at 10,000 × *g* for 15 min. All centrifugations were performed in a swinging-bucket rotor at 4° C. The resulting pellet was washed with 70% ethanol, dried, and resuspended in 1–2 ml of water. Nuclear DNA was then purified in two successive CsCl-bisbenzamide equilibrium gradients as described by Garber and Yoder (1983).

Plasmids pCG1 and pCG7, which contain a β-tubulin gene cloned from *C. graminicola* strain CgM2BmlR3, and plasmid pBT6, which contains a *tub-2* allele cloned by Orbach *et al.* (1985, 1986) from a benomyl-resistant mutant of *N. crassa*, were propagated in *E. coli* strain TB-1 (Bethesda Research Laboratories, Gaithersburg, MD). Plasmid DNA was isolated from stain TB-1 by lysis in alkali (Maniatis *et al.* 1982) and purified by RPC-5 ANALOG chromatography (Thompson *et al.* 1983) on a 1.0 × 15 cm column containing NACS-37 (Bethesda Research

Laboratories). Standard procedures (Maniatis *et al.* 1982) were used to propagate bacteriophage λ in *E. coli* strains LE392 and P2392 (Stratagene, San Diego, CA) and to prepare λ DNA.

DNA manipulations. A genomic library of *C. graminicola* strain CgM2BmlR3 was constructed in the λ replacement vector, EMBL3 (Frischauf *et al.* 1983). Partially digested, size fractionated, *Mbo*I fragments of CgM2BmlR3 nuclear DNA were ligated with *Bam*HI-digested EMBL3 arms. The reaction products were then packaged *in vitro* by incubation with commercial bacteriophage λ extracts (Promega Biotec, Madison, WI). The library was plated on *E. coli* strain P2392 and recombinant phage that contained sequences with homology to the *N. crassa tub-2* gene were identified by plaque hybridization (Benton and Davis 1977) to pBT6 DNA. DNA used for hybridization probes was labeled with [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, IL) by nick translation (Rigby *et al.* 1977). DNA sequencing was performed by using the dideoxy chain termination method (Sanger *et al.* 1977). All restriction digests and subcloning were done by standard techniques (Maniatis *et al.* 1982).

Protoplast preparation and transformation. Protoplasts were prepared from cultures of CgM2 that were grown and harvested as described. Mycelia and conidia were suspended in 150 and 75 ml, respectively, of sterile 0.7 M NaCl that contained 12.5 mg/ml of Novozyme 234 (Nova Biolabs, Wilton, CT). Cell wall digests were performed by incubating the suspensions at 30° C for 60–90 min on a gyratory shaker (100 rpm). After digestion, cell debris was separated from protoplasts by filtering through a nylon screen that contained 20 μm pores. The protoplasts were pelleted at 3,000 × *g* and suspended in 15 ml of 0.7 M NaCl. The protoplasts were pelleted again, resuspended in 5 ml of STC (1.2 M sorbitol, 10 mM Tris-Cl, pH 7.5, 10 mM CaCl₂; Yelton *et al.* 1984), and counted. Protoplasts were pelleted once more and resuspended in STC at a final concentration of 5 × 10^8 protoplasts per milliliter.

The transformation procedure was that as described for *Cochliobolus heterostrophus* by Turgeon *et al.* (1985). Each transformation was performed in a 100 × 13 mm borosilicate tube that contained 1–20 μg of either pCG1, pCG7, or pBT6 circular DNA in 25 μl of STC and 5 × 10^7 protoplasts in 100 μl of STC. After incubation in polyethylene glycol and its subsequent removal by aspiration, each protoplast-DNA mixture was suspended in 200–300 μl of STC. The suspension was diluted by adding a 3-ml volume of molten top agar (48° C) consisting of 1 M sucrose, 0.1% yeast extract, 0.1% casein hydrolysate, and 6 g/L of agar (Turgeon *et al.* 1987). This mixture was then overlayed on a 100 × 15 mm plate containing 30 ml of similar medium, which differed only in that it contained 15 g/L of agar and 0.5 μg/ml of benomyl (E. I. du Pont de Nemours, Wilmington, DE). The benomyl was freshly prepared as a 0.5 mg/ml stock solution in ethanol and added just before pouring the medium. Transformants were selected by incubating the plates at 30° C. Benomyl-resistant colonies appeared in 7–10 days and individual conidial isolates were obtained by plating dilutions of conidial suspensions on PDA.

Genomic blots and hybridizations. DNA was transferred to MSI Magna Nylon 66 membrane filters (Fisher Scientific, Pittsburgh, PA) from agarose gels by the method of Southern (1975). Hybridizations were performed at 68° C for 8–14 hr in the presence of 0.08% bovine serum albumin, 0.08% polyvinylpyrrolidone, 0.08% Ficoll, 10 μg/ml of *E.*

coli tRNA, 10 µg/ml of sonicated calf thymus DNA, and different concentrations of saline sodium citrate (SSC; 150 mM NaCl, 15 mM NaCitrate, pH 7.0) to modify the stringency. To obtain high stringency, hybridizations were performed in 3× SSC, and washed with several changes of 0.1× SSC plus 0.1% SDS at 68° C for 90 min. Low-stringency hybridizations were performed in 6× SSC and washed with 2× SSC plus 0.1% SDS. Nick-translated probe (spec. act. = 10^8 cpm/µg of DNA) was present at 200,000 cpm/ml in the hybridizations.

Inoculation of plants. Fourteen-day-old corn seedlings of cultivars 33-16 (resistant) and Mo940 (susceptible) were inoculated by applying 0.5 ml of a conidial suspension (5×10^4 conidia per milliliter in sterile water containing 0.1% Tween-20) directly into the whorl of the plant. *C. graminicola* was isolated from infected plants by surface sterilizing excised leaf pieces in 20% bleach and then using the leaf pieces containing the fungus to inoculate PDA prepared with and without benomyl.

RESULTS AND DISCUSSION

Cloning of a *C. graminicola* β-tubulin gene. A *C. graminicola* β-tubulin gene was identified in an EMBL3 library of CgM2BmlR3 genomic DNA fragments by specific hybridization to *N. crassa tub-2* sequences in nick-translated pBT6 DNA. Hybridizations were conducted at low stringency, and six positive clones were detected among 7,000 plaques that had been screened. Three of the clones were purified and their DNA was examined by Southern blot analysis. The results indicated that inserts from two of the clones were overlapping and contained a common 5.2-kb *Sal*I-*Hind*III fragment that hybridized with the *N. crassa* sequences in pBT6 (Fig. 1). None of the cloned sequences flanking the 5.2-kb *Sal*I-*Hind*III fragment hybridized with the probe, suggesting that this internal fragment contained a complete β-tubulin gene. Analysis of the third clone indicated that it had a unique restriction pattern and only a terminal portion of the insert, which was truncated upon cloning, hybridized to pBT6 DNA (data not shown).

The 5.2-kb *Sal*I-*Hind*III fragment that was present in λCG1 (one of the two overlapping clones) was subcloned into pUC19 to construct pCG1 (Fig. 1, A and B). To determine the orientation and approximate the position of the β-tubulin gene in the cloned fragment, we sequenced a small segment (225 nucleotides) beginning at the *Bam*HI site in the middle of the pCG1 insert (Fig. 1B). Analysis of the sequence (data not shown) indicated that this region was 79% identical to β-tubulin coding sequences between nucleotides 1,610 and 1,824 in the 3′ end of the *N. crassa tub-2* gene (Orbach *et al.* 1986). If the sizes of the *N. crassa* and *C. graminicola* β-tubulin genes are comparable, the nucleotide sequence obtained from pCG1 predicts that the 3′ end of the *C. graminicola* β-tubulin coding sequence is approximately located at nucleotide 5,500 on the pCG1 restriction map (Fig. 1B). Furthermore, the orientation of the sequence in pCG1 is such that the 5′ end of the β-tubulin coding sequences would be expected to be in the *Sal*I-proximal portion of the insert, at about nucleotide 3,600 on the pCG1 restriction map (Fig. 1B).

The smaller transformation vector, pCG7, was derived by cleaving pCG1 with *Hind*III followed by limited digestion with nuclease Bal-31. This resulted in the removal of approximately 1,100 base pairs that included the *Hind*III site (Fig. 1C). The *Hind*III site was deleted to facilitate Southern blot analysis of integration events in transformants

obtained with the *C. graminicola* β-tubulin gene.

Transformation of *C. graminicola*. *C. graminicola* strain CgM2 was transformed to benomyl resistance with plasmids pBT6, pCG1, and pCG7. As shown in Figure 2, approximately 250 benomyl-resistant colonies were selected on benomyl-amended regeneration medium in transformations with 20 µg of pBT6 DNA. These results indicate an efficiency of about 13 transformants per microgram of plasmid DNA. In some experiments, as many as 28 transformants per microgram of DNA were obtained and comparable efficiencies were routinely achieved in transformations with pCG1 and pCG7 (data not shown). Furthermore, transformation efficiencies in this range were obtained with less DNA; however, we consistently observed a two- to threefold decrease in the number of transformants when the amount of plasmid exceeded 20 µg (data not shown). For example, in individual transformations with 50–100 µg of plasmid, fewer than 100 benomyl-resistant colonies were recovered, and this was presumably due to precipitation of plasmid DNA observed during the addition of polyethylene glycol. Thus, in using the procedure, we find transformation to be optimal when the amount of DNA is maintained at about 20 µg. Our results, indicating that the same transformation efficiency can be obtained with the *N. crassa* and *C. graminicola* genes, suggest that heterologous genes from other Ascomycetes may function in *C. graminicola*.

Evidence for two β-tubulin genes in *C. graminicola* and the lack of plasmid integration at these loci. Gel blots containing undigested nuclear DNA from untransformed CgM2, eight pCG7 transformants, and eight pBT6 transformants were probed with pUC19, which shares extensive homology with vector sequences in pCG7 and pBT6. The results showed that the probe hybridized with the high molecular weight band (about 50-kb pairs), corresponding to chromosomal sequences of the transformants; no homology was detected between sequences in pUC19 and DNA from untransformed CgM2
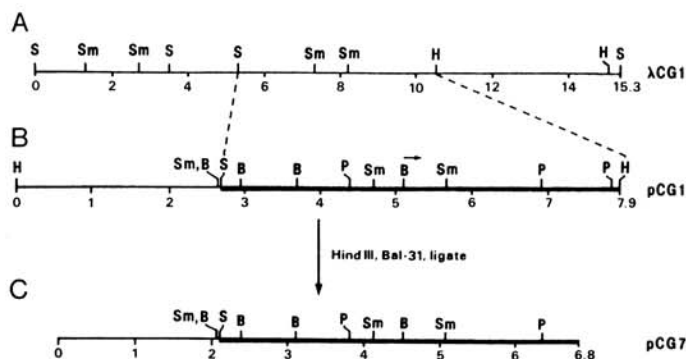

Fig.1. Cloning of the CgM2BmlR3 restriction fragment containing β-tubulin sequences. **A**, 15.3-kb *Sal*I insert of recombinant clone, λCG1. The internal 5.2-kb *Sal*I-*Hind*III fragment bracketed by the dashed lines indicates a portion of the insert that includes the sequences with homology to the *Neurospora crassa tub-2* allele in pBT6. **B**, The fragment was subcloned into pUC19 that had been cleaved with *Sal*I and *Hind*III to create plasmid pCG1; the arrow represents the direction and extent to which the insert was sequenced. **C**, pCG1 was modified with *Hind*III and Bal-31 to create plasmid pCG7. **B** and **C**, Bold lines represent *Colletotrichum graminicola* sequences, light lines represent pUC19 sequences. **A**, **B**, and **C**, The numbers below each map indicate the length and position of the coordinates in kilobase pairs. The relative positions of the restriction sites were deduced from the electrophoretic mobilities of fragments produced in single and double digests. Sites for restriction enzymes have the following designations: B, *Bam*HI; H, *Hind*III; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.
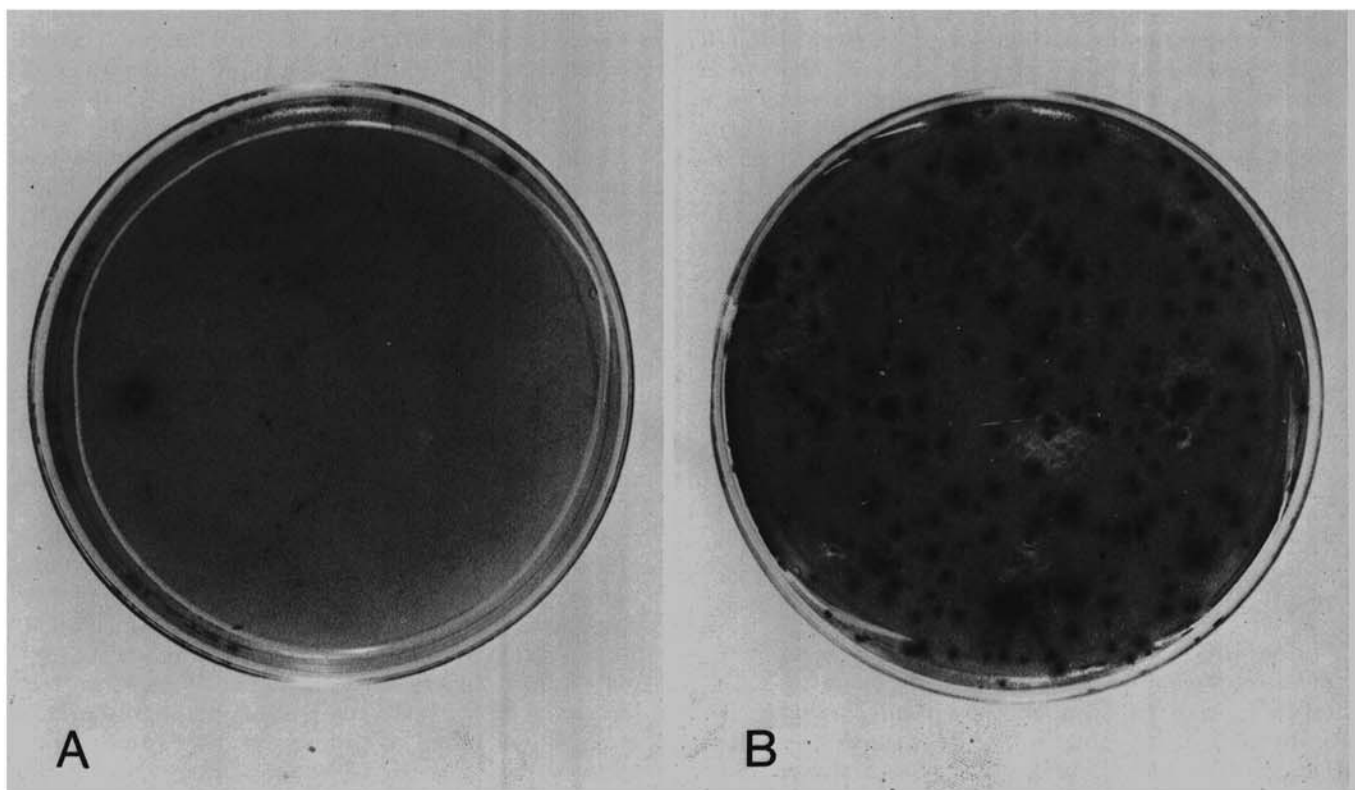
Fig. 2. Appearance of benomyl-resistant colonies regenerated from *C. graminicola* protoplasts after transformation. Plates with regeneration medium containing 0.5 μg/ml of benomyl were each overlayed with 5×10^7 protoplasts and incubated. **A,** Untreated control; **B,** Protoplasts were treated with 20 μg of pBT6 DNA.

(data not shown). This indicated that pCG7 and pBT6 integrated into chromosomal sequences and were not maintained as autonomously replicating plasmids.

The DNA sequences in *C. graminicola* strain CgM2 that hybridize with pCG7 and pBT6 represent potential integration sites if homologous recombination between the introduced DNA and regions of the genome were to occur. To examine this possibility, we prepared blots from two gels that each contained *Hind*III-digested DNA from CgM2 and the eight pCG7 transformants. Because *Hind*III does not cleave pCG7, its integration within a homologous sequence would be expected to reduce the electrophoretic mobility of a *Hind*III fragment containing the site of integration. Subsequently, one blot was probed with pCG7 and the other with pBT6 under conditions of high stringency. A comparison of the hybridization patterns obtained with DNA from each transformant, as represented by M4051, and those obtained with DNA from CgM2 are shown in Figure 3. When pCG7 was used as the probe, a 15-kb fragment detected in DNA from the CgM2 control was also detected in DNA from each transformant (Fig. 3, compare lanes 1 and 2). The hybridization to a single DNA fragment from CgM2, with a size of at least 10.5 kb, was expected based on the restriction map of the λCG1 insert (see Fig. 1A). In the case of each transformant, pCG7 appeared to have integrated within sequences represented by a strongly hybridizing region of the blot containing fragments larger than 15 kb (Fig. 3, lane 1). However, when the additional blot containing identical digests was probed with pBT6, we detected a sole, 2.3-kb fragment in DNA from the CgM2 control that was unaltered in the DNA from each transformant (Fig. 3, lanes 3 and 4). In this case, pCG7 sequences in each transformant were detected in a region of the blot containing fragments in the 9- to 22-kb size range
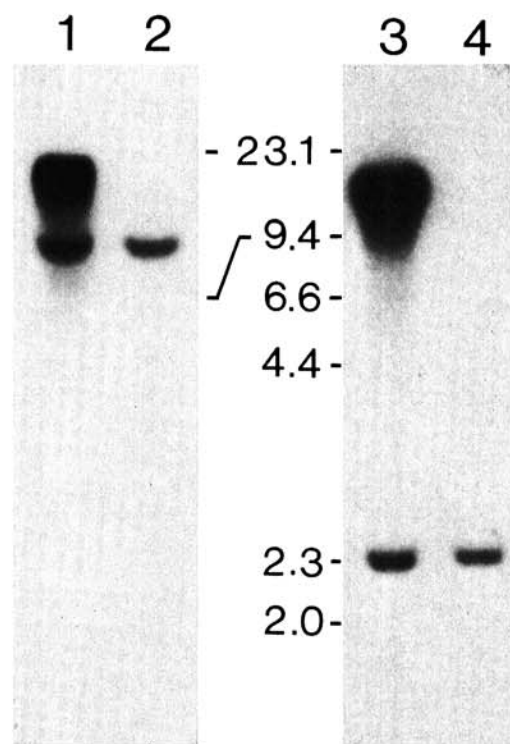

Fig. 3. Autoradiograms of gel blots containing *Hind*III-digested DNA from CgM2 and M4051, a representative pCG7 transformant. Lanes 1 and 2 contain DNA from M4051 and CgM2, respectively, and were probed with pCG7. Lanes 3 and 4 contain DNA from M4051 and CgM2, respectively, and were probed with pBT6. The samples contained 1 μg of DNA and were separated on 0.6% agarose gels before transfer and hybridization. The relative positions of size standards are in kilobase pairs. The gel used to prepare the blot probed with pCG7 was run for an extended period in order to separate the 15-kb fragment from higher molecular weight sequences detected in the hybridizations.

(Fig. 3, lane 3). Whereas these results indicate that pCG7 did not integrate in regions of the genome with homology to pCG7 or pBT6, they also suggest that the 2.3-kb band detected with the pBT6 probe may contain a β-tubulin gene that differs from that in pCG7.

A similar approach, involving *Sma*I digests, was used to determine whether pBT6 transformants contained plasmid DNA integrated within homologous sequences. Because *Sma*I does not cleave pBT6, its integration within a homologous sequence would be expected to reduce the electrophoretic mobility of a *Sma*I fragment containing the site of integration. Therefore, we prepared blots from duplicate gels that contained *Sma*I-digested DNA from CgM2 and each of the eight pBT6 transformants. One blot was then probed with pBT6 and the other was probed with pCG7. A comparison of the hybridization patterns obtained with DNA from each transformant, as represented by M2019, and those obtained with DNA from CgM2, are shown in Figure 4A. When *Sma*I digests from each of the eight pBT6 transformants were probed with pBT6 under conditions of high stringency, a 4-kb fragment detected in hybridizations with CgM2 DNA was also detected in DNA

Fig. 4. Autoradiograms from gel blots containing *Sma*I-digested DNA from CgM2 and M2019, a representative pBT6 transformant. **A,** Lanes 1 and 2 contain DNA from CgM2 and M2019, respectively, and were probed with pCG7. Lanes 3 and 4 contain DNA from CgM2 and M2019, respectively, and were probed with pBT6. **B,** Hybridizations were performed using conditions of lowered stringency. Lanes 1 and 2 both contain DNA from CgM2 and were probed with pCG7 and pBT6, respectively. **A** and **B,** The position of the 0.9-kb fragment with homology to pCG7 is indicated. Size standards and electrophoresis are described in Figure 3.

from each of the transformants (Fig. 4A, compare lanes 3 and 4). Integrations were detected based on hybridization in regions of the blot containing fragments in the 8.5- to 25-kb size range (Fig. 4A, lane 4). When identical blots were probed with pCG7 under the same conditions, fragments with approximate sizes of 0.9, 4.6, and 10 kb were detected in DNA from the CgM2 control and from each of the transformants (Fig. 4A, compare lanes 1 and 2). Hybridization to DNA fragments from CgM2 with sizes of 0.9 and 4.6 kb, and another with a size of at least 7.1 kb, was expected based on the restriction map of the λCG1 insert (see Fig. 1A). In the case of the transformants, integrations were detected in regions of the blot containing fragments in the 18- to 23-kb size range (Fig. 4A, lane 2). As with pCG7, transformation with pBT6 did not appear to involve integration within genomic sequences homologous to pBT6 or pCG7. Furthermore, these analyses of the pBT6 transformants also suggest the presence of distinct β-tubulin genes in *C. graminicola*.

As previously described and as illustrated in Figure 1, plaque hybridizations between *C. graminicola* genomic clones and pBT6 were used to isolate λCG1, from which pCG7 was derived. This inferred that the inserts in pBT6 and pCG7 were related, which was subsequently confirmed by DNA-sequence analysis. Accordingly, the results from hybridizations (Figs. 3 and 4A) that indicated the separate identity of *C. graminicola*-DNA sequences with homology to either pBT6 or pCG7 were unexpected. Presumably, the amount of homology between the inserts in pBT6 and λCG1, and plaque hybridization conditions involving lowered stringency, were sufficient to allow detection of *C. graminicola* sequences in λCG1 during the screening of the genomic clones. To examine the relationship further between cloned sequences in pBT6 and those from λCG1 in pCG7, additional Southern blot hybridizations were performed. Blots containing *Sma*I digests of DNA from untransformed CgM2 were individually probed with pCG7 and pBT6 under conditions of low stringency. Cross-hybridization was evident with either probe, but it was restricted to the 0.9-, 4.0-, and 4.6-kb fragments (Fig. 4B). Based on differences in the intensity of bands in the autoradiograms, however, pCG7 preferentially hybridized with the 0.9-, 4.6-, and 10-kb fragments (Fig. 4B, lane 1), whereas pBT6 preferentially hybridized with the 4-kb fragment (Fig. 4B, lane 2). That pBT6 did not hybridize to the 10-kb fragment with homology to pCG7 (Fig. 4B, compare lanes 1 and 2) can be explained on the basis of previous results. As shown in Figure 1C, the insert in pCG7 contains 1.7 kb of DNA that maps between the *Sma*I site at about nucleotide 5,100 and the end of the insert at nucleotide 6,800. This region, based on nucleotide-sequence data from pCG1, is believed to contain little, if any, β-tubulin coding sequence and is believed to reside downstream from the gene. Furthermore, it maps between nucleotides at about 8,200 and 9,900 in the λCG1 insert (Fig. 1A), indicating that it is contained within an *Sma*I fragment of at least 7.1 kb. Therefore, it is plausible that these additional sequences in pCG7 would be unrelated to any in pBT6 and that they would hybridize to a fragment the size of 10 kb. Thus, the results from these hybridizations provide additional evidence that *C. graminicola* contains two β-tubulin genes with somewhat divergent nucleotide sequence. Similar studies have demonstrated that *A. nidulans* has two divergent β-tubulin genes (May *et al.* 1985; May *et al.* 1987), whereas *N. crassa* (Orbach *et al.* 1986), *S. cerevisiae* (Neff *et al.* 1983), and *Schizosaccharomyces*
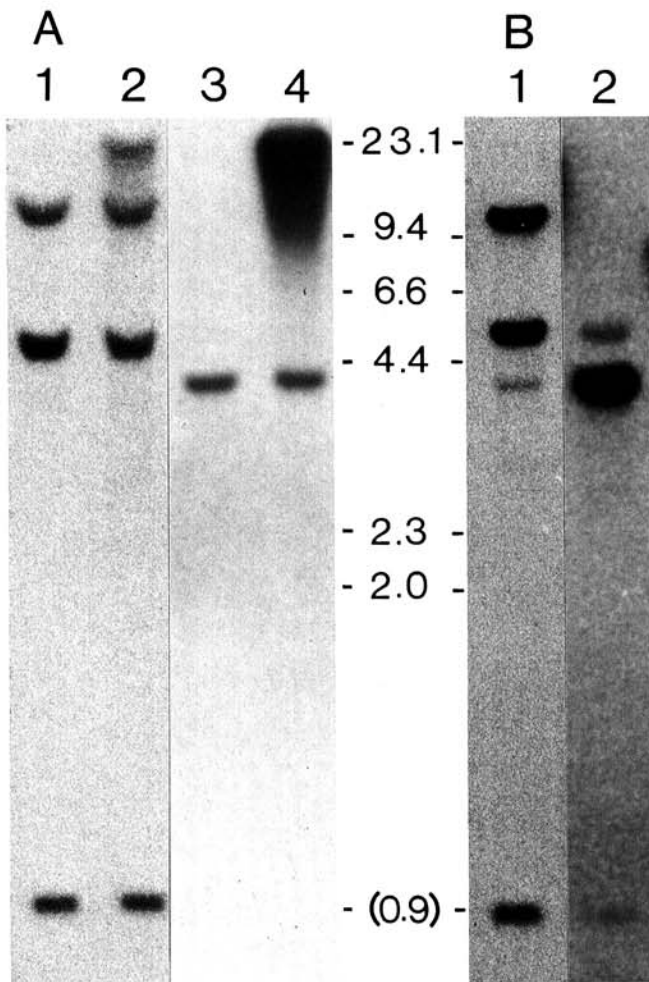
pombe (Hiraoka *et al.* 1984) appear to have only one. To verify that *C. graminicola* has more than one β-tubulin gene, we are currently cloning and characterizing those sequences that demonstrate greater homology with the *N. crassa tub-2* gene in pBT6.

Besides providing evidence for the existence of two β-tubulin genes in *C. graminicola*, these results clearly indicate that pBT6 and pCG7 have not integrated into sites of homology shared with the *C. graminicola* genome. However, we cannot rule out the possibility of gene conversion events (i.e., double-crossovers) that may have occurred between sequences in the plasmid inserts and the homologous sequences in the genome. Thus, these data suggest that the probability of achieving site-specific integrations with intact plasmid DNA is very low in this system. Results from studies of transformation in *A. nidulans* (Miller *et al.* 1985), *N. crassa* (Paietta and Marzluf 1985), and *M. grisea* (Parsons *et al.* 1987) have indicated that the frequency of site-specific integration can be increased when linear molecules, with free ends that are homologous to genomic sequences, are used in transformation. We have initiated experiments to determine if this approach can be used to produce gene replacements in *C. graminicola*.

Organization of introduced pCG7 and pBT6 sequences in *C. graminicola* transformants. To characterize the integration of plasmid sequences further, five of the eight

pCG7 transformants and five of the eight pBT6 transformants were randomly chosen for additional hybridization studies. In these experiments, pUC19 was used as a probe to avoid hybridization with indigenous β-tubulin sequences, thus simplifying the detection of fragments that contained the integrated plasmid DNA. In the case of the pCG7 transformants, nuclear DNA was digested with *Sal*I, which cleaves pCG7 in one location. The hybridization pattern from gel blots containing the digests indicated that integrations were different in each of the five transformants (Fig. 5A). Moreover, these results showed that multiple insertions occurred in most, if not all, transformants, with transformant M4043 as a possible exception (Fig. 5A, lane 1). Similar results were obtained in hybridizations involving DNA from pBT6 transformants. In this experiment, digests were performed with *Pst*I, which cleaves pBT6 in one location. As was the case with the pCG7 transformants, the hybridization patterns from gel blots containing the digests indicated that the pBT6 transformants differed from one another with respect to the number and sites of plasmid integration (Fig. 5B). In Figure 5, the presence of a strongly hybridizing band that is the same size as the plasmid used in transformation (i.e., 6.8 kb for pCG7 and 5.8 kb for pBT6) is evident in each case with the exception of transformant M2019 (Fig. 5B, lane 4). A band of this nature, which appears in digests in which plasmid sequences are cleaved once, is consistent with integration of
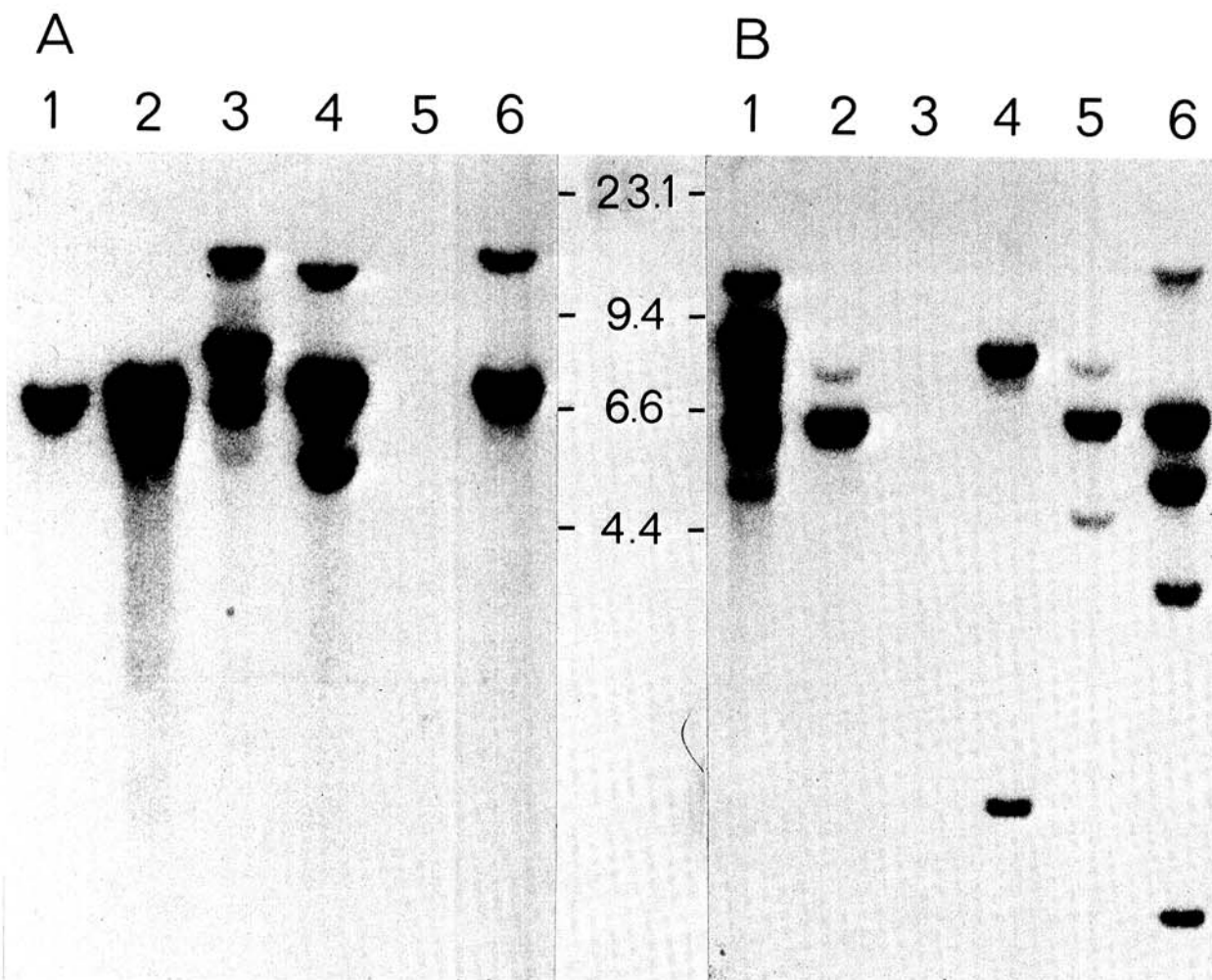

Fig. 5. Autoradiograms of gel blots probed with pUC19 showing differences in integration events among pCG7 and pBT6 transformants. **A,** *Sal*I digests of nuclear DNA from pCG7 transformants M4043, M4072, M4104, M4031, M4051 (lanes 1, 2, 3, 4, and 6, respectively) and CgM2 (lane 5). **B,** *Pst*I digests of nuclear DNA from pBT6 transformants M2014, M2018, M2019, M2020, M2021 (lanes 1, 2, 4, 5, and 6, respectively) and CgM2 (lane 3). **A** and **B,** Size standards and electrophoresis are described in Figure 3.

two or more tandem-arrayed copies of the plasmid at a single locus.

Mitotic stability and pathogenicity of transformants. Three of the eight pCG7 transformants and three of the eight pBT6 transformants were randomly chosen to study the mitotic stability of the benomyl-resistant phenotype. Each transformant was cultured on PDA in the absence of benomyl for 5 wk. Conidial suspensions prepared from the cultures were then used to subculture each transformant on identical medium; appropriate dilutions were employed to permit subsequent recovery of individual colonies, each derived from a single conidium. After germination of the conidia and limited colony growth, 25 colonies from each transformant were transferred to PDA that contained benomyl (0.5 μg/ml). For each of the three pCG7 transformants tested, all of the single conidial isolates maintained the benomyl-resistant phenotype. Of the three pBT6 transformants tested, 23 of 25, 24 of 25, and 25 of 25 single conidial isolates maintained the transformed phenotype. The high stability of the transformed phenotype indicates that pBT6 and pCG7 have excellent potential as vectors in experiments designed to study intraspecific complementation in *C. graminicola*. This is further supported by results obtained from greenhouse studies. In these experiments, we randomly chose five of the eight pBT6 transformants and five of the eight pCG7 transformants. Conidial suspensions from each were used to inoculate susceptible (Mo940) and resistant (33-16) cultivars. All of the transformants tested produced symptoms indistinguishable from untransformed CgM2 on cultivar Mo940, and all elicited the typical resistance response on cultivar 33-16 (data not shown). In addition, benomyl-resistant *C. graminicola* was isolated from all Mo940 plants that had been inoculated with transformants. Despite the random and multiple plasmid integrations that appear to be associated with most transformations, none of the transformants displayed differences in pathogenicity. Thus, it seems likely that these vectors will be suitable for future studies involving transfer of pathogenicity and host specificity genes between isolates of the fungus.

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