

## Transformation and Cotransformation of *Gaeumannomyces graminis* to Phleomycin Resistance

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### ABSTRACT

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*Gaeumannomyces graminis* var. *graminis* and *G. g. tritici* were transformed to phleomycin resistance by an improved transformation procedure with pAN8-1, a plasmid encoding the *ble* gene of *Streptoalloteichus hindustanus*. *G. g. graminis* was cotransformed with pAN8-1 and pBT3, a plasmid encoding resistance to benomyl. Vector

DNA apparently was integrated into the fungal genome in all transformants analyzed, at different sites in the genome and with varying copy numbers. The selected phenotypes (Phleo<sup>R</sup> or Ben<sup>R</sup>) were stable through mitosis in most transformants. Integrated plasmid DNA was stable through meiosis in all transformants tested.

*Additional keywords:* benomyl resistance, take-all, bleomycin.

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*Gaeumannomyces graminis* (Sacc.) von Arx & Oliver is a soil-borne, filamentous Ascomycete that parasitizes the roots, crowns, lower stems, and stolens of many members of the Gramineae

(reviewed in 6). *G. g. var. tritici*, the etiologic agent of take-all disease in wheat and barley, is considered a major root pathogen and limits cereal production in many areas of the world. *G. g. graminis* causes crown sheath rot of rice and has recently been implicated as a causative agent of spring dead spot of bermuda-

grass (17).

*G. graminis* is a homothallic organism that produces perithecia during its sexual stage (reviewed in 1). Each ascus contains eight randomly ordered ascospores. Genetic studies of filamentous fungi are facilitated by easily scored genetic markers, and the lack of defined mutants of *G. graminis* has hindered progress in this area. Only a single auxotroph of *G. graminis* has been reported (3). Selfed or crossed perithecia could be more easily distinguished by introducing selectable genetic markers into one or both parents of a cross. *G. graminis* also undergoes anastomosis (hyphal fusion), and complementation analysis could be conducted with appropriately marked parental strains.

We recently described a procedure (10) to transform *Gaeumannomyces* to benomyl resistance (Ben<sup>R</sup>) with pBT3 (19), a plasmid encoding fungicide-resistant  $\beta$ -tubulin. However, preliminary attempts to transform *G. graminis* to hygromycin- or G418-resistance with various vectors (5,20,25,28) were unsuccessful.

Bleomycin and phleomycin are closely related broad-spectrum antibiotics produced by strains of *Streptomyces verticillus*; they are effective against both prokaryotic and eukaryotic organisms (2). Phleomycin interacts with DNA of susceptible organisms and cleaves preferentially at inverted repeat sites in single-stranded DNA (26) and at unmethylated sites in double-stranded DNA (11). Bleomycin resistance genes from transposon Tn5 (4), *Staphylococcus aureus* plasmid pUB110 (23), and the chromosome of *Streptoalloteichus hindustanus* (8) recently have been characterized. The product of the bleomycin resistance gene from *S. hindustanus* reversibly binds bleomycin and prevents it from cleaving DNA (8). The proteins encoded by the *ble* genes from Tn5 and pUB110 exhibit a high degree of homology with the resistance protein from *S. hindustanus* (8).

Several vectors encoding phleomycin resistance have been used to transform or cotransform yeast (7) and filamentous fungi (12,15,16,27). pAN8-1 contains the *ble* gene of *S. hindustanus* flanked by the promoter region of the highly expressed *gpd* gene of *Aspergillus nidulans* and the terminator region of the *trpC* gene of *A. nidulans* (15). We report here improvement of our transformation procedure and successful transformation and cotransformation of *G. graminis* to phleomycin resistance (Phleo<sup>R</sup>) with pAN8-1. Phenotypic and genotypic stability of phleomycin and benomyl resistance through mitotic and meiotic divisions also is reported.

## MATERIALS AND METHODS

**Strains and media.** Strains of *G. g. tritici* (strain DM528) and *G. g. graminis* (strain DM562) were provided by Dr. D. E. Mathre and grown in complex L medium (18). Protoplasts were obtained as described previously and regenerated on protoplast regeneration medium (PRM) (24). Glucose-asparagine medium was used for perithecia development (13).

**Transformation to benomyl resistance.** Protoplasts of *Gaeumannomyces* sp. were transformed with plasmid pBT3 as described previously (10), with the following modifications. After transformation, protoplasts were washed once with stabilizing buffer, resuspended in 3 ml of PRM broth and incubated with shaking overnight at 28°C. Transformed protoplasts were pelleted for 5 min at 730 g and resuspended in 0.1 ml of PRM broth. Concentrated protoplasts then were spread onto PRM plates containing 1.0  $\mu$ g or 0.75  $\mu$ g of benomyl per milliliter, respectively, for *G. g. graminis* and *G. g. tritici*. Selective media were prepared from a 0.1% (w/v) solution of technical grade benomyl (a gift from Dupont, Wilmington, DE) in 95% ethanol. Protoplasts of *G. g. tritici* were overlaid with 10 ml of PRM containing 0.5  $\mu$ g benomyl per milliliter 3–5 days after plating. Protoplasts of *G. g. graminis* were not overlaid. Four transformants (strains JH2437-2439 and JH2442) of *G. g. tritici* were obtained following transformation with linear pBT3.

**Transformation to phleomycin resistance.** Protoplasts of *G. graminis* were transformed with approximately 3  $\mu$ g of uncut pAN8-1 or pAN8-1 linearized by digestion with *EcoRI*, a restriction enzyme that does not cut within the phleomycin

resistance gene, with the procedure developed for transformation with pBT3. Transformants of *G. g. graminis* were obtained from circular pAN8-1 (strains JH2000-2002, JH2014, JH2016, and JH2026-2028) and linear pAN8-1 (strains JH2023-2025). Transformants of *G. g. tritici* were also obtained from both circular pAN8-1 (strains JH2020-2021 and JH2031-2032) and linear pAN8-1 (strains JH2018-2019 and JH2029-2030). Transformants were selected on PRM containing 50  $\mu$ g of phleomycin per milliliter, prepared from a 1% solution of phleomycin (Cayla Laboratories, Toulouse, France) in sterile water. It was not necessary to overlay phleomycin plates. Resistant colonies, which grew larger than small background colonies, were transferred to solid L medium containing 20  $\mu$ g of phleomycin per milliliter.

**Cotransformation.** Protoplasts of *G. g. graminis* were cotransformed with 3  $\mu$ g of circular pAN8-1 and 3  $\mu$ g of circular pBT3. Transformants (strains JH1902-1911) were selected on PRM containing 1.0  $\mu$ g of benomyl per milliliter. Ben<sup>R</sup> colonies were then scored for phleomycin resistance on solid L medium containing 20  $\mu$ g of phleomycin per milliliter.

**Perithecia development.** Meiosis was induced on glucose-asparagine plates with a 12-hr photoperiod from one General Electric cool-white bulb, F40CW, and one General Electric warm-white bulb, F40WW, approximately 35 cm from plates, which were incubated right side up (13). Ascospores were isolated by a modification of Rossman's single-spore isolation technique (22). Mature perithecia were placed in a drop of water on a sterile microscope slide and squashed under a sterile coverslip with a pencil eraser. The drop of water then was spread onto 1% agar plates. After 24 hr, germinating ascospores were transferred to nonselective L medium. Following growth they were tested for resistance on selective L medium (0.8  $\mu$ g of benomyl per milliliter or 20  $\mu$ g of phleomycin per milliliter).

**Plant inoculation and fungal reisolation.** Wheat or rice seeds were surface-sterilized in 1% AgNO<sub>3</sub> for 3 min and washed with distilled water. Sterile seeds were placed in glass jars (200 ml) containing 50 ml of 1% agar and several 1-mm  $\times$  1-cm plugs of *G. g. graminis* or *G. g. tritici*. Plants were grown with the same light regimen used to produce fungal perithecia. Disease symptoms were observed within 2–3 wk. Fungi were reisolated by removing crown sheath leaves and placing them directly onto selective L medium with 0.8  $\mu$ g of benomyl per milliliter or 20  $\mu$ g of phleomycin per milliliter or onto nonselective L medium followed by transfer to selective L medium.

**Isolation and analysis of DNA.** DNA was extracted from mycelia grown in 100-ml cultures of L broth without selective pressure as described previously (10). DNA samples were digested with restriction enzymes according to the manufacturers' directions. Electrophoresis and hybridization of dried gels were performed as described (9). Each lane of agarose gels contained 1–2  $\mu$ g of fungal genomic DNA. Plasmid DNA was purified according to standard procedures (14) and was nick translated with <sup>32</sup>P-thymidine triphosphate (21).

## RESULTS

*G. graminis* was inhibited by low concentrations of benomyl (0.75–1.2  $\mu$ g/ml) (10) and phleomycin (20  $\mu$ g/ml). We transformed and cotransformed *G. graminis* with pAN8-1, a plasmid that carries a phleomycin resistance gene (*ble*) from *S. hindustanus* (15) and with pBT3, a plasmid that carries a benomyl-resistance gene (*tub-2*) from *Neurospora crassa* (19). We included the following alterations in the transformation procedure used in earlier experiments (10). Instead of Benlate (a water-soluble compound with 50% active benomyl), we used benomyl solubilized in ethanol. Protoplasts were spread directly onto the surface of selective plates instead of adding protoplasts to molten agar overlays. Finally, we allowed resistance genes to be expressed overnight before we plated protoplasts. These modifications of the original transformation procedure improved the consistency of transformation experiments, and, although the frequency of transformation of *G. g. graminis* was not higher than previously observed (10), we were able to obtain greater numbers of trans-

formants of *G. g. tritici* using the improved protocol. The frequency of transformation of both *G. g. tritici* and *G. g. graminis* with pAN8-1 was approximately 1 transformant per microgram of circular DNA. Results with linear DNA were not significantly different.

DNA was isolated from wild-type *G. g. graminis* and *G. g. tritici* and from 11 Phleo<sup>R</sup> *G. g. graminis* and nine Phleo<sup>R</sup> *G. g. tritici* colonies transformed with linear or circular pAN8-1. Uncut DNA was probed with pAN8-1 to demonstrate the presence of the transforming vector in the Phleo<sup>R</sup> transformants and its absence in nontransformed wild-type mycelium (data not shown). Homology was present only in high molecular weight DNA from Phleo<sup>R</sup> transformants, suggesting that the vector integrated into the fungal genome of each transformant and did not replicate autonomously.

To determine the number of sites of integrated pAN8-1 in transformants, DNA was digested with *Hind*III, an enzyme that does not cut within the vector, and hybridized with <sup>32</sup>P-labeled pAN8-1 (Fig. 1 and less exposed autoradiographs of Fig. 1, data not shown). Many of the pAN8-1 transformants (e.g., strains JH2016,

JH2029, and JH2030) had only a single *Hind*III fragment that showed homology with the probe, suggesting that these transformants had a single site of plasmid integration. Strains JH2018, JH2002, and JH2028 contained at least two fragments homologous to pAN8-1, suggesting that they had at least two sites of vector integration. The sites of integration varied in transformants as evidenced by the different sizes of homologous fragments. Because pAN8-1 (~5.9 kb) is not cut by *Hind*III, homologous fragments were, as expected, larger than the transforming vector. However, one transformant, strain JH2027, displayed only a smaller 4.4-kb homologous fragment, suggesting that a complete copy of pAN8-1 was not present in this strain.

DNA from phleomycin-resistant transformants also was digested with *Nco*I, an enzyme that cuts once within pAN8-1, and hybridized with labeled pAN8-1 (Fig. 2). If only one copy of the vector DNA was integrated into the fungal genome, two homologous fragments would be expected in transformant DNA. Transformants with three or more homologous *Nco*I fragments probably contained multiple copies of integrated vector DNA. Both single (e.g., strains JH2016, JH2026, JH2023) and multiple

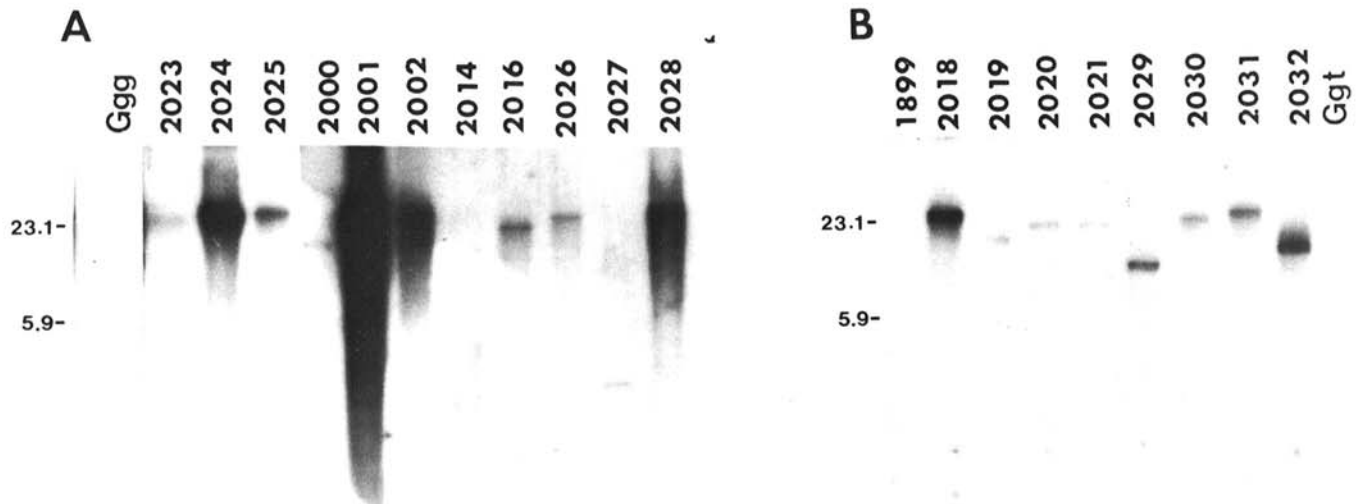


Fig. 1. Hybridization of labeled pAN8-1 with control *Gaeumannomyces graminis* and transformant genomic DNA, which was digested with *Hind*III, an enzyme that does not cut pAN8-1. **A**, *G. g. graminis* (Ggg) control and transformants. **B**, *G. g. tritici* (Ggt) control and transformants. Molecular weight markers are in kilobase pairs.

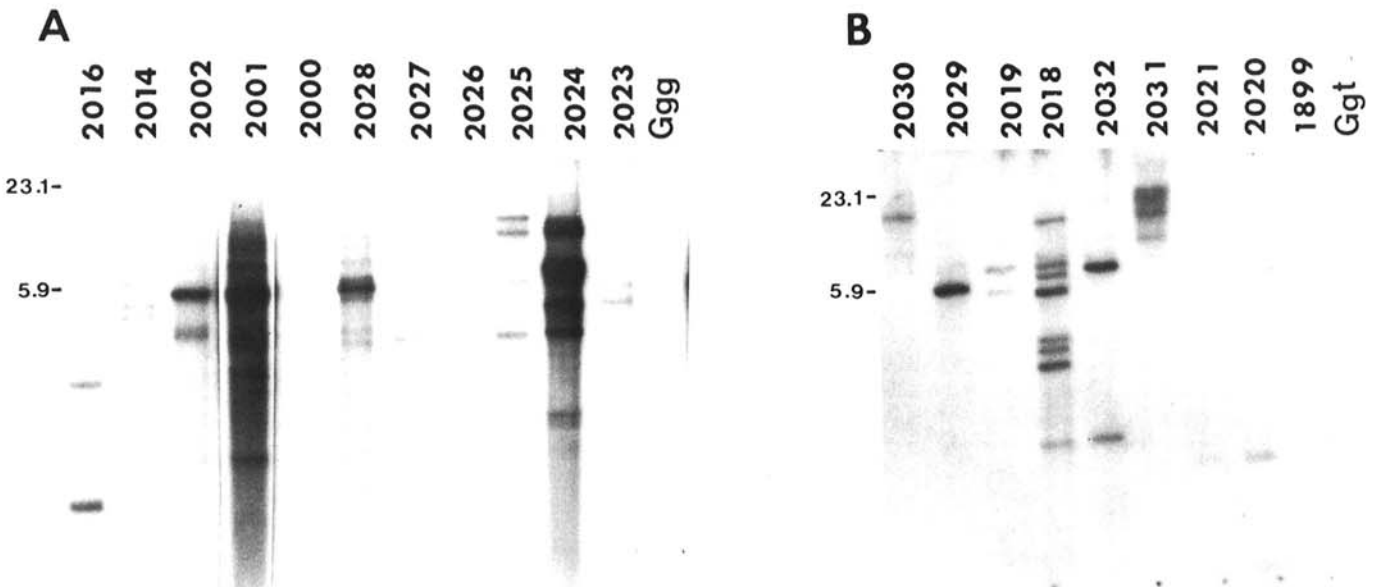
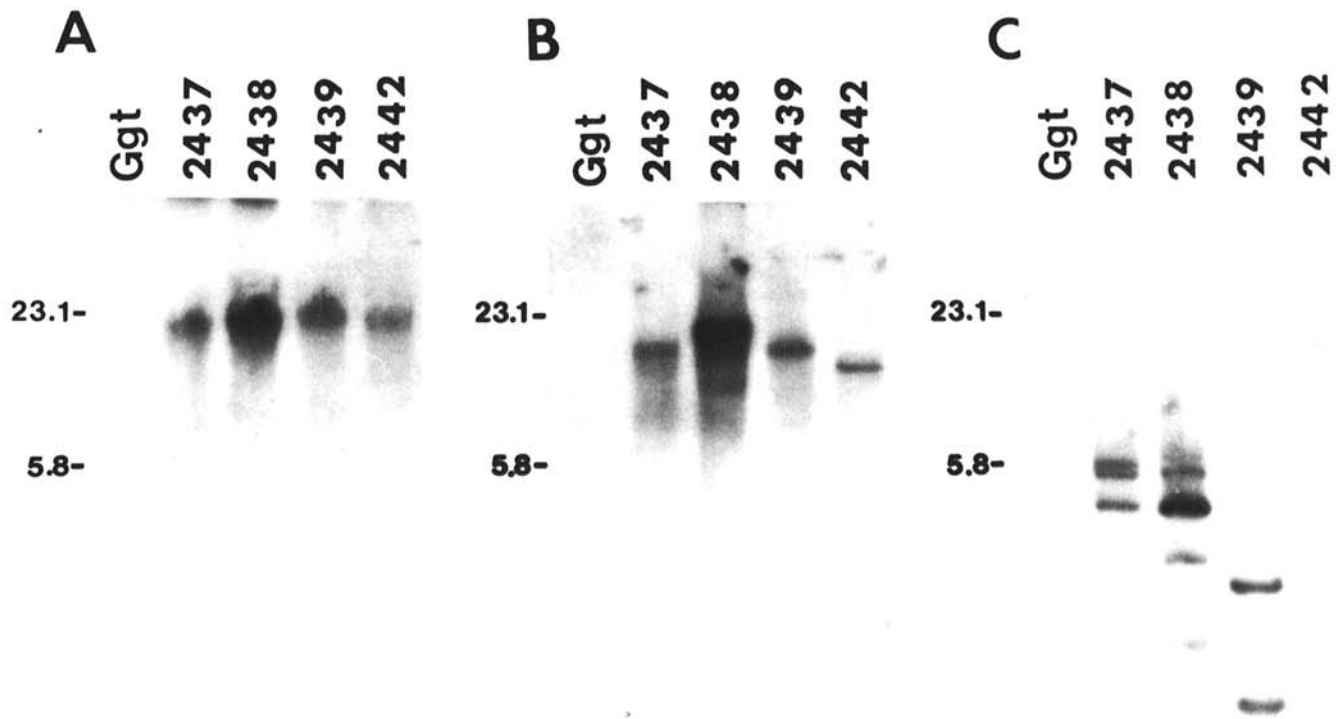


Fig. 2. Hybridization of labeled pAN8-1 with control *Gaeumannomyces graminis* and transformant DNA, which was digested with *Nco*I, an enzyme that cuts once within pAN8-1. **A**, *G. g. graminis* (Ggg) control and pAN8-1 transformants. **B**, *G. g. tritici* (Ggt) control and pAN8-1 transformants. Molecular weight markers are in kilobase pairs.



**Fig. 3.** Hybridization of labeled pBT3 with genomic DNA from control *Gaeumannomyces graminis* var. *tritici* (Ggt) and pBT3 transformants. **A**, Uncut DNA. **B**, DNA digested with *EcoRV*, an enzyme that does not cut pBT3. **C**, DNA digested with *NcoI*, an enzyme that cuts pBT3 once. Molecular weight markers on the left are in kilobase pairs.

(e.g., strains JH2025, JH2024, JH2018) insertions of pAN8-1 were observed in transformants. Which type of insertion occurred did not correlate well with whether circular or linear transforming DNA was used. This is in contrast to earlier transformations of *G. g. graminis* with pBT3, where circular transforming DNA resulted in more transformants with multiple, tandemly repeated plasmid insertions (10).

We previously had difficulty obtaining transformants of *G. g. tritici* (10). Improvements described in this paper allowed us to transform *G. g. tritici* to Ben<sup>R</sup> as well as to Phleo<sup>R</sup> with greater efficiency. DNA from four Ben<sup>R</sup> isolates of *G. g. tritici* transformed with linear pBT3 also was analyzed (Fig. 3). Homology was present only in high molecular weight uncut DNA of all four transformants, suggesting that pBT3 had integrated into the fungal genome. DNA also was restricted with *EcoRV*, an enzyme that does not cut pBT3, and probed with pBT3 (Fig. 3). Each of the transformants appeared to have pBT3 integrated at a single site in the fungal genome. To determine the copy number of the transforming DNA, the DNA was cut with *NcoI*, an enzyme that cuts once within pBT3 (Fig. 3). Strains JH2439 and JH2442 were apparently transformed with a single copy of pBT3, whereas strains JH2437 and JH2438 contained multiple copies of pBT3.

We also successfully cotransformed *G. g. graminis* to Phleo<sup>R</sup>. When pAN8-1 and pBT3 plasmids were used together to transform protoplasts of *G. g. graminis* to Ben<sup>R</sup>, 15/115 transformants also were Phleo<sup>R</sup>. Phleo<sup>R</sup>-Ben<sup>R</sup> cotransformants grew on L medium containing 0.8 μg of benomyl and 20 μg of phleomycin per milliliter.

To demonstrate the presence of pAN8-1 DNA in the cotransformants, genomic DNA from 10 Phleo<sup>R</sup>-Ben<sup>R</sup> cotransformants was digested with *NcoI* and *EcoRI*. pAN8-1 digested with *NcoI* and *EcoRI* is cut into two fragments of 3.5 and 2.4 kb, whereas pBT3 is cut into a 4.0-kb fragment and a 1.8-kb fragment. Digested DNA was then probed with pAN8-1. A 3.5-kb band and a 2.4-kb band were observed in all cotransformants tested, confirming the presence of pAN8-1 (data not shown). When gels were probed

**TABLE 1.** Mitotic stability of selected phenotype in transformants of *Gaeumannomyces graminis*

Transformant	Axenic culture		Reisolated from infected host	
	Phleo <sup>R</sup>	Ben <sup>R</sup>	Phleo <sup>R</sup>	Ben <sup>R</sup>
JH1902-1903	—	+	+	+
JH1904-1908	+	+	+	+
JH2023-2028, JH2000-2002, JH2016,2018	+	NA <sup>a</sup>	+	NA
JH2020-2021, JH1899	+	NA	NT <sup>b</sup>	NA
JH2031-2032	—	NA	NT	NA
JH2018-2019, JH2029-2030	+	NA	NT	NA
JH2437-3439, JH2442	NA	+	NA	NT

<sup>a</sup>NA = not applicable.

<sup>b</sup>NT = not tested.

with pBT3, the expected pBT3-homologous fragments were detected in all but one cotransformant (strain JH1909).

The axenic mitotic stability of phleomycin-resistance and benomyl-resistance was determined by transferring 1-mm × 1-cm plugs from edges of transformant colonies at least three times onto complex L medium in the absence of selective pressure and then transferring them back onto appropriate selective media. Phleomycin resistance was mitotically stable in 18/20 (90%) of the pAN8-1 transformants tested (Table 1). Benomyl resistance

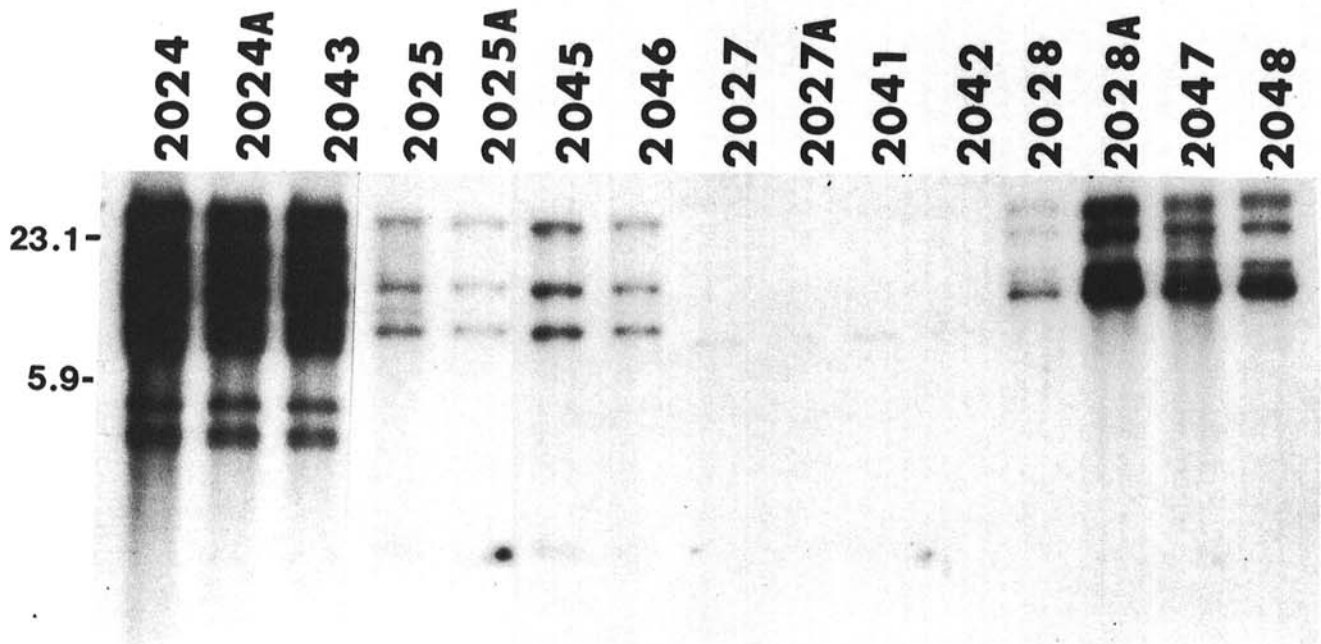


Fig. 4. Hybridization of labeled pAN8-1 with genomic DNA from pAN8-1 transformants and ascospore cultures derived from them. Strains JH2024A and JH2043 are ascospore derivatives of strain JH2024; strains JH2025A, JH2045, and JH2046 are ascospore derivatives of strain JH2025; strains JH2027A, JH2041, and JH2042 are ascospore derivatives of strain JH2027; and strains JH2028A, JH2047, and JH2048 are ascospore derivatives of strain JH2028. DNA has been digested with *Bgl*II, an enzyme that cuts once within pAN8-1. Molecular weight markers are in kilobase pairs.

was mitotically stable in all four pBT3 transformants tested. Twenty-five percent (2/8) of the cotransformants lost phleomycin resistance but not benomyl resistance following axenic culturing.

The stability of the transformant and cotransformant phenotype also was determined following plant inoculation and fungal reisolation (Table 1). Both phleomycin and benomyl resistance appeared to be stable in fungi reisolated from infected plants.

Ascospore cultures of transformants and cotransformants were obtained on nonselective medium and transferred onto selective media to determine if the selected phenotypes were stable through meiosis. DNA from 11 ascospore cultures derived from pAN8-1 transformants was cut with *Bgl*II and probed with pAN8-1 (Fig. 4). Hybridization patterns were compared with the original transformant to determine the meiotic stability of the transformant genotype. In each transformant tested, ascospore cultures apparently retained the hybridization pattern of the original transformant.

Transformants were also tested for their pathogenic potential on host (rice or wheat) plants. All transformants appeared to be as pathogenic as their wild-type parent of *G. g. tritici* or *G. g. graminis*. Disease symptoms were observed on host plants 2 wk after inoculation and included blackening of crown and root tissue. Wilting also was evident in wheat seedlings infected with *G. g. tritici*.

## DISCUSSION

Modifications of the original transformation protocol enabled us consistently to obtain Phleo<sup>R</sup> or Ben<sup>R</sup> transformants of *G. g. graminis* and *G. g. tritici* with pAN8-1 or pBT3, respectively. Integration of transforming sequences occurred at different sites and with various copy numbers in the fungal genome. We did not observe a correlation between copy number and the use of linear versus circular transforming DNA. We did, however, note a correlation between copy number and drug resistance. In general, transformants that displayed better growth on benomyl or phleomycin contained more copies of pBT3 or pAN8-1, respectively. Autonomously replicating vectors were not observed.

*G. g. graminis* also was cotransformed with pAN8-1 and pBT3. Approximately 13% (15/115) of the transformants selected on media containing benomyl also were resistant to phleomycin. With

other plasmids, cotransformation with pBT3 was observed in up to 80% of the transformants (unpublished data).

The Phleo<sup>R</sup> and Ben<sup>R</sup> phenotypes of transformants and cotransformants were stable through mitosis in most transformants, although two strains lost Phleo<sup>R</sup> following several transfers onto nonselective media. Phleo<sup>R</sup> was also phenotypically stable through meiosis in all transformants tested.

Very few genetic studies of *G. graminis* have been conducted. The availability of selectable markers should facilitate genetic analysis and provide a basis to study pathogenic determinants and host-parasite interactions of this fungus. The meiotic stability of vector DNA in transformants will enable us to distinguish between crossed and selfed perithecia in matings between two genetically marked strains. The use of marked strains also will allow us to select heterokaryons and thus conduct complementation analysis. Finally, integration of the transforming vector into different sites of the fungal genome may permit isolation of additional mutants and characterization of specific genes.

**Note in added proof:** DNA from parent transformants and ascospore cultures derived from them also was cut with *Hpa*II, a methylation sensitive enzyme and *msp*I, a methylation insensitive enzyme. In all cases the hybridization pattern observed for the parental culture was also observed for the derived ascospore cultures (data not shown).

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