Transformation of Gaeumannomyces graminis and the fate of transforming DNA
by Alice LaRayne Pilgeram

A thesis submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy Plant Pathology
Montana State University

Abstract:
Gaeumannomyces graminis, a homothallic plant pathogenic ascomycete, was recently transformed to benomyl resistance using plasmid pBT3 or to phleomycin resistance using plasmid pAN8-l. Outcrossing is infrequent in G. graminis, but could be detected using our marked strains. One to one segregations of both benomyl and phleomycin resistance were observed in the ascospore progeny. Transforming DNA also segregated in the outcrossed progeny, although DNA homologous to pAN8-1 was occasionally deleted or rearranged.

Integration of transforming DNA into a gene would most likely disrupt its expression. A benomyl resistant auxotroph of G g. tritici transformed with a single copy of the benomyl resistance gene (TUB2) was identified and found to require nicotinic acid (Nic2). TUB2 and Nic2 were inseparable in crosses between the auxotroph and a wild-type strain or in crosses between the auxotroph and a phleomycin resistant transformant with a single integrated copy of the phleomycin resistance gene (BLE). Hence, the two genes are tightly linked and the requirement for nicotinic acid could be due to insertional inactivation of the Nic2 gene by transforming DNA.

The meiotic stability of transforming DNA in selfed progeny of G. graminis was also investigated. The level of phleomycin resistance was unaltered in selfed progeny from G. graminis transformed with plasmid pAN8-l, but rearrangements and deletions of transforming DNA were observed in progeny. The meiotic stability of transforming DNA in BenR transformants was dependent upon the copy number of the transforming plasmid. The level of benomyl resistance was unchanged in selfed progeny from a G. g. tritici transformant with a single integrated copy of TUB2, whereas, ascospore derivatives of G. graminis transformants with multiple integrated copies of pBT3 were less resistant to benomyl than their parent. As expected transforming DNA was rearranged or deleted in progeny with decreased levels of resistance. Benomyl resistance continued to diminish in the second generation and deletions of transforming DNA continued to occur.

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A thesis submitted in partial fulfillment
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Doctor of Philosophy

in

Plant Pathology

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 1991

APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ACKNOWLEDGEMENTS

I would like to express my appreciation to my husband, Dan, for his support and patience and to my parents, Ann and Rayne Pilgeram, for the encouragement they have given me my entire life.

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ABSTRACT

Gaeumannomyces graminis, a homothallic plant pathogenic ascomycete, was recently transformed to benomyl resistance using plasmid pBT3 or to phleomycin resistance using plasmid pAN8-1. Outcrossing is infrequent in *G. graminis*, but could be detected using our marked strains. One to one segregations of both benomyl and phleomycin resistance were observed in the ascospore progeny. Transforming DNA also segregated in the outcrossed progeny, although DNA homologous to pAN8-1 was occasionally deleted or rearranged.

Integration of transforming DNA into a gene would most likely disrupt its expression. A benomyl resistant auxotroph of *G. g. tritici* transformed with a single copy of the benomyl resistance gene (*TUB2*) was identified and found to require nicotinic acid (*Nic2*). *TUB2* and *Nic2* were inseparable in crosses between the auxotroph and a wild-type strain or in crosses between the auxotroph and a phleomycin resistant transformant with a single integrated copy of the phleomycin resistance gene (*BLE*). Hence, the two genes are tightly linked and the requirement for nicotinic acid could be due to insertional inactivation of the *Nic2* gene by transforming DNA.

The meiotic stability of transforming DNA in selfed progeny of *G. graminis* was also investigated. The level of phleomycin resistance was unaltered in selfed progeny from *G. graminis* transformed with plasmid pAN8-1, but rearrangements and deletions of transforming DNA were observed in progeny. The meiotic stability of transforming DNA in BenR transformants was dependent upon the copy number of the transforming plasmid. The level of benomyl resistance was unchanged in selfed progeny from a *G. g. tritici* transformant with a single integrated copy of *TUB2*, whereas, ascospore derivatives of *G. graminis* transformants with multiple integrated copies of pBT3 were less resistant to benomyl than their parent. As expected transforming DNA was rearranged or deleted in progeny with decreased levels of resistance. Benomyl resistance continued to diminish in the second generation and deletions of transforming DNA continued to occur.

CHAPTER ONE

INTRODUCTION

Molecular Genetics of Plant Pathogenic Fungi

DNA of interest can be incorporated into plasmid constructs containing selectable markers and introduced into fungal genomes by transformation or cotransformation (Fincham 1989). Selectable markers include genes that complement specific mutations in the recipient, genes which confer resistance to antibiotics or fungicides, and genes which permit growth on novel substrates. A number of plant pathogenic fungi have been transformed with constructs encoding hygromycin B resistance (*hygB*), benomyl resistance (*TUB2*), or phleomycin resistance (*ble*) (Table 1). Prototrophy has been restored to arginine auxotrophs of *Magnaporthe grisea* (Parsons *et al.* 1987) and *Nectria haematococca* (Van Etten and Kistler 1988) following transformation with the *argB* gene of *Aspergillus nidulans*. The *AmdS* gene of *A. nidulans* codes for acetamidase, which cleaves acetamide into acetate and ammonia (Hynes *et al.* 1983). Acquisition of *AmdS* enables a fungus to utilize acetamide as the sole source of carbon and/or nitrogen. *Cochliobolus heterostrophus* (Turgeon *et al.* 1985) and *Glomerella cingulata* f.sp.

phaseoli (*Colletotrichum lindemuthian*) (Rodriguez and Yoder 1987) have been transformed with *AmdS*.

Transforming DNA usually integrates into the chromosomal DNA of filamentous fungi (Fincham 1989; Wang and Leong 1989). Integration of transforming DNA can occur at homologous or heterologous sites in the fungal genome (Fincham 1989). Homologous recombination is the result of crossing over between a resident sequence in the fungal genome and its homolog on the transforming vector, generating a tandem array of native and transforming sequences. The resident gene also can be replaced by its homolog without integration of vector sequences (gene conversion). Transforming DNA can integrate into nonhomologous regions of the chromosome. The relative frequency of the mode of integration is dependent upon the fungus and the transforming vector.

Gene transfer systems facilitate the identification and characterization of genes necessary for the growth and pathogenicity of filamentous fungi. Genes which complement defined mutations in recipient strains can be isolated following transformation. The *Ustilago maydis PYR3* gene, which encodes an enzyme in the pyrimidine biosynthetic pathway, has been cloned (Banks and Taylor 1988). A *U. maydis* genomic library was constructed and transformed into a *pyrC* mutant of *Escherichia coli*. The clone containing the *PYR3* gene was reisolated from three different *E. coli* prototrophs. The reisolated clone complemented a *Saccharomyces cerevisae ura4* mutation, as well as a *U. maydis pyr3-1* pyrimidine auxotroph.

Uncharacterized DNA fragments can be incorporated into transforming vectors encoding a selectable marker. Fungi, transformed with these vectors, can be identified by selecting for the dominant marker, and the influence of the unknown DNA fragment determined. *Colletotrichum lagenarium* is the causative agent of anthracnose of cucumber. Mutants incapable of melanin biosynthesis, cellulase synthesis and secretion, or penetration peg formation are avirulent on cucumber (Kubo *et al.* 1991). An albino mutant (melanin-) of *C. lagenarium* was transformed with a cosmid library of wild-type DNA, constructed in pKVβ, which contains a benomyl resistance gene (*TUB2*) as a selectable marker. Seven BenR, dark brown (melanin+) transformants, which formed melanized apressoria were isolated and all were able to penetrate host tissue.

In response to stress, injury or invading organisms, *Pisum sativum* (pea) produces pisatin, a phytoalexin inhibitory to most fungi. *N. haematococca* detoxifies pisatin to a less toxic compound thus allowing the fungus to infect the plant and cause a stem and root rot. The fungal gene encoding pisatin demethylating activity (*PDA*) was isolated by transforming an auxotroph of *A. nidulans* (Trp-/pisatin sensitive) with a genomic library of *N. haematococca* DNA constructed in a cosmid carrying the *A. nidulans trpC* gene and selecting for prototrophy (Weltring *et al.* 1988). Prototrophs were screened for sensitivity to pisatin and a single transformant carrying a *PDA* gene was isolated. *C. heterostrophus*, a fungal pathogen of maize, and *A. nidulans*, a saprophyte, were transformed with the *PDA* gene and inoculated onto pea plants (Schäfer *et al.*

1989). Symptoms were observed on plants inoculated with recombinant *C. heterostrophus* but not on plants inoculated with recombinant *A. nidulans*. Evidently *PDA* can expand the host range of an existing fungal pathogen but does not confer pathogenicity on a saprophyte.

The diploid formed by the fusion of two haploid strains of *U. maydis* of opposite mating type is pathogenic and causes corn boil smut. The *a* mating type locus controls the fusion of two haploids, whereas, the *b* mating type locus determines pathogenicity and sexual development. Diploids homozygous at the *b* locus are nonpathogenic and form yeast-like colonies. Heterozygous diploids are pathogenic and form mycelia. Kronstad and Leong (1989) constructed a cosmid library from a *b1* haploid and transformed a *a1/a1 b2/b2* diploid strain. A single mycelial colony was isolated among 960 transformants. A subclone of the cosmid was used to screen a library prepared from a *b2* haploid and a single homologous fragment identified, presumably the *b2* locus. The identities of the *b1* and *b2* loci were confirmed by the colony morphology and pathogenicity of transformed homozygous diploids.

Nonselectable genes can also be introduced into fungal genomes via cotransformation. Cells capable of taking up a selectable plasmid will frequently take up additional plasmids (Fincham 1989; Wang and Leong 1989; Werners *et al.* 1987). A cDNA clone of the cutinase gene was isolated from *N. haematococca* and cotransformed along with a plasmid encoding the hygromycin resistance gene (*hygB*) into *Mycosphaerella* spp., a pathogen of injured papaya fruit (Dickman *et*

al. 1989). Hygromycin resistant transformants were screened for the cutinase gene. Four transformants expressed cutinase and acquired the ability to attack papaya in the absence of preexisting wounds.

Integration of transforming DNA into a gene or its regulatory sequences would most likely disrupt its expression. The flanking sequences of two genes, *uapA* (uric acid-xanthine permease) (Diallinas and Scazzocchio 1989) and the *wA* (white conidiospore) (Tilburn *et al.* 1990), from *A. nidulans* and two genes, *preg* and *pgov* (phosphorous acquisition) (Kang and Metzenberg, personal communication), from *N. crassa* have been cloned following insertional inactivation. The intact genes of interest have then been isolated from genomic libraries using hybridization.

Stability of Transforming DNA

Transforming DNA is mitotically stable in most ascomycetes following axenic culturing (Keller *et al.* 1991; Panaccione *et al.* 1988; Selker and Garrett 1988; Tilburn *et al* 1983). Royer *et al.* (1991) observed that integrated DNA in transformants of *Ophiostoma ulmi* with a single copy of transforming vector were mitotically stable following growth in minimal media, whereas the hybridization pattern of multiple-copy transformants was altered. However, instability of transforming sequences has been observed following disease cycles. Deletion of integrated *hygB* DNA (hygromycin resistance) was observed in *C. heterostrophus* following seven disease cycles on maize (Keller *et al.* 1991). Alterations in the

hybridization patterns of integrated DNA in transformants of *F. moniliforme* and *F. graminearum* were observed following infection of maize plants (Dickman and Partridge 1989).

Transforming DNA is not always meiotically stable in filamentous fungi (Fincham 1989). Gene duplications arise when a strain is transformed with a gene it already possesses or multiple copies of transforming DNA integrate into a genome. These duplications are frequently modified during meiosis in several filamentous ascomycetes.

Duplicated sequences in *N. crassa* were both deleted and heavily methylated between fertilization and karyogamy leading to numerous G:C to A:T transitions which are irreversible (RIP, repeat induced point mutations (Selker and Garrett 1988)). Introduction of a non-duplicated sequence into *N. crassa* did not lead to RIP.

Duplicated sequences were also methylated in *Ascobolus immersus*, but not irreversibly (Goyon and Faugeron 1989). Ascospore cultures derived from *met2+* transformants of *A.immersus* with a nonfunctional resident copy of the *met2* were frequently methionine auxotrophs, due to extensive methylation of both copies of the *met2+* gene. Methylation was not accurately maintained during subsequent mitotic growth and the *met2+* gene could be reactivated. Premeiotic pairing of duplicated sequences was a prerequisite for inactivation (Faugeron *et al.* 1990). A methylated copy could pair with a nonmethylated copy, so even odd numbers of copies of a gene could be inactivated.

In *P. anserina*, cosmid DNA integrated predominantly by additive recombination, resulting in a sequence duplication separated by transforming vector (Coppin-Raynal *et al.* 1989). The vector and one copy of the duplication were frequently excised from the genome during sexual crosses. Such a mechanism leads to the stable replacement of a resident DNA sequence with a transforming DNA sequence.

Ascospore progeny from a cross between hygromycin-resistant transformants of *O. ulmi* containing multiple copies of the hygromycin resistance gene and hygromycin sensitive strains were less resistant to hygromycin than their transformed parent suggesting that DNA modification had occurred (Royer *et al.* 1991). Duplicated genes were inactivated in *G. fujikuroi* but could be reactivated after one or more meiotic divisions (Leslie and Dickman 1991). The mechanism of the inactivation was not determined.

Duplicated genes introduced by transformation in *Sordaria macrospora* were not modified during meiosis or mitosis (Le Chevanton *et al.* 1989). Deletion or methylation of duplicated *ura5-1* genes was not observed.

Gaeumannomyces graminis

Gaeumannomyces graminis (Sacc.) von Arx & Oliver parasitizes the roots of many members of the Gramineae plant family (Walker 1981). *G. g. tritici* is the primary cause of take-all in wheat and barley. *G. g. avenae* causes take-all patch of bentgrass, and take-all in oats, but is occasionally isolated from infected wheat

or barley. *G. g. graminis* causes crown sheath rot of rice and is one of the causative agents of spring deadspot of bermudagrass (McCarty and Lucas 1989).

The roots or germinating seeds of host plants are colonized by hyphae of *G. graminis* which survives as a saprophyte of plant debris in infested soil (reviewed in Skou 1981). Contact between the parasite and root is established by trophical growth of hyphae toward the root. On rare occasions germinating ascospores may also contribute to the establishment of disease. Runner hyphae extend from the initial point of infection over the root surface and can give rise to infection hyphae. When an infection hypha contacts the wall of a susceptible cell, it forms a narrow infection or penetration peg, which produces cell-wall degrading enzymes enabling the fungus to penetrate into epidermal cells of the plant. The infection hyphae grow through the penetrated cells and proceed to the next layer. The mycelia eventually penetrate the vascular tissue of the root leading to occlusion of the vessel and rapid destruction of the stele. Runner hyphae may colonize the roots of non-susceptible host plants but penetration is limited or nonexistent. *G. g. graminis* colonizes root tissue of wheat and barley but is unable to penetrate the stele. *G. graminis* grows parasitically until the end of the growing season, when the saprophytic phase of its lifestyle is resumed on plant debris. Perithecia can be observed on dead tissue.

Molecular Genetics of *Gaeumannomyces graminis*

G. graminis is a homothallic ascomycete which produces perithecia containing asci with eight non-ordered fusiform ascospores during its sexual stage (reviewed in Asher 1981). Discharged ascospores may be responsible for initiating disease in isolated wheat and barley fields, but their importance as inoculum sources in established epidemics is controversial (Hornby 1981). Perithecia develop on the bases of stems of infected plants late in the growing season, but can be produced on inoculated plant tissue (Pilgeram and Henson 1990; Holden and Hornby 1981) or in culture (Holden and Hornby 1981; Weste 1981). Nongerminating asexual conidia (phialospores) are produced by germinating ascospores or in the soil near infected plant tissue. Phialospores capable of germination have been produced under laboratory conditions.

Until recently, only a single defined mutant of *G. graminis* had been reported in the literature. Blanch *et al.* (1981) crossed highly pathogenic strains of *G. g. tritici* with a weakly pathogenic *PABA* auxotroph and analyzed the virulence of outcrossed ascospore progeny. The genetic marker (PABA auxotrophy) facilitated the identification of hybrid perithecia, which contained prototrophic and auxotrophic ascospores. *G. graminis* was transformed to benomyl resistance (Henson *et al.* 1988) using plasmid pBT3, a plasmid encoding fungicide-resistant β-tubulin (TUB2) (Orbach *et al.* 1986). Transformants were obtained with either circular or linear plasmid DNA, and we observed nonhomologous integration of

transforming DNA into the fungal genome in all cases. Linearization of vector DNA with restriction enzymes prior to transformation, resulted in more transformants with a single copy of integrated DNA, although 75% of the transformants analyzed still had multiple copies of transforming DNA. Transformants with multiple sites of integration and tandem duplications were isolated.

Henson (1989) cloned a 4.3 kilobase mitochondrial fragment (pMSU315) from *G. g. tritici* which hybridized to DNA isolated from *G. g. graminis*, *G. g. tritici* or *G. g. avenae*. The probe weakly hybridized with DNA from *Phialophora* spp. and *N. crassa*. The imperfect stages of all three varieties of *G. graminis* are classified within Phialophora (Walker 1981). *G. g. tritici* and *G. g. graminis* can be differentiated on the basis of hybridization patterns (Henson 1989). The diagnostic nature of the probe was confirmed by Bateman, Ward, and Antoniw (personal communication), who used the probe to identity *G. g. tritici* and *G. g. avenae*. The two varieties can then be differentiated on the basis of host range; *G. g. tritici* is usually isolated from cereals and *G. g. avenae* is usually isolated from turf grass.

Oligonucleotide primers which flanked a region of PMSU315 were used in the polymerase chain reaction (PCR) to amplify *G. graminis* DNA (Schesser *et al.* 1991). *G. graminis* DNA was detected in total DNA from infected seedlings. Negative results were obtained with uninfected seedlings or seedlings infected with other pathogenic fungi.

Additional isolates of *Gaeumannomyces* and *Phialophora* and isolates of *Magnaporthe* were screened with plasmid pMSU315 and their DNA amplified using PCR (Henson, personal communication). DNA from *Gaeumannomyces* spp., *Phialophora* spp. and *M. poae*, the causative agent of hot-weather patch of Kentucky bluegrass (Landshoot and Jackson) had strong homology with the probe and was amplified using primers developed for *G. g. tritici*. PCR results varied with reaction conditions (Henson, personal communication).

Phytopathogenic Fungus	Marker	Reference
Aspergillus oryzae	*PYR*	Mattern *et al.* 1987
Claviceps purpea	*ble*	van Engelenburg *et al.* 1989
Cochliobolus heterostrophus	*hyg*B	Turgeon *et al.* 1987
	*Amd*S	Turgeon *et al.* 1985
Colletotrichum graminicola	*TUB2*	Panaccione *et al.* 1988
Colletotrichum lagenarium	*TUB2*	Kubo *et al.* 1991
Colletotrichum trifolii	*hyg*B	Dickman 1988
	TUB2	Dickman 1988
Cryphonectria parasitica	*hyg*B	Churchhill *et al.* 1990
	TUB2	Churchhill *et al.* 1990
Fulvia fulva	*hyg*B	Oliver *et al.* 1987
Fusarium graminearum	*hygB*	Dickman and Partridge 1989
	TUB2	Dickman and Partridge 1989
Fusarium moniliforme	*hygB*	Dickman and Partridge 1989
	TUB2	Dickman and Partridge 1989
Fusarium oxysporum	*hyg*B	Kistler and Benny 1988
	niaD	Malardier *et al.* 1989
Gaeumannomyces graminis	*TUB2*	Henson *et al.* 1988
	ble	Pilgeram and Henson 1990
Gibberella fujikuroi	*hyg*B	Leslie and Dickman 1991
Glomerella cingulata	*hyg*B	Rodriguez and Yoder 1987
	*Amd*S	Rodriguez and Yoder 1987
Magnaporthe grisea	*hyg*B	Leung *et al.* 1990
	*Arg*B	Parsons *et al* 1987
Mycosphaerella spp.	*hyg*B	Dickman *et al.* 1989
Nectria haematococca	*hyg*B	Van Etten and Kistler 1988
	*Arg*B	Van Etton and Kistler, 1988

Table 1. Phytopathogenic fungi which have been transformed and selectable marker utilized.

Phytopathogenic Fungus	Marker	Reference
Ophiostoma ulmi	*hyg*B	Royer *et al.* 1991
Septoria nodorum	*hyg*B	Cooley *et al.* 1988
	TUB2	Cooley *et al.* 1990
Ustilago hordei	*hyg*B	Holden *et al.* 1988
Ustilago maydis	*hyg*B	Wang *et al.* 1988
		Tsukuda *et al.* 1988
	PYR	Banks and Taylor 1988
Ustilago nigra	*hyg*B	Holden *et al.* 1988

Table 1 (cont). Phytopathogenic fungi which have been transformed and selectable marker utilized.

CHAPTER TWO

TRANSFORMATION AND COTRANSFORMATION OF *GAEUMANNOMYCES GRAMINIS* TO PHLEOMYCIN RESISTANCE.[1]

Gaeumannomyces graminis (Sacc.) von Arx & Oliver is a soilborne, filamentous ascomycete that parasitizes the roots, crowns, lower stems and stolens of many members of the Gramineae plant family (reviewed in Garrett 1981). *G. graminis* var. *tritici* (*Ggt*), 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. graminis* var. *graminis* (*Ggg*) causes crown sheath rot of rice and has recently been implicated as a causative agent of spring dead spot of bermudagrass (McCarty and Lucas 1989).

G. graminis is a homothallic organism that produces perithecia during its sexual stage (reviewed in Asher 1981). 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 (Blanch *et al.* 1981). Selfed and crossed perithecia could be more easily distinguished by introducing selectable genetic markers into one or both parents of

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[1] Pilgeram and Henson, 1990
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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 (Henson *et al.* 1988) to transform *Gaeumannomyces* to benomyl resistance (BenR) using pBT3 (Orbach *et al.* 1986), a plasmid encoding fungicide-resistant β-tubulin. However, preliminary attempts to transform *G. graminis* to hygromycin or G418 resistance with various vectors (Cullen *et al.* 1987, Queener *et al.* 1985, Turgeon *et al.* 1987, Webster and Dickson 1983) 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 (Berdy 1980). Phleomycin interacts with DNA of susceptible organisms and cleaves preferentially at inverted repeat sites in single stranded DNA (Ueda *et al.* 1985) and at unmethylated sites in double stranded DNA (Hertzberg *et al.* 1985). Bleomycin resistance genes from transposon Tn5 (Collis and Hall, 1985), *Staphylococcus aureus* plasmid pUB110 (Semen *et al.* 1987), and the chromosome of *Streptoalloteichus hindustanus* (Gatignol *et al.* 1988) recently have been characterized. The product of the bleomycin resistance genes from *S. hindustanus* reversibly binds bleomycin and prevents it from cleaving DNA (Gatignol *et al.* 1988). The proteins encoded by the *ble* genes from Tn5 and pUB110 exhibit a high degree of homology with the resistance protein from *S. hindustanus* (Gatignol *et al.* 1988).

Several vectors encoding phleomycin resistance have been used to transform or cotransform yeast (Gatignol *et al.* 1987) and filamentous fungi (Kolar *et al.* 1988, Mattern *et al.* 1988, Mattern *et al.* 1987, van Engelenburg *et al.* 1989). Plasmid 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 *trp*C gene of *A. nidulans* (Mattern *et al.* 1988). We report here improvement of our transformation procedure and successful transformation and cotransformation of *G.* to phleomycin resistance (PhleoR) with pAN8-1. Phenotypic and genotypic stability of phleomycin and benomyl resistance through mitotic and meiotic divisions is also reported.

Materials and Methods

Strains and Media

G. g. tritici (strain DM528) and *G. g. graminis* (strain DM562) were provided by Dr. D.E. Mathre and grown in complex L medium (Miller 1972). Protoplasts were obtained as described previously and regenerated on protoplast regeneration medium (PRM) (Stanway and Buck 1984). Glucose-asparagine medium was used for perithecia development (Lily and Barnett 1951).

Transformation to Benomyl Resistance

Protoplasts of *Gaeumannomyces* sp. were transformed with plasmid pBT3 as described previously with the following modifications (Henson *et al.* 1988).

After transformation, protoplasts were washed once with stabilizing buffer, resuspended in 3 ml of PRM broth and incubated 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 were then spread onto PRM plates containing 1.0 μg or 0.75 μg 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, Del.) in 95% ethanol. Protoplasts of *G. g. tritici* were overlayed with 10 ml PRM containing 0.5 μg benomyl per milliliter 3-5 days after plating. Protoplasts of *G. g. graminis* were not overlayed. 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. g. graminis* were transformed with approximately 3 μg of pAN8-1 or pAN8-1 linearized by digestion with *Eco*RI, 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 2000-2002, 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 μg 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 μg of phleomycin per milliliter.

Cotransformation

Protoplasts of *G. g. graminis* were cotransformed with 3 μg of circular pAN8-1 and 3 μg of circular pBT3. Transformants (strains JH1902-1911) were selected on PRM containing 1.0 μg of benomyl per milliliter. BenR colonies were then scored for phleomycin resistance on solid L medium containing 20 μg 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, F4OCW, and one General Electric warm white bulb, F40WW, approximately 35 cm from plates, which were incubated right side up (Lily and Barnett 1951). Ascospores were isolated by a modification of Rossman's single-spore isolation technique (Rossman 1985). 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 hours, germinating ascospores were transferred to nonselective L medium. Following growth they were tested for

resistance on selective L medium (0.8 μg of benomyl per milliliter or 20 μg of phleomycin per milliliter).

Plant Inoculation and Fungal Reisolation

Wheat or rice seeds were surface sterilized in 1% $AgNO_3$ for three minutes and washed with distilled water. Sterile seeds were placed in glass jars (200 ml) containing 50 ml of 1% agar and several 1-mm x 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 to 3 wk. Fungi were reisolated by removing crown sheath leaves and placing them directly onto selective L medium with 0.8 μg of benomyl per milliliter or 20 μg 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 previously described (Henson *et al.* 1988). DNA samples were digested with restriction enzymes according to the manufacturers' directions. Electrophoresis and hybridization of dried gels were performed as described (Henson *et al.* 1987). Each lane of agarose gels contained 1-2 μg of fungal genomic DNA. Plasmid DNA was purified according to standard

procedures (Maniatis *et al.* 1982) and was nick translated with 32P-thymidine triphosphate (Rigby *et al.* 1977).

Results

G. graminis was inhibited by low concentrations of benomyl (0.75-1.2 μg/ml) (Henson *et al.* 1988) and phleomycin (20 μg/ml). We transformed and cotransformed *G. graminis* with pAN8-1, a plasmid which carries a phleomycin resistance gene (*ble*) from *S. hindustanus* (Mattern *et al.* 1988) and with pBT3, a plasmid which carries a benomyl-resistance gene (*TUB2*) from *Neurospora crassa* (Orbach *et al.* 1986). We included the following alterations in the transformation procedure used in earlier experiments (Henson *et al.* 1988). 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 plating 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 (Henson *et al* 1988), we were able to obtain greater numbers of *G. g. tritici* transformants 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 PhleoR *G. g. graminis* and nine PhleoR *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 PhleoR transformants and its absence in non-transformed wild-type mycelium (data not shown). Homology was present only in high molecular weight DNA from PhleoR 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, the DNA was digested with *Hin*dIII, an enzyme which does not cut within the vector, and hybridized with ^{32}P-labeled pAN8-1 (Fig. 1 and less exposed autoradiographs of Fig. 1, data not shown). Many of the pAN8-1 transformants (e.g. JH2016, JH2029, JH2030) had only a single *Hin*dIII fragment that showed homology with the probe, suggesting that these transformants had a single site of plasmid integration. Strains JH2018, JH2002, 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 *Hin*dIII, homologous fragments were, as expected, larger than the

transforming vector. However, one transformant, 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 which 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. JH2016, JH2026, JH2023) and multiple (e.g. 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 (Henson *et al.* 1988).

We previously had difficulty obtaining transformants of *G. g. tritici* (Henson *et al.* 1988). Improvements described in this paper allowed us to transform *G. g. tritici* to BenR as well as to PhleoR with greater efficiency. DNA from four BenR *G. g. tritici* isolates transformed with linear pBT3 was also 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

was also restricted with *Eco*RV, 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 *Nco*I, an enzyme which 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 PhleoR. When pAN8-1 and pBT3 plasmids were used together to transform protoplasts of *G. g. graminis* to BenR, 15/115 transformants were also PhleoR. PhleoR-BenR 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 PhleoR-BenR cotransformants was digested *Nco*I and *Eco*RI. Plasmid pAN8-1 digested with *Nco*I and *Eco*RI 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 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 X 1-cm plugs from edges of transformant colonies at least three times on 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 2). Benomyl resistance 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 was also determined following plant inoculation and fungal reisolation (Table 2). 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 two weeks after inoculation and included blackening of crown and root tissue. Wilting was also evident in wheat seedlings infect with *G. g. tritici*.

Discussion

Modifications of the original transformation protocol enabled us to consistently obtain PhleoR or BenR 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 number 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 was also cotransformed with pAN8-1 and pBT3. Approximately 13% (15/115) of the transformants selected on media containing benomyl were also resistant to phleomycin. With other plasmids, cotransformation was observed in up to 80% of the transformants (unpublished data).

The PhleoR and BenR phenotypes of transformants and cotransformants were stable through mitosis in most transformants, although two cotransformants lost PhleoR following several transfers onto non-selective media. PhleoR 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 the 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 will also allow us to select heterokaryons and thus conduct complementation analysis. Finally, integration of the transforming vector into the fungal genome may facilitate 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).

Transformant	Axenic culture		Reisolated from infected host	
	PhleoR	BenR	PhleoR	BenR
JH1902-1903	-	+	+	+
JH1904-1908	+	+	+	+
JH2023-2028, JH2000-2002, JH2016,2018	+	NA[a]	+	NA
JH2020-2021, JH1899	+	NA	NT[b]	NA
JH2031-2032	-	NA	NT	NA
JH2018-2019, JH2029-2030	+	NA	NT	NA
JH2437-2439, JH2442	NA	+	NA	NT

[a] NA = not applicable
[b] NT = not tested

Table 2. Mitotic stability of selected phenotype in transformants of *Gaeumannomyces graminis*.

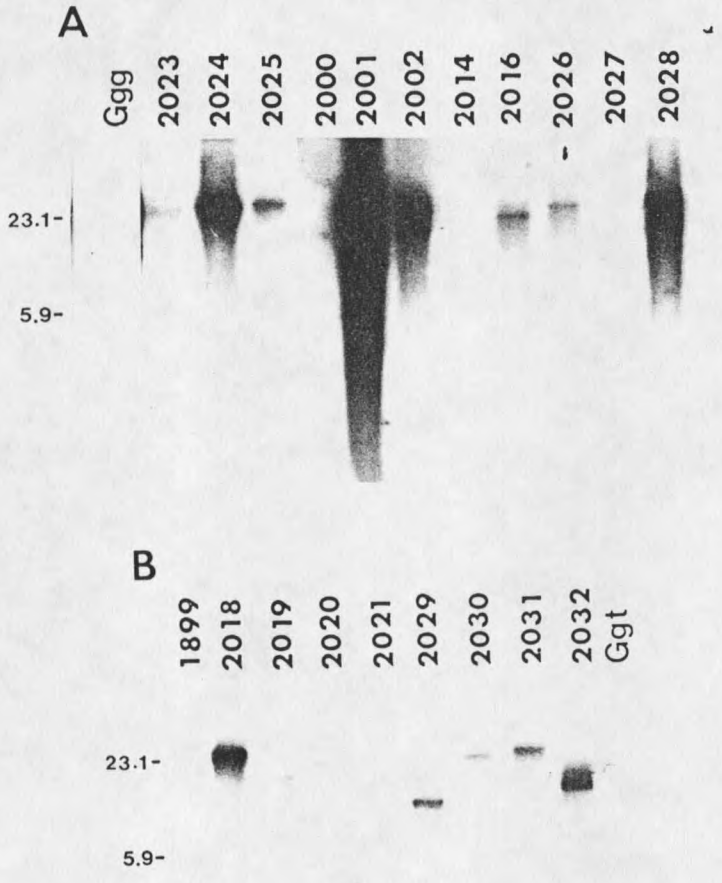

Fig. 1. Hybridization of labeled pAN8-1 with control *Gaeumannomyces graminis* and transformant genomic DNA, which was digested with *Hin*dIII, an enzyme which does not cut pAN8-1. **A.** *G. g. graminis* (*Ggg*) control and transformants. **B.** *G. g. tritici* (*Ggt*) control and transformants. Molecular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

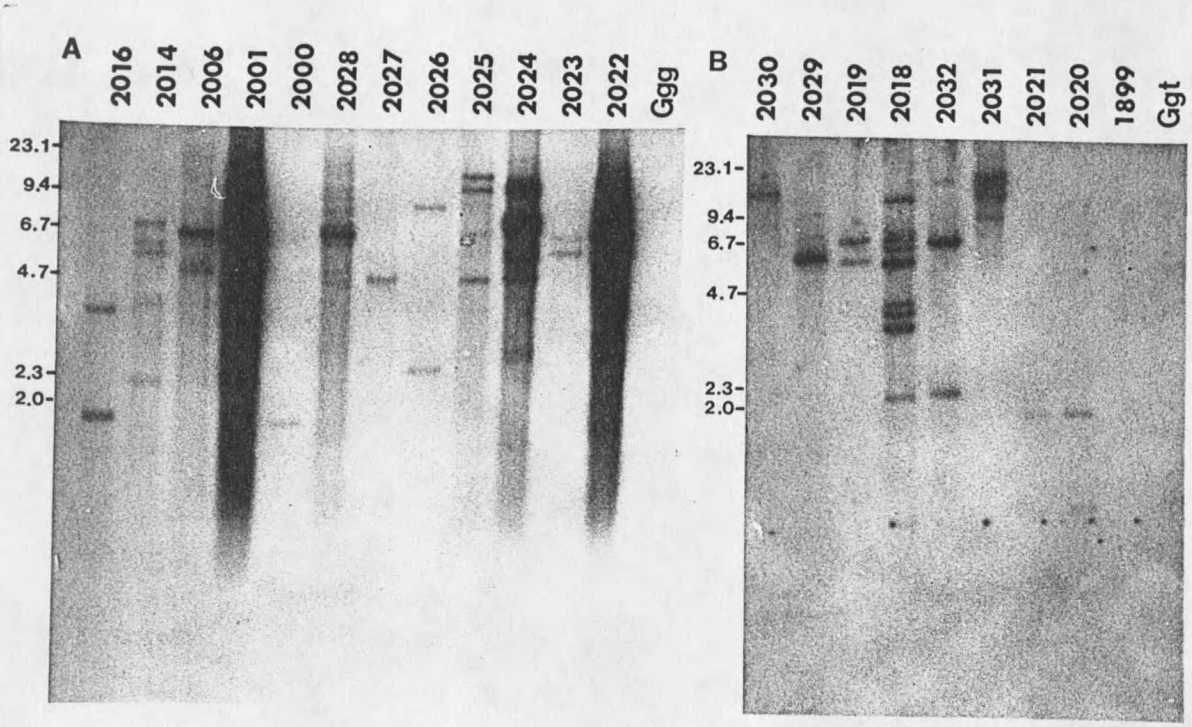

Fig. 2. Hybridization of labeled pAN8-1 with control *Gaeumannomyces graminis* and transformant DNA, which was digested with *Nco*I, an enzyme that cuts once within pAN8-1. **A.** *G. g. graminis* (*Ggg*) control and pAN8-1 transformants. **B.** *G. g. tritici* (*Ggt*) control and pAN8-1 transformants.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

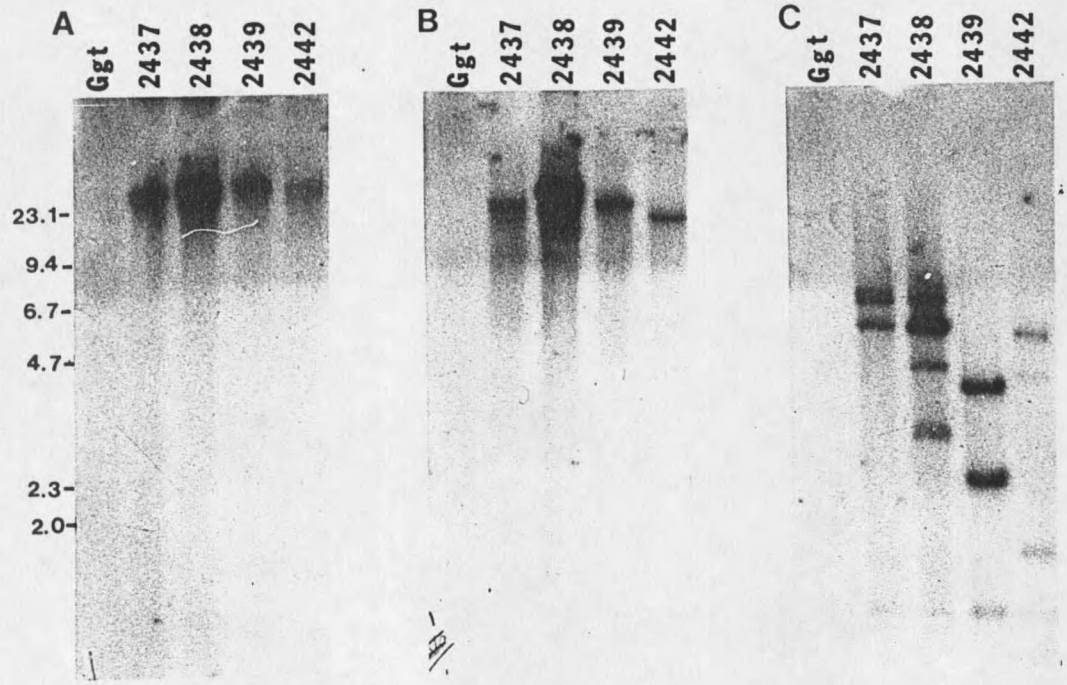

Fig. 3. Hybridization of labeled pBT3 with genomic DNA from control *Gaeumannomyces graminis* var. *tritici* (*Ggt*) and pBT3 transformants. **A.** Uncut DNA. **B.** DNA digested with *Eco*RV, an enzyme that does not cut pBT3. **C.** DNA digested with *Nco*I, an enzyme that cuts pBT3 once. Molecular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

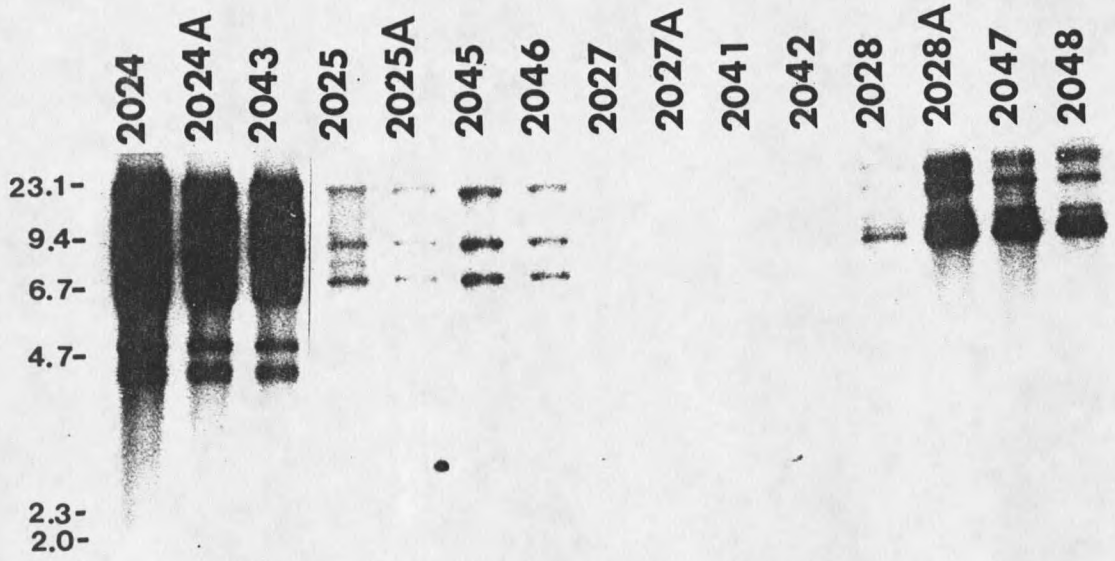

Fig. 4. Hybridization of labeled pAN8-1 with genomic DNA from pAN8-1 transformants and ascospore cultures derived from them. Strains JH2024A and JH2043 are ascospore derivatives of strain JH2024; strains JH2025A, JH2045, and JH2046 are ascospore derivatives of strain JH2025; strains JH2027A, JH2041,and 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.

CHAPTER THREE

SEXUAL CROSSES OF THE HOMOTHALLIC FUNGUS *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI* BASED ON USE OF AN AUXOTROPH OBTAINED BY TRANSFORMATION[2]

Gaeumannomyces graminis var. *tritici* (Sacc.) von Arx & Oliver, the etiologic agent of take-all disease in wheat and barley, is a soilborne, filamentous ascomycete that produces perithecia during its homothallic sexual stage. Blanch *et al.* (1981) described crosses between an ultraviolet light-generated, p-aminobenzoic acid auxotroph and unmarked, wild-type *G. graminis* var. *tritici* strains. However, no other auxotrophic mutants or outcrosses have been reported, and the lack of markers has impeded genetic studies of this fungus.

Recently we transformed *G. graminis* to benomyl resistance (BenR) (Henson *et al.* 1988) with plasmid pBT3 (Orbach *et al.* 1986) or phleomycin resistance (PhleoR) (Pilgeram and Henson 1990) with plasmid pAN8-1 (Mattern *et al.* 1987). Some transformants had only one or two copies of vector DNA which was

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[2] Pilgeram and Henson 1991
Exper. Mycol. (accepted).

integrated at various sites in different transformants (Henson *et al.* 1988; Pilgeram and Henson 1990). Homologous integration was not observed in either *G. g. graminis* or *G. g. tritici.*

Heterologous integration of transforming DNA into a gene would most likely disrupt its expression. Insertional mutagenesis has been used to generate mutants of *Neurospora crassa* (Kang, S. and R. L. Metzenberg, personal communication) and *Aspergillus nidulans* (Tilburn *et al.* 1990; Diallinas and Scazzocchio 1989). Thus, we screened all *G. graminis* transformants for auxotrophy, that is, for plasmid insertions into biosynthetic genes.

We describe here the isolation and characterization of a nicotinic acid auxotroph (Nic-) of *G. graminis* var. *tritici* following transformation with pBT3. Genetic crosses between the auxotroph and a wild-type strain or a PhleoR transformant of *G. g. tritici* demonstrated that auxotrophy and BenR were closely linked in ascospore progeny; hence, the requirement for nicotinic acid could be due to the insertional inactivation of a biosynthetic gene by transforming DNA. In addition, both benomyl and phleomycin resistance segregated one to one in ascospore progeny.

Materials and Methods

Strains and Media

G. graminis var. *tritici* (strain DM528) (*Nic2+*) was provided by Dr. D.E. Mathre (Table 1). Strain JH2446 was a BenR transformant that was isolated as previously described (Henson *et al.* 1988) by transformation of strain DM528 with a benomyl resistance plasmid, pBT3 (Orbach *et al.* 1986) that was linearized by digestion with *Pst*I. Strain JH2029 was a PhleoR transformant that was isolated as previously described (Pilgeram and Henson 1990) by transformation of strain DM528 with a phleomycin resistance plasmid, pAN8-1 (Mattern *et al.* 1987) that was linearized with *Eco*RI. Strains were cultured on complex L medium (Miller 1972) at room temperature. BenR was scored on L medium supplemented with 0.8 μg of benomyl (a gift from Dupont, Wilmington, DE) per ml and PhleoR was scored on L medium supplemented with 20 μg phleomycin (Cayla Laboratories, Toulouse, France) per ml. Czapek-Dox medium (Difco Laboratories, Detroit, MI) supplemented with 1.0 μg biotin per ml and 1.0 μg thiamine per ml was used as minimal media for scoring auxotrophy. One quarter strength Hewitt's solution was made by combining 50 ml macronutrient solution (20 mM KNO^3, 14 mM $Ca(NO_3)_2$, 7.5 mM $MgSO_4$, 6.7 mM NaH_2PO_4), 25 μl micronutrient solution (14.8 mM $MnSO_4$, 10 mM $CuSO_4$, 10 mM $ZnSO_4$, 0.5 M $H_{3BO}3$, 1 M NaCl, 5 mM

Na_2MoO_4, 0.07 mM $(NH_4)_6Mo_7O_{24}$), and 2.5 ml iron solution (0.1 M Fe (K or Na) ethylenediaminetetra-acetic acid) into a final volume of one liter (Hewitt 1966).

Isolation and Analysis of DNA

Fungal DNA extractions, electrophoresis, and hybridization of dried gels were performed as described (Henson *et al.* 1987; Henson *et al.* 1988). Plasmid DNA was purified and nick-translated with ^{32}P-thymidine according to standard procedures (Maniatis *et al.* 1982).

Identification and Isolation of an Auxotrophic Transformant

Transformants were screened for auxotrophy on minimal medium. Strain JH2446 (BenR) was one of several *G. graminis* mutants that failed to grow on minimal medium and was transferred onto a series of minimal plates containing various vitamins and amino acids to identify the nutrient required for growth (Holliday 1956).

Perithecia Production

Pondera wheat seeds were surface sterilized for 7 minutes with 1% $AgNO_3$, washed 3X with sterile distilled H_2O, and germinated in petri dishes containing 1% agar. Approximately 50 ml of 1/4 strength Hewitt's solution was added to 200 ml bottles lined with filter paper (Weste, 1970). Bottles were autoclaved for 20

minutes and 2-3 day old germinated seeds were placed in the bottles between the filter paper and the glass wall of the bottle.

Agar plugs of strains to be crossed were transferred onto a plate of nonselective L medium and incubated at room temperature until the two strains grew together. Roots of germinated wheat seedlings in sterile bottles were inoculated with agar plugs taken from the junction of the two colonies. Infected plants were incubated at room temperature with a twelve hour photoperiod from one General Electric cool-white bulb, F40CW, and one General Electric warm-white bulb, F40WW approximately 20 cm above the plants (Weste, 1970). Mature perithecia could be isolated 6 to 10 weeks after inoculation.

During the course of this study, a new method for perithecia production was developed. Ten ml of 1% agar prepared from a 1:10 dilution of 1/4 strength Hewitt's solution was placed in a 40 ml test tube along with 4 to 5 wheat leaves harvested from one week old plants. The tubes were autoclaved (15 minutes), allowed to solidify and inoculated with cultures of *G. graminis*. The tubes were incubated under lights as described. Mature perithecia could be isolated from the leaf tissue 2 to 4 weeks following inoculation.

Ascospore Isolation

Ascospores were isolated by a modification of Rossman's single-spore isolation technique (Rossman 1985). Individual perithecia were isolated from infected plant leaves, roots, or the walls of the bottle and placed in a drop of water on a sterile microscope slide. A perithecium was observed under a light microscope (10X) to confirm that a single perithecium was present. The perithecium was crushed under a coverslip with a pencil eraser and exuded ascospores were washed into a sterile tube with 100 μl of sterile water.

Ten μl aliquots of the ascospore suspension were plated onto L medium supplemented with 0.8 μg benomyl per ml (selective for BenR) and onto minimal medium (selective for *Nic2*+). Growth was observed after 2-3 days at room temperature. Perithecia which contained ascospores capable of growing on medium containing benomyl, as well as ascospores capable of growing on minimal medium, were presumed to be crossed. Remaining ascospores from these perithecia, which were stored at 4C, were germinated on non-selective L medium and individual colonies transferred to non-selective L medium. Colonies were scored for benomyl resistance on L medium supplemented with 0.8 μg benomyl per ml and for auxotrophy on minimal medium (Table 3). Ascospore colonies

from crosses between strain JH2446 and strain JH2029 were also scored for phleomycin resistance on L medium with 20 μg phleomycin per ml (Table 3).

Pathogenicity Testing

G. graminis isolates were grown in complex L broth for 7-10 days with shaking at room temperature. The mycelium was collected by centrifugation (7000 RPM, 10 min.) and washed 2X with distilled water. After resuspension in distilled water, the mycelium was spread onto vermiculite in 4 in. by 4 in. pots. Pondera wheat seeds (approximately 100/pot) were spread over mycelium and covered with vermiculite. Plants were grown in natural sunlight and watered daily with distilled water. Inoculated plants showed symptoms of take-all (wilted, yellow leaves and blackened roots) within two to three weeks.

Results

Characterization of an Auxotrophic Transformant

Among our collection of BenR transformants, we identified several strains which were auxotrophic as evidenced by their lack of growth on minimal medium. The requirement of strain JH2446 was identified as nicotinic acid on a series of auxotrophy tester plates (data not shown) (Holliday 1956). Nicotinamide adenine dinucleotide (NAD) can be synthesized from tryptophan by *N. crassa* (Henderson

1949), mammals (Gholson *et al.* 1964; Henderson 1949), *Xanthomonas pruni* (Wilson and Henderson 1963), *Fusarium oxysporum* (Desaty and Vining 1967), *Rhodotorula glutinis* (Birnbaum *et al.* 1972) and *Saccharomyces cerevisiae* under aerobic conditions (Ahmed and Moat 1966) (Fig. 5). Quinolinic acid is derived from 3-hydroxyanthranilic acid by 3-hydroxyanthranilate 3,4 dioxygenase (EC 1.13.11.6) (Decker *et al.* 1961). Nicotinamide is converted into nicotinic acid which is converted into nicotinic acid mononucleotide.

Assuming that *G. graminis* utilizes a similar pathway for nicotinic acid biosynthesis, minimal media containing each of the intermediates in the nicotinic acid biosynthetic pathway were inoculated with strain JH2446 (Nic-), strain DM528 (Nic+) or strain JH2029 (Nic+) (Table 4). Plates were incubated at room temperature and radial growth was measured every two days. Strains DM528 and JH2029 were able to grow on minimal media with or without nicotinic acid or its intermediates, although growth was inhibited at high concentrations of nicotinamide (18 mM). The radial growth of strain JH2446 was indistinguishable from that of wild type on media supplemented with nicotinamide or nicotinic acid. Growth was also observed on media supplemented with 4.5 mM quinolinic acid but not on medium supplemented with tryptophan, kynurine, hydroxyanthranilic acid or 10 μM quinolinic acid. The lack of growth of strain JH2446 on minimal media supplemented with 10 μM quinolinic acid was not surprising as low levels of

quinolinic acid also failed to support the growth of a *Nic2* mutant of *N. crassa* (Henderson, 1949). In addition, the mycelium became reddish-brown as strain JH2446 aged, indicative of a build up of 3-hydroxyanthranilic acid (Perkins *et al.* 1982). Thus, strain JH2446 was unable to convert hydroxyanthranilic acid into quinolinic acid and therefore could not synthesize NAD. The auxotrophy was designated *Nic2* in keeping with *N. crassa* nomenclature (Perkins *et al.* 1982).

The pathogenicity of the auxotrophic mutant was also tested in pot assays and found to be similar to strain DM528 (wild-type) and strain JH2029 (a PhleoR transformant). Evidently there was sufficient nicotinic acid to support the growth of the auxotroph in vermiculite-grown wheat.

Perithecia Production

Several methods were used to obtain mature perithecia. *G. graminis* var. *tritici* and *G. graminis* var. *graminis* perithecia were reliably produced by the filter paper method of Weste (1970). Inoculated wheat seedlings on water-agar plates were also a source of perithecia (Osbourne, A. personal communication). However, despite harsh surface sterilization, seedlings were often contaminated with other fungi and bacteria. Moreover, mature perithecia were seldom produced before 8 weeks after seedling inoculation. Other plate methods (Speakman 1984; Lily and Barnett 1951), which did not require host plants, gave inconsistent results and

required extended incubation periods. Our modified method for perithecia production, as described in Materials and Methods, was an improvement because mature ascospores could be obtained 2-4 weeks following inoculation of autoclaved wheat leaves. In addition, contaminants from unsterile host tissue were eliminated.

Crosses with a Nic2 mutant

Twenty four perithecia containing mature ascospores were isolated from crosses of wild type strain DM528 (*Nic2*+) and transformant JH2446 (*TUB2 Nic2*) (Table 5). Aliquots of ascospores from each perithecium were plated onto L medium supplemented with 0.8 μg benomyl per ml and onto minimal medium. Three perithecia contained ascospores capable of growing on minimal medium and L medium with benomyl and were presumed to be products of a cross. The phenotypes of ascospore colonies from these perithecia were determined. In the progeny of these crosses *TUB2* and the wild-type showed a 1:1 segregation (69 BenR:54 BenS, χ^2=1.8, p>0.1) and BenR and nicotinic acid auxotrophy were inseparable in all progeny analyzed.

In addition to the 6.6 kb resident β-tubulin gene, *Hin*dIII-restricted DNA from strain JH2446 and all BenR progeny analyzed contained two fragments which hybridized to ^{32}P-labeled pBT3 (18.0 kb and 4.8 kb) (Fig. 6). The β-tubulin

gene weakly hybridizes with pBT3, and was visible in all lanes in longer exposures of X-Ray film (data not shown). The 18.0 or 4.8 kb fragments were not observed in *Hin*dIII-digested DNA from the wild-type or BenS progeny.

It was possible that benomyl sensitivity in the above progeny was due to deletion of transforming DNA, rather than crossing. Therefore, we also crossed strain JH2446 with another transformant, strain JH2029, which had a single integrated copy of pAN8-1, encoding phleomycin resistance. Ten perithecia with viable ascospores were isolated from wheat inoculated with strain JH2446 (*TUB2 Nic2*) and strain JH2029 (*Nic2+ BLE*), and two of these perithecia gave rise to both BenR and PhleoR progeny (Table 5). One to one segregations of both the *BLE*:wild-type (29 PhleoS:27 PhleoR) and *TUB2*:wild-type (29 BenS:27 BenR) were observed. Moreover, BenR and PhleoR sorted independently; that is, a 1:1:1:1 distribution of the resistance genes was observed in the progeny (χ^2 =2.7, p>0.1). Again, no separation of *TUB2* and *Nic2* was observed in the 56 progeny tested.

DNA from progeny of the cross between strains JH2446 and JH2029 was examined (Fig. 7). DNA from strain JH2446, restricted with *Nco*I, contained four fragments (1.5 kb, 1.7 kb, 5.7 kb and 7.9 kb) which hybridized to ^{32}P-labeled pBT3. The 1.5 kb fragment corresponds to the native β-tubulin gene and, again could be observed in all lanes with longer exposures of X-ray film (data not shown). The

7.9 kb band observed in strain JH2446 was not observed in any of the progeny tested and its size suggested that it was a partially digested fragment containing the 1.7 and 5.7 kb fragments. The 1.7 and 5.7 kb fragments were observed in all BenR progeny of the cross.

PhleoR was inherited in a 1:1 fashion in crosses with strain JH2029. However, the *Nco*I fragment containing the *BLE* gene sometimes had a deletion in the PhleoR progeny (Fig. 7). For example, the *BLE* fragments in strains JH2698 and JH2701 were 3.6 kb, whereas the *BLE* fragment in strain JH2029 was 10.4 kb. Transforming DNA was also modified in selfed progeny of strain JH2029 (manuscript in preparation).

Strain JH2658 (BenS PhleoS) contained a *Nco*I fragment which hybridized to labeled pBT3 (Fig. 7). The fragment (7.0 kb) was smaller than the 10.4 kb fragment observed in strain JH2029 or the 7.9 kb fragment observed in strain JH2446 and probably contained modified DNA from either pBT3 or pAN8-1.

Discussion

The introduction of selectable markers into *G. graminis* has enabled us to distinguish hybrid from selfed perithecia. Approximately 15% (5/34) of perithecia tested were products of outcrossing (Table 5). The significance of the sexual cycle in field outbreaks of take-all is controversial (reviewed in Hornby 1981), but

perithecia with viable ascospores can be produced on the remains of infected host plants in the field (reviewed in Skou 1981). Therefore, outcrossing and subsequent exchange of genetic information between isolates of *G. graminis* could generate strains with altered virulence or host range.

We isolated a BenR transformant of *G. graminis* var. *tritici* which required nicotinic acid for growth. Further characterization revealed that the auxotroph was unable to convert hydroxyanthranilic acid into quinolinic acid, an intermediate reaction in the NAD biosynthetic pathway catalyzed by 3-hydroxyanthranilate 2,3 dioxygenase (*Nic2*). Segregation of the observed auxotrophy (*Nic2*) and the integrated *TUB2* gene were examined in progeny from a cross between the auxotroph and the wild-type (Table 5). BenR and auxotrophy did not separate in any of the progeny. Hence, *Nic2* and *TUB2* segregated as a single gene or tightly linked genes. The maximum calculated distance separating *TUB2* and *Nic2* was 0.56 map units. Although PhleoR and BenR segregated as expected in crosses between the auxotroph and a PhleoR transformant, deletions of transforming sequences were observed in some PhleoR progeny. Again no separation of BenR and nicotinic acid auxotrophy was observed.

The actual relationship between the *TUB2* gene and the *Nic2* gene in strain JH2446 was not resolved using sexual crosses. The auxotrophy could be the result of spontaneous mutation of the *Nic2* gene independent of the integration of

transforming DNA or could be a direct result of the integration of transforming DNA. One could differentiate between the two using pBT3, a cloned *Nic2* gene and a restriction enzyme which cuts once within pBT3. If pBT3 is integrated within the *Nic2* gene, pBT3 and *Nic2* will hybridize to the same two fragments. If pBT3 and *Nic2* are separate sequences, pBT3 would again hybridize with two fragments, but *Nic2* would only hybridize with one of them. We are unaware of any cloned *Nic2* genes from fungi, or other organisms that might be useful for this hybridization analysis.

Insertional inactivation has facilitated cloning of the *uapA* (uric acid-xanthine permease) gene (Diallinas and Scazzocchio 1989) and the *wA* (white conidiospore) gene (Tilburn *et al.* 1990) of *Aspergillus nidulans* and the phosphorus acquisition genes, *preg* and *pgov*, of *N. crassa* (Kang, S. and Metzenberg, R.L., personal communication). Transforming DNA was recovered from the fungal genome and used to clone the flanking sequences of the inactivated genes, which were used to screen wild-type libraries for the genes of interest. The inactive *Nic2* gene could be rescued from strain JH2446 by a similar cloning scheme. If pBT3 is integrated within the gene or is located nearby, recovery of pBT3 should yield flanking DNA consisting of *Nic2* sequences and could be used to isolate the intact gene from a wild-type library.

STRAIN	SOURCE	GENOTYPE	PHENOTYPE
DM528	D.M. Mathre, MSU	*Nic2+*	BenS Nic+ PhleoS
JH2446	pBT3 transformant	*TUB2 Nic2-*	BenR Nic- PhleoS
JH2029	pAN8-1 transformant	*Nic2+ BLE*	BenS Nic+ PhleoR
JH2863	JH2446/DM528 progeny	*Nic2+*	BenS Nic+
JH2864	"	*TUB2 Nic2-*	BenR Nic-
JH2865	"	*Nic2+*	BenS Nic+
JH2867	"	*TUB2 Nic2-*	BenR Nic-
JH2870	"	*Nic2+*	BenS Nic+
JH2871	"	*TUB2 Nic2-*	BenR Nic-
JH2656	JH2446/JH2029 progeny	*TUB2 Nic2-*	BenR Nic- PhleoS
JH2658	"	*Nic2+*	BenS Nic+ PhleoS
JH2659	"	*TUB2 Nic2- BLE*	BenR Nic- PhleoR
JH2660	"	*Nic2+ BLE*	BenS Nic+ PhleoR
JH2661	"	*TUB2 Nic2-*	BenR Nic- PhleoS
JH2664	"	*TUB2 Nic- BLE*	BenR Nic- PhleoR
JH2698	"	*Nic2+ BLE*	BenS Nic+ PhleoR
JH2699	"	*TUB2 Nic2- BLE*	BenR Nic- PhleoR
JH2701	"	*TUB2 Nic2- BLE*	BenR Nic- PhleoR

Table 3. Genotypes and phenotypes of *Gaeumannomyces graminis* var. *tritici* transformants and ascospore progeny. Genetic nomenclature of Yoder *et al.*, 1986 was used to describe the genotypes and phenotypes of all strains.

STRAIN	NICOTINIC ACID INTERMEDIATE													GENO-TYPE
	NONE	TRP		KN		HAA		QA		NCA		NA		
		10 μM	10 mM	10 μM	0.7 mM	10 μM	1.4 mM	10 μM	4.5 mM	10 μM	18 mM	10 μM	20 mM	
DM528	+	+	+	+	+	+	+	+	+	+	-	+	+	*Nic2+*
JH2446	-	-	-	-	-	-	-	-	+	+	-	+	+	*Nic2-*
JH2029	+	+	+	+	+	+	+	+	+	+	-	+	+	*Nic2+*

Table 4. Growth on minimal plates supplemented with intermediates in the nicotinic acid biosynthetic pathway. TRP-tryptophan, KN-kynurine, HAA-hydroxyanthranilic acid, QA-quinolinic acid, NCA-nicotinamide, NA-nicotinic acid.

CROSS	TOTAL PERITHECIA	HYBRID PERITHECIA	TOTAL ASCOSPORES	PHENOTYPE	NUMBER PROGENY
JH2446 X DM528 *TUB2 Nic2 X Nic2+*	24	3	123	BenR Nic-	69
				BenS Nic+	54
				BenR Nic+	0
				BenS Nic-	0
JH2446 X JH2029 *TUB2 Nic2 X Nic2+ BLE*	10	2	56	BenR Nic- PhleoR	10
				BenR Nic- PhleoS	17
				BenS Nic+ PhleoR	17
				BenS Nic+ PhleoS	11
				BenR Nic+	0
				BenS Nic-	0

Table 5. Genetic crosses with transformant JH2446 as a parent and phenotypic characterization of progeny.

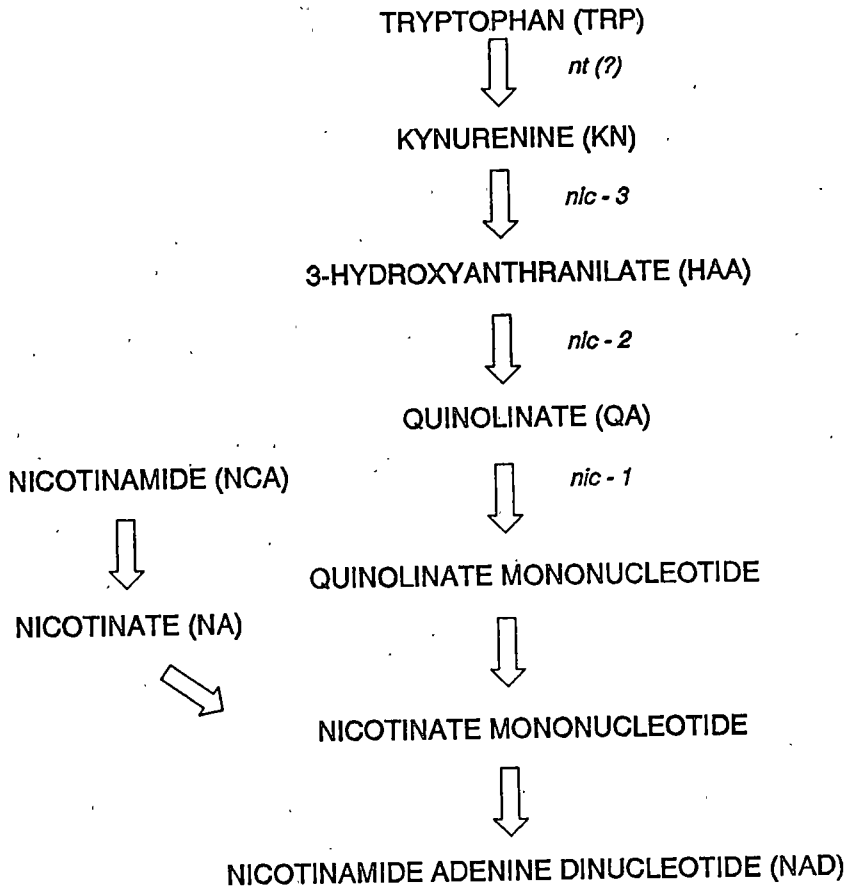

Fig. 5. Biosynthetic pathway from tryptophan to NAD, showing sites of gene action in *N. crassa*.

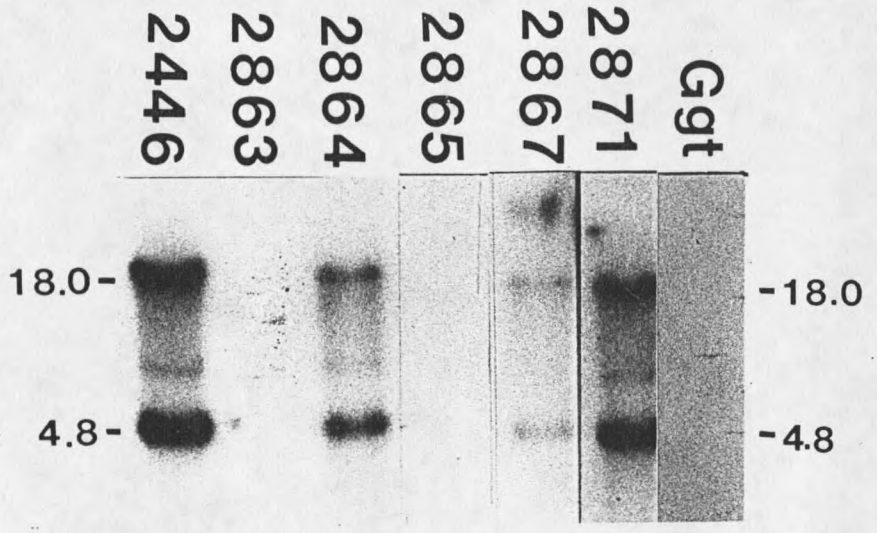

Fig. 6. Southern hybridization of *Hin*dIII-digested DNAs from strain DM528 (wild-type), strain JH2446 (BenR transformant), and progeny from a cross between strains JH2446 and DM528 (strains) probed with labeled pBT3. *Hin*dIII cuts once within pBT3 and does not cut pAN8-1. Molecular weight markers are in kilobase pairs.

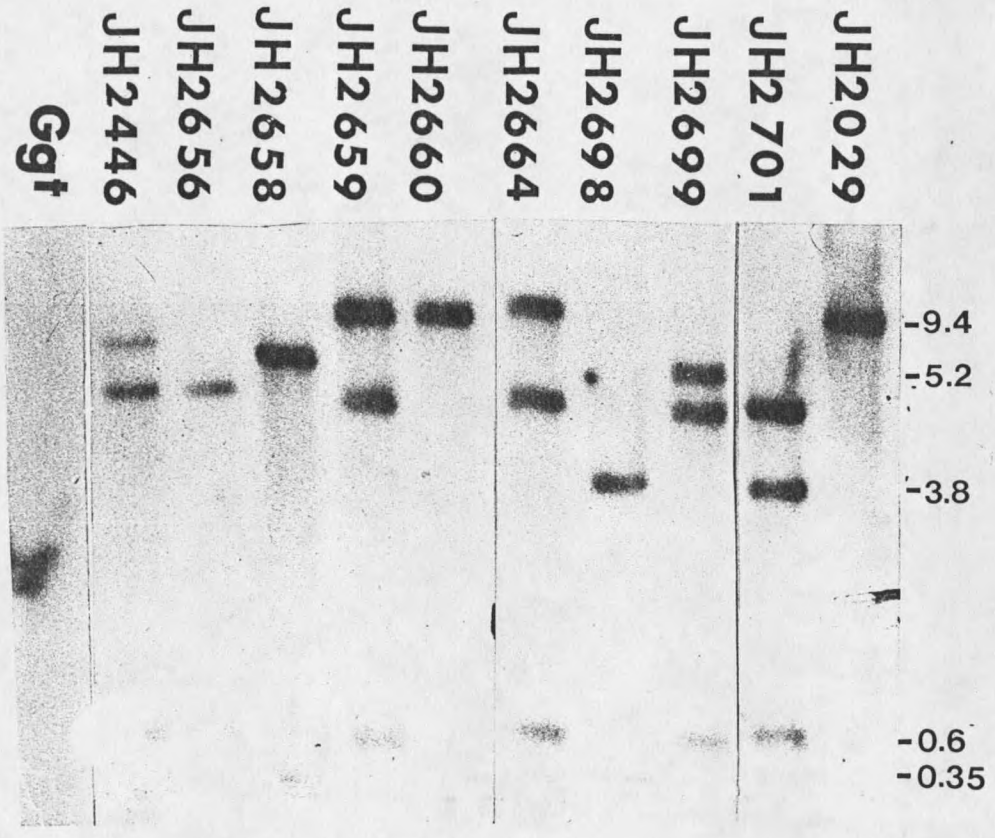

Fig. 7. Southern hybridization of *Nco*I-digested genomic DNAs from strain JH2446 (BenR transformant), strain JH2029 (PhleoR transformant) and progeny from a cross between strains JH2446 and JH2029 (strains JH2656, JH2658, JH2659, JH2660, JH2664, JH2698, JH2699 and JH2701) probed with labeled pBT3. *Nco*I cuts once within pBT3 and does not cut pAN8-1. Molecular weight markers are in kilobase pairs.

CHAPTER FOUR

FATE OF INTEGRATED DNA IN TRANSFORMANTS OF GAEUMANNOMYCES GRAMINIS

Gaeumannomyces graminis is a soilborne ascomycete. Three varieties which cause root diseases on Graminacious plants have been described (reviewed in Walker 1981). *G. g. tritici* is the causative agent of take-all of wheat and barley. *G. g. graminis* is a saprophyte of many grasses and a pathogen of rice (black sheath rot) (Walker 1981) and bermudagrass (spring dead spot) (McCarty and Lucas 1989).

G. graminis has been transformed to benomyl resistance (BenR) (Henson *et al.* 1988) and to phleomycin resistance (PhleoR) (Pilgeram and Henson 1990). The stability of transforming DNA has been investigated in several filamentous fungi. Fungicide resistance was expressed following serial transfer of transformants of *Ustilago maydis* (Wang *et al.* 1988) and *Ascobolus immersus* (Goyon and Faugeron 1989) on nonselective media. Loss of selectable marker was observed in a few transformants of *Cochliobolus heterostrophus* (Keller *et al.* 1991), *Glomerella cingulata* f.sp *phaseoli* (Rodriguez and Yoder 1987), *Fusarium* spp. (Dickman and Partridge 1989) and *G. graminis* (Pilgeram and Henson, 1990). Genetic rearrangements were observed following axenic culturing in multicopy

transformants of *G.c.phaseoli* (Rodriguez and Yoder 1987) and *Ophiostoma ulmi* (Royer *et al.* 1991).

Transformants of Fusarium were resistant to hygromycin following mitotic growth on host plants, but rearrangements and deletions of transforming DNA had occurred (Dickman and Partridge 1989). Deletions of the *hygB* gene could be detected in certain transformants of *C. heterostrophus* following multiple disease cycles on maize (Keller *et al.* 1991).

Meiotic instability of transforming DNA has also been observed in filamentous fungi. DNA sequences can be duplicated when multiple copies of transforming DNA integrate into a fungal genome, tandem duplications of the transforming vector arise, or a fungus is transformed with homologous DNA. Many filamentous fungi modify duplicated sequences during meiosis resulting in reduced expression or skewed segregations. In *Neurospora crassa* sequence duplications are heavily methylated between fertilization and karyogamy leading to numerous G:C to A:T transitions (RIP, repeat induced point mutations (Selker and Garrett 1988)). Nonduplicated transforming sequences are not subject to RIP. Duplicated sequences were also methylated in *Ascobolus immersus*, but not mutated (Goyon and Faugeron 1989). Methylation is not accurately maintained during subsequent mitotic growth, and the silenced genes can be reactivated.

A normal segregation of hygromycin resistance (HygR) was not observed in crosses with multicopy transformants of *Gibberella fujikuroi* (*F. moniliforme*) (Leslie and Dickman 1991). A high ratio of the progeny were HygS, but several

contained *hygB* sequences, which could be expressed in the next generation. The percentage of HygR progeny from crosses of *O. ulmi* transformants crossed with a compatible wild-type strains ranged from 2% to 55% (Royer *et al.* 1991). The basis for the variation was not determined.

In contrast, *Sordaria macrospora* does not modify duplicated genes introduced by transformation during meiosis or mitosis (Le Chevanton *et al.* 1989). Meiotic stability of transforming DNA was also observed in *C. heterostrophus* (Keller *et al.* 1991).

The segregation of *TUB2*, which confers resistance to benomyl and *BLE*, which confers resistance to both bleomycin and phleomycin, in progeny from crosses between a BenR transformant of *G. g. tritici* with a single integrated copy of pBT3 (Orbach *et al.* 1986) and a wild type strain or between the BenR transformant and a PhleoR transformant of *G. g. tritici* with a single integrated copy of pAN8-1 (Mattern *et al.* 1987) have been described (Pilgeram and Henson 1991). Modifications of the *TUB2* gene were not detected in any of the BenR progeny, but deletions and rearrangements of transforming DNA were observed in the transforming DNA of some PhleoR progeny. However, BenR and PhleoR segregated normally. The effect of copy number and transforming vector on the phenotypic and genotypic stability of integrated DNA in *G. g. graminis* and *G. g.tritici* transformants were investigated.

Materials and Methods

Fungal Strains and Media

G. g. tritici (strain DM528) and *G. g. graminis* (strain DM562) were provided by Dr. D.E. Mathre and transformed to benomyl resistance or phleomycin resistance as described (Table. 6). *G. graminis* was routinely cultured in complex L broth (Miller 1972) at room temperature. SM-7, a selective media for *G. g. tritici*, was used to isolate *G. graminis* from infected plants (Elliot Juhnke *et al.* 1984).

Isolation and Analysis of DNA

Fungal DNA extractions, electrophoresis and hybridization of dried gels were performed as described (Henson *et al.* 1987; Henson *et al.* 1988). Plasmid DNA was purified and nick-translated with ^{32}P-thymidine according to standard procedures (Maniatis *et al.* 1982).

Analysis of Fungicide Resistance

Fungicide sensitivity was determined by measuring the radial growth of a culture on L media containing increasing concentrations of benomyl, bleomycin, or phleomycin. BenR was measured on media with 0.0, 0.5, 0.75 or 1.0 μg/ml benomyl (a gift from DuPont, Wilmington, DE) and PhleoR was measured on media with 0.0, 1.0 or 2.0 μg/ml bleomycin (a generous gift from Bristol-Meyer, Evansville, IN) or 22 μg/ml phleomycin (Cayla Laboratories, Toulouse, France). The radial growth of *G. g. tritici* was measured eight days after inoculation at room

temperature and the radial growth of *G. g. graminis* was measured four days after inoculation.

Analysis of Mitotic Stability

BenR transformants of *G. g. tritici* (strains JH2446 and JH872) were inoculated onto L media without benomyl and incubated for two days at room temperature. A plug of each strain was transferred to L media with and without 0.8 μg/ml benomyl and incubated at room temperature. Strains were transferred to fresh media (with or without selection) every four days. The mitotic stability of BenR was assessed after the fourth serial transfer by comparing the radial growth of a transformant cultured with and without selective pressure on increasing concentrations of benomyl.

The stability of fungicide expression in *G. g. tritici* transformants (strains JH2446, JH872 and JH2029) was also determined following a disease cycle. Surface sterilized Pondera wheat seeds were inoculated with mycelia from each transformant. The plants were watered as needed and grown at room temperature with natural lighting from an eastern exposure. Symptoms of take-all (wilting, stem and root discoloration) were apparent in two to three weeks. Fungi were reisolated by placing infected roots or crowns directly onto SM-7 medium. Recovered *G. g. tritici* were then cultured on nonselective L medium prior to analyzing fungicide sensitivity.

Analysis of Meiotic Stability

Mycelial plugs of transformants of *G. graminis* were transferred to tubes containing autoclaved wheat leaves and incubated as described (Pilgeram and Henson 1991). A minimum of four ascospores were isolated from two or three mature perithecia from each transformant and cultured on nonselective L medium. The first generation or S1 refers to ascospores derived directly from *G. graminis* transformants. S1 progeny are referred to by the transformant and perithecia from which they were derived. Strain JH2446-B1 is a S1 ascospore isolated from perithecium B of strain JH2446. Second generation or S2 ascospores are selfed progeny of S1 ascospores. Thus, strain JH872-A1-B3 is a S2 ascospore isolated from perithecium B of JH872-A1.

Results

Mitotic Stability of Transformant Phenotype

Benomyl resistance of *G. graminis* transformants was mitotically stable regardless of the copy number of transforming DNA. For example, as shown in Fig. 8A, the fungicide resistance of transformants JH2446 and JH872 was unaltered following multiple transfers on selective or nonselective media. Similar results were obtained with *G. g. graminis* transformants JH962 and JH1097 (data not shown). Fungicide resistance was also phenotypically stable in *G. g. tritici* transformants reisolated from host plants. Differences in the fungicide sensitivity

of transformant JH872 before and after a disease cycle were insignificant (Fig. 8B). Resistance in strains JH2446 and JH2029 was also mitotically stable after host infection (data not shown).

Meiotic Stability of Transforming DNA

Both BenR and PhleoR were meiotically stable in *G. g. tritici* transformants with a single integrated copy of transforming DNA (strains JH2446 and JH2029). First generation ascospores were as resistant to fungicide as their parent (Fig. 9). PhleoR was also phenotypically stable in progeny from *G. g. graminis* transformed with pAN8-1 (strains JH2016 and JH2028) (Fig. 10).

DNA was isolated from each transformant and its S1 progeny, restricted and probed with transforming vector. DNA from transformant JH2446, restricted with *Hin*dIII, contained three bands (21.0 kb, 7.1 kb and 4.7 kb) (Fig. 11A). The 7.1 kb band was observed in all strains and corresponds to the native β-tubulin gene. The 21.0 and 4.7 kb *Hin*dIII fragments were present in DNA isolated from BenR progeny and were absent from benomyl sensitive strains (i.e. *G. g. tritici*).

An additional hybridizing fragment (3.4 kb) was present in *Hin*dIII DNA from non-transformed *G. g. tritici* and ascospores derived from strain JH2446 (Fig. 11A), but was not observed in DNA from strain JH2446. A similar phenomenon was observed in a *G. g. tritici* blot probed with pAN8-1, where an intense band (3.4 kb) was present in *Hin*dIII digested DNA from non-transformed wild-type, transformant JH2029, and most JH2029 progeny, but not all progeny (Fig. 11B). The presence or absence of the hybridizing band in *G. g. tritici* correlated with

when the DNA was isolated (i.e. when the strain was cultured). *G. g. tritici* will not survive at temperatures above 30 C. In July and August, the lab approached the non-permissive temperature and DNA from *G. g. tritici* cultures grown under these conditions contain the hybridizing fragment. The fragment is absent from DNA isolated from strains cultured at lower temperatures. Thus, the hybridizing fragment appears to be a response to heat stress, but will require further characterization. Additional hybridizing DNA was not observed in strains of *G. g. graminis*, which are tolerant of higher temperatures.

In addition to the 3.4 kb *Hin*dIII fragment, pAN8-1 hybridized to a 14.0 kb fragment in transformant JH2029 (Fig. 11B). The intact 14.0 kb fragment was observed in some PhleoR progeny (i.e. strains JH2029-B3 and JH2029-D1), but was partially deleted or rearranged in others (i.e. strains JH2029-B4 and JH2029-C1). The DNA modifications did not affect PhleoR (Fig. 10B), indicating that the *ble* gene itself was unaltered.

When DNA from strain JH2016 was restricted with *Hin*dIII and probed with ^{32}P-labeled pAN8-1, two hybridizing fragments (16.0 kb and 3.5 kb) were observed (Fig. 12A). The 16.0 Kb homologous fragment was observed in all progeny analyzed, but the 3.5 kb fragment was frequently deleted. Hybridizing DNA was not present in untransformed *G. g. graminis*.

Strain JH2028 contained three fragments which hybridized to ^{32}P-labeled pAN8-1 when the DNA was restricted with *Hin*dIII (18.0 kb, 11.0 kb and 3.5 kb) (Fig. 12B). The 18.0 kb and 11.0 kb fragments were present in all ascospore

derivatives of strain JH2028, but the presence of the 3.5 kb fragment was variable and could be homologous to the 3.5 kb fragment observed in strain JH2016. Additional polymorphisms were not observed when the DNA was restricted with *Pst*I or *Eco*RI (data not shown).

Duplicated copies of the *TUB2* gene were not meiotically stable in BenR transformants of *G. g. tritici* or *G. g. graminis*. Progeny were more susceptible to benomyl than their transformed parent but less susceptible than the untransformed wild-type. The genotype of transforming DNA was also altered in the progeny.

G. g. graminis transformants JH964 and JH2974 both contained multiple copies of *TUB2*. Strain JH964 contained more fragments homologous to ^{32}P-labeled pBT3 than strain JH2794 and was more resistant to benomyl (Figs. 13 and 14). Ascospore progeny from strain JH2974 were equally susceptible to benomyl, but the susceptibility of progeny from strain JH964 was quite variable (Fig. 13).

DNA sequences within the 1.5 kb, 3.5 kb and 5.1 kb *Hin*dIII fragments observed in strain JH964 contribute to benomyl resistance (Fig. 14A). Strains JH964-C2 and C3 lack the 1.5 kb fragment and were slightly more susceptible to benomyl than strain JH964 (Figs. 13A and 14A). Benomyl resistance was further reduced in strains JH964-A3 and C4 in which both the 1.5 kb fragment and the 3.5 kb were absent. The 3.5 kb fragment and 5.1 kb fragment were deleted from the genome of strains JH964-F1, F2 and F3, and although the 1.5 kb fragment was present, they were as susceptible to benomyl as the wild-type.

Radial growth on media containing benomyl was fairly uniform among ascospore progeny from strain JH2974 (Fig. 13B). The genotypes of transforming DNA in the progeny were also similar. Four hybridizing bands were observed in transformant JH2794 (Fig. 14B). The 6.0 kb band was present in all of the ascospore progeny and encoded a *TUB2* gene because the progeny were more resistant to benomyl than the wild-type. The absence of the 3.7 kb band in some of the progeny (strain JH2974-A1 and B1) did not impact benomyl resistance. The 3.4 kb and/or the 2.9 kb fragments of strain JH2974 also encoded BenR and were not present in ascospore progeny. A 3.0 kb fragment was observed in some progeny but it did not increase benomyl resistance.

Meiotic instability of transforming DNA was also observed in a transformant of *G. g. tritici* containing multiple copies of pBT3 (strain JH872). Again, S1 progeny were less resistant to benomyl than strain JH872 (Fig. 15A). Differences in levels of resistance were not alleviated by 5-azacytidine which inhibits cytosine methylation, indicating cytosine methylation was not suppressing gene expression (data not shown). Alternatively, the 5-azacytidine may not have been taken up by the organism.

DNA from strain JH872 and six S1 progeny isolated from a single perithecium were cut with several restriction enzymes including *Hin*dIII (Fig. 15B). DNA deletions were detected in the DNA from all six progeny. Several bands observed in strain JH872 were unaltered in the S1 progeny and one or several encoded benomyl resistance. The reduction in benomyl resistance would indicate

that at least one *TUB2* gene had been inactivated in the progeny. DNA modifications in some of the progeny were indistinguishable from each other (strain JH872-A1 and JH872-A5; strains JH872-A3 and JH872-A6).

The stability of transforming DNA in second generation ascospore progeny of strain JH872 was also analyzed. Some S2 progeny (JH872-A1-A1 and JH872-A1-A3) were as resistant to benomyl as their S1 parent (Fig. 16). Others were less resistant but none were more resistant. More variation in levels of BenR was observed in S2 progeny derived from a S1 parent with a higher level of resistance (i.e. JH872-A3) (Fig.16A) than in S2 progeny derived from a S1 parent with a lower level of resistance (i.e. JH872-A2) (Fig. 16B). Sequences homologous to pBT3 continued to rearrange in the second generation of strain JH872, although some fragments remained unaltered in different progeny (Fig. 17).

Discussion

Fungicide resistance was phenotypically stable in *G. g. tritici* following serial transfer on selective or nonselective media and following reisolation from an infected host regardless of the transforming vector or number of integrated copies (Fig. 8). Following a disease cycle, the fungicide sensitivity of G. g. tritici isolates was unchanged from that of the original transformant.

Following a single disease cycle, transformants of *C. heterostrophus* reisolated from diseased maize plants were still HygBR, but deletions of the integrated DNA from transformants with a single copy of transforming vector

integrated at a homologous site could be observed following eight or more disease cycles (Keller *et al.* 1991). Dickman and Partridge (1989) reisolated fungicide resistant transformants of *F. graminearum* and *F.moniliforme* from inoculated maize plants, and although the transforming phenotype was retained, rearrangements and deletions of transforming DNA were observed.

The meiotic stability of transforming DNA in *G. graminis* was dependent upon the transforming vector, the copy number of that vector and the variety transformed. PhleoR was phenotypically stable in *G. graminis* transformed with pAN8-1 regardless of copy number (Figs. 9B, 10A, and 10B), but was not genotypically stable (Fig. 11B, 12A, and 12B). Rearrangements and deletions were observed in genomic DNA from transformant progeny probed with ^{32}P-labeled pAN8-1. The stability of PhleoR would indicate that the *ble* gene itself is not extensively modified.

The level of BenR was unaltered in the progeny of a *G. g. tritici* transformant with a single integrated copy of pBT3 (Fig. 9A). Fragments in genomic DNA from strain JH2446 which hybridized with ^{32}P-labeled pBT3 were unchanged in DNA from ascospore progeny (Fig. 11A).

Both the phenotype and genotype conferred by transforming DNA were altered in the progeny derived from *G. graminis* with multiple integrated copies of pBT3. Cultures derived from selfed ascospores were less resistant to benomyl than their parent transformant (Figs. 13A, 13B and 15A). Progeny with increased levels of resistance were not observed.

The loss of fungicide resistance in progeny from multicopy pBT3 transformants was apparently due to deletions of or within a *TUB2* gene. Transforming DNA was more extensively deleted in strain JH872, a *G. g. tritici* transformant, than in strains JH964 or JH2974, *G. g. graminis* transformants. Additional transformants will need to be analyzed to confirm varietal differences.

DNA homologous to ^{32}P-labeled pBT3 was deleted in both the first and second generation ascospores derived from strain JH872 (Fig. 16). Some of the S2 progeny were as resistant to benomyl as their S1 parent, others were as susceptible as the wild-type. S2 progeny contained DNA which hybridized to ^{32}P-labeled pBT3 regardless of its level of resistance. The stability of transforming DNA in the third generation is under investigation.

The instability of multiple copies of *TUB2* could result from its homology with the native β- tubulin gene or the toxicity of surplus β-tubulin. The *A. nidulans benA* (β-tubulin) gene was used to identify the *TUB2* gene in a λ library from a BenR mutant of *N. crassa* (Orbach *et al.* 1986). The *TUB2* protein of *N. crassa* differs from native β-tubulin at a single amino acid and forms functional microtubules in the presence or absence of benomyl. Excess β-tubulin was toxic to *Saccharomyces cerevisiae*, resulting in loss of microtubule structure and eventual loss of viability (Katz *et al.* 1990; Weinstein and Solomon 1990).

The *ble* gene product binds to bleomycins or phleomycins and prevents them from cleaving DNA (Gatignol *et al.* 1988). The *ble* gene does not have a

homolog within the fungal genome nor does its expression appear to be detrimental to filamentous fungi.

Transformants of *G. graminis* tolerated multiple copies of the *ble* gene better than multiple copies of the *TUB2* gene. Multiple copies of the *TUB2* gene triggered gene deletions and decreased benomyl resistance in sexual progeny. Although, when pAN8-1 DNA was deleted, sensitivity to bleomycin or phleomycin was not altered. Consequently, we conclude that active copies of the *ble* gene were not modified.

The relative stabilities of various transformation constructs could affect their usefulness as dominant markers in releases of recombinant organisms, but the instability of duplicated sequences has been a useful tool to characterize genes in filamentous fungi. Wild-type *N. crassa* was cotransformed with the wild-type *nmr* (negative nitrogen regulatory gene) and *hygB* genes and crossed with wild-type (Jarai and Marzluf 1991). The duplicated *nmr* genes were RIPed creating chlorate sensitive (nmr-) progeny. Marathe *et al.* (1990) recovered *N. crassa* progeny incapable of utilizing acetate as a nutrient source from a cross between transformant with multiple copies of a gene preferentially transcribed when acetate was the sole carbon source and a wild strain.

Deletion of transforming DNA was not observed in *G. graminis* during mitotic growth. Tandem duplications are mitotically unstable in *C. heterostrophus*, where a single copy of the duplication is occasionally excised following multiple disease cycles on maize (Keller *et al.* 1991). Excision of transforming DNA is

detected by the loss of HygR, but the homologous wild-type sequences could also have been deleted in other isolates. Homologous integration of a mutated gene into the wild-type locus and the subsequent excision of the wild-type gene would result gene replacement in some of the conidia. Reciprocal recombination of duplicated sequences during mitotic growth also resulted in gene replacement in conidia of *A. nidulans* (Dunne and Oakley 1988). Multiple copies of transforming DNA may be deleted in *G. graminis* following multiple disease cycles or in the germinating phialidic conidia produced by G. g. graminis (Wong and Walker 1975).

VARIETY	TRANSFORMANT	VECTOR	INTEGRATED COPIES	REFERENCE
Ggt	JH2446	pBT3	single	Pilgeram and Henson 1991
	JH872	pBT3	multiple	This study.
	JH2029	pAN8-1	single	Pilgeram and Henson 1991
Ggg	JH1097	pBT3	single	Henson, Blake and Pilgeram 1988
	JH962	pBT3	multiple	Henson, Blake and Pilgeram 1988
	JH964	pBT3	multiple	Henson, Blake and Pilgeram 1988
	JH2974	pBT3	multiple	This study.
	JH2016	pAN8-1	single	Pilgeram and Henson 1990
	JH2028	pAN8-1	multiple	Pilgeram and Henson 1990

Table 6. Characterization of transformants of *Gaeumannomyces graminis*.

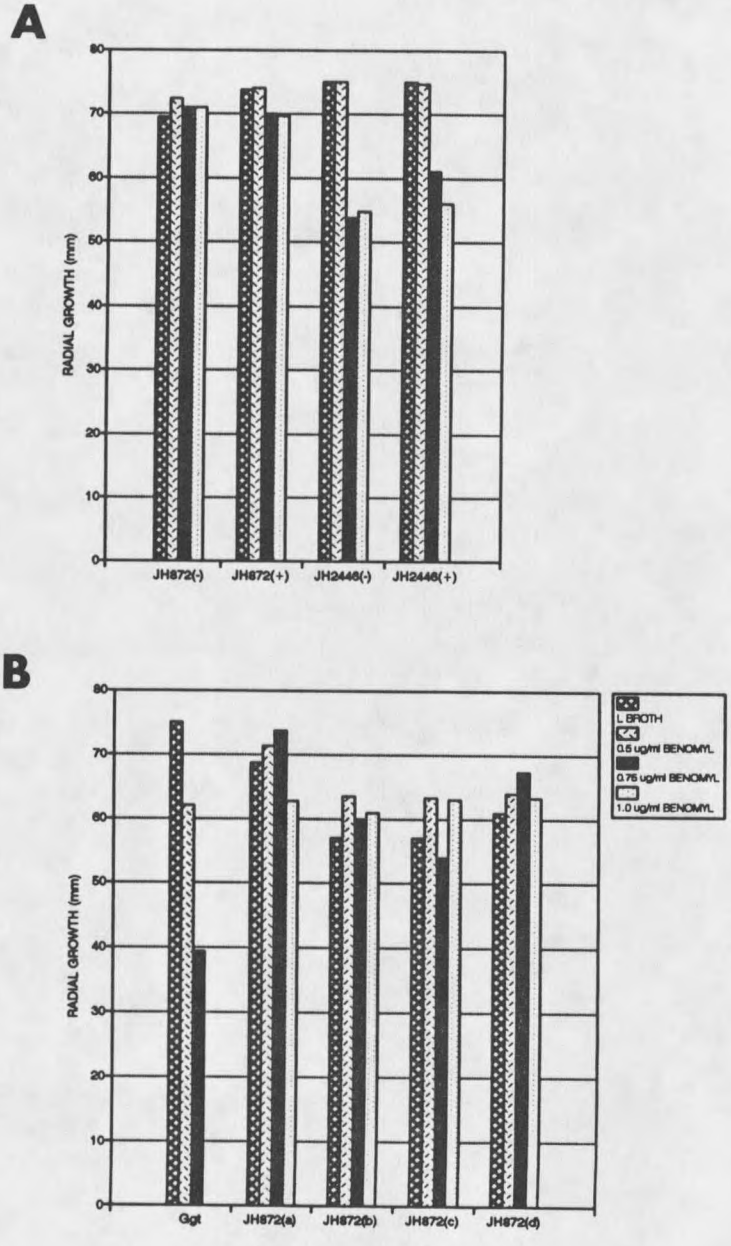

Fig. 8. Mitotic stability of *G. g.tritici* transformants. **A** Comparison of ben^R following four serial transfers of strains JH872 and JH2446 on nonselective L medium (-) or selective L medium (0.8 μg/ml benomyl (+)). **B** Benomyl resistance of strain JH872 before (a) and after (b,c,d) a disease cycle. Radial growth was measured eight days after inoculation.

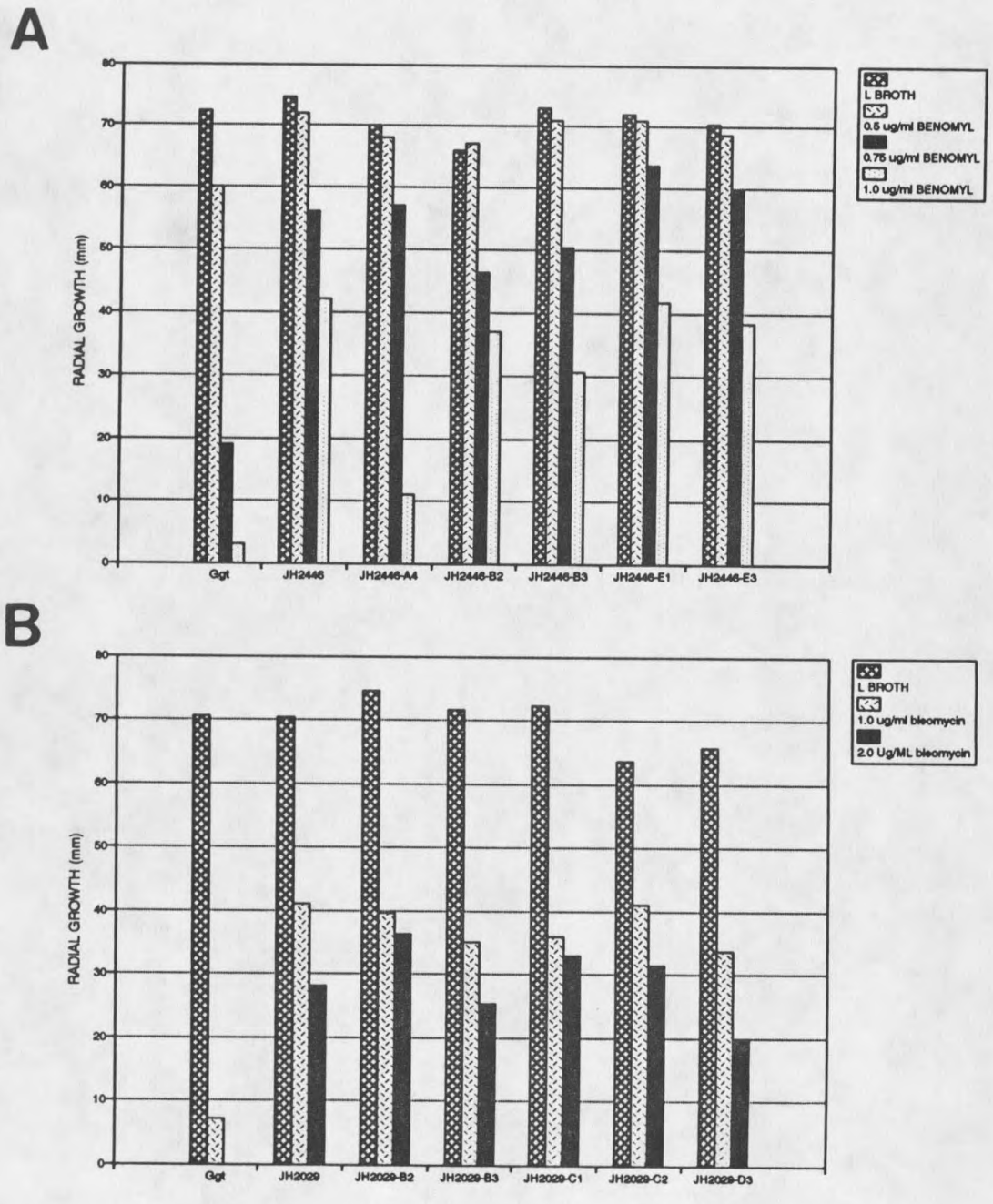

Fig. 9. Meiotic stability of fungicide resistance in *G. g. tritici (Ggt)* transformants containing a single copy of transforming DNA. *A* Benomyl resistance of transformant JH2446 which contains a single integrated copy of pBT3 and its ascospore progeny. **B** Bleomycin resistance of strain JH2029 which contains with a single integrated copy of pAN8-1 and its ascospore progeny. Radial growth was measured eight days after inoculation.

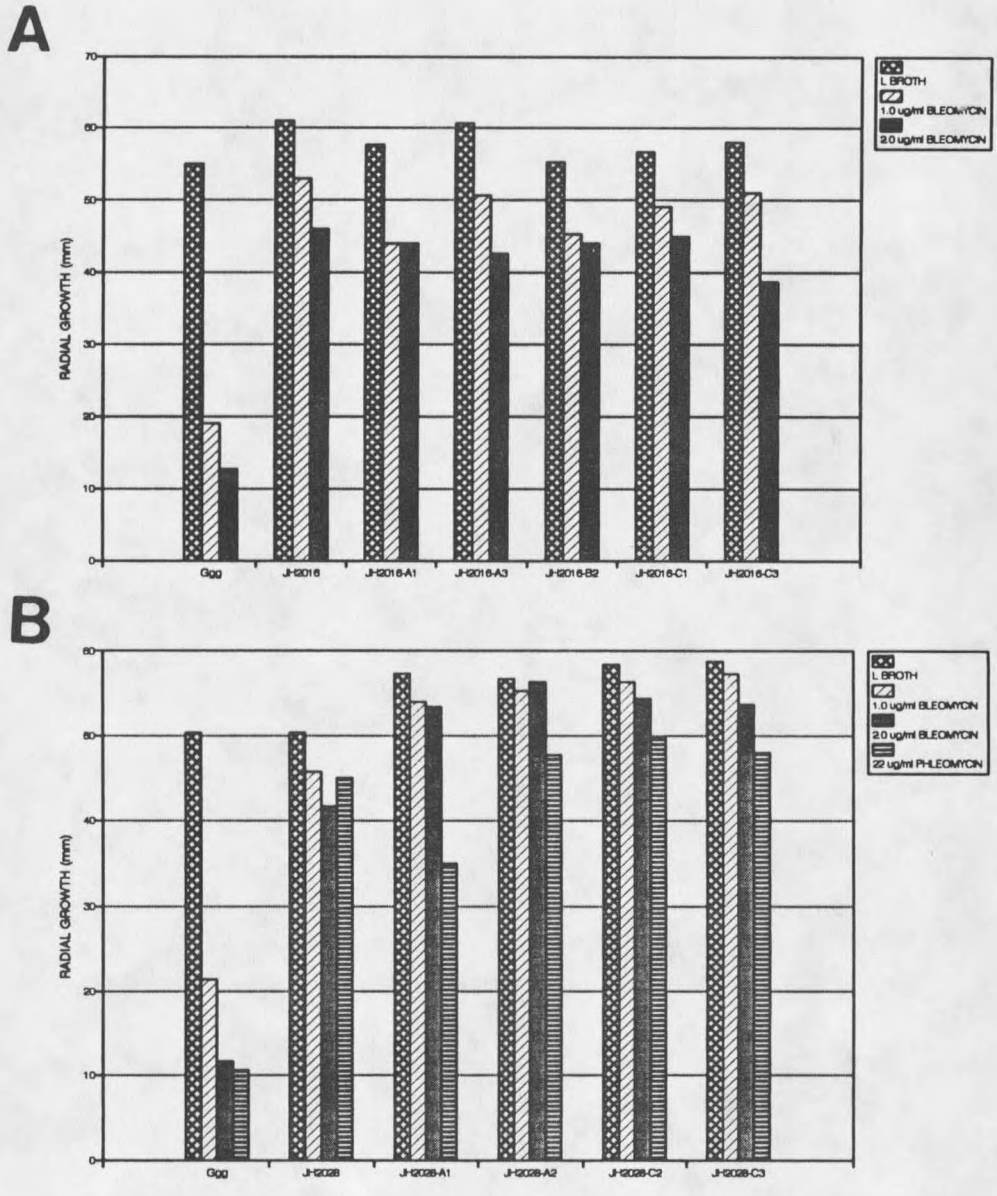

Fig. 10. Meiotic stability of bleomycin resistance in **A** *G. g. graminis* (*Ggg*) transformed with a single copy of pAN8-1 (strain JH2016) or **B** *G. g. graminis* transformed with multiple copies of pAN8-1 (strain JH2028). Radial growth was measured eight days after inoculation.

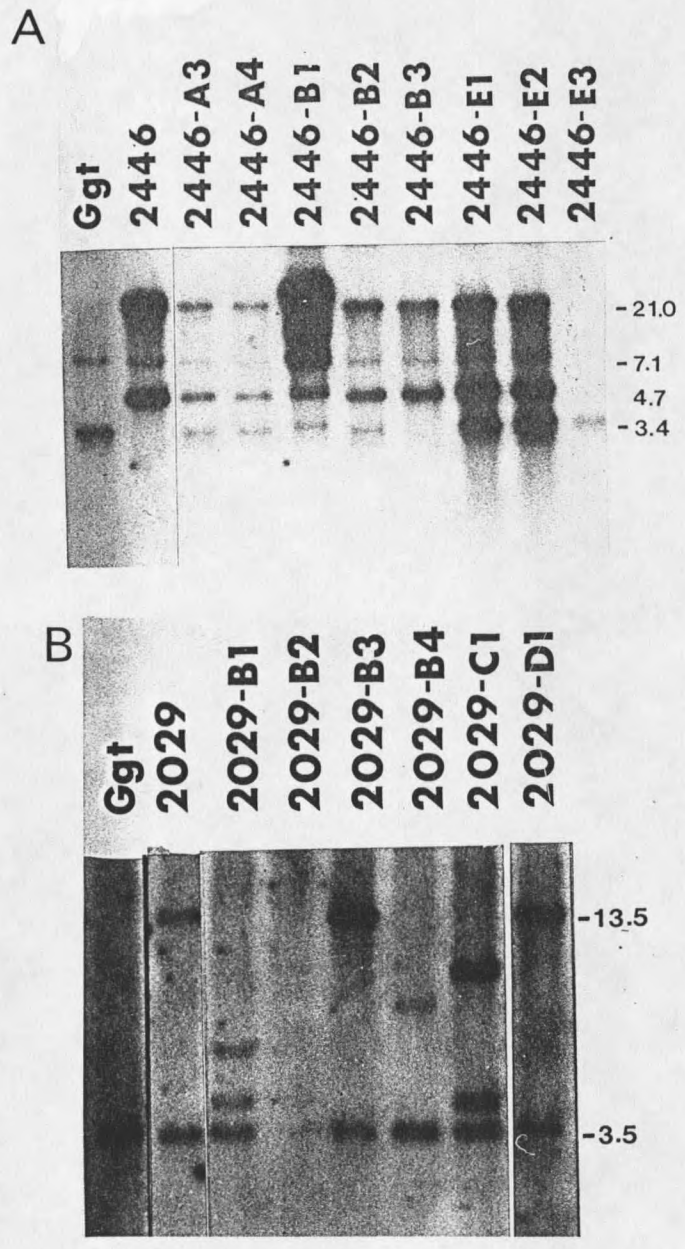

Fig. 11. Meiotic stability of the *TUB2* gene or *ble* gene in *G. g. tritici* (*Ggt*) transformants containing a single copy of transforming DNA. **A** Genomic DNA from wild-type *Ggt*, transformant JH2446 and its ascospore progeny, restricted with *Hin*dIII and probed with ^{32}P-labeled pBT3. **B** Genomic DNA from wild-type *Ggt*, transformant JH2029 and its ascospore progeny, restricted with *Hin*dIII and probed with ^{32}P-labeled pAN8-1. Molecular weight markers are in kilobase pairs.

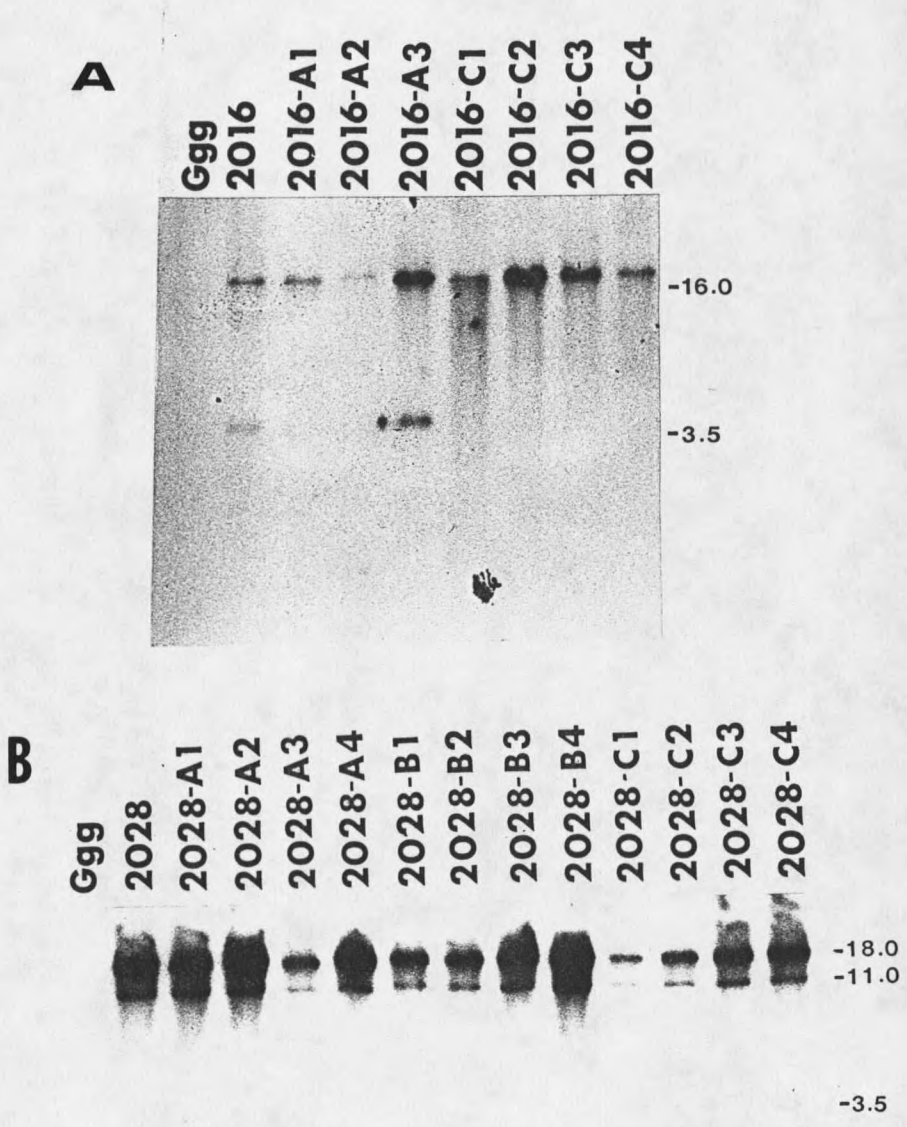

Fig. 12. Meiotic stability of the *ble* gene in *Hin*dIII restricted DNA from *Ggg* transformants probed with ^{32}P-labeled pAN8-1. **A** Transformant JH2016, which contained a single integrated copy of pAN8-1, and its ascospore progeny. **B** Transformant JH2028, which contained multiple copies of pAN8-1, and its ascospore progeny. Molecular weight markers are in kilobase pairs.

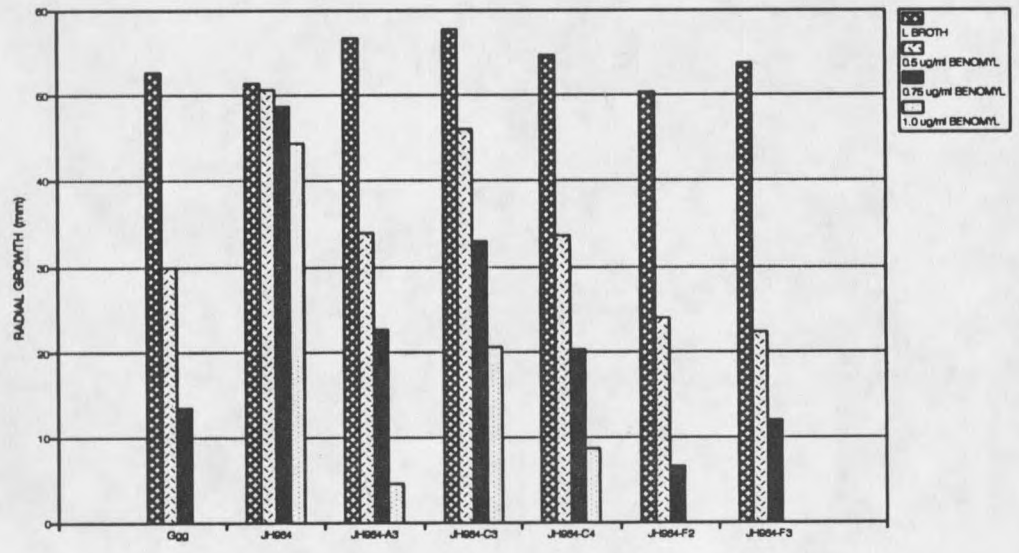

Fig. 13. Benomyl resistance of a *G. g. graminis* (*Ggg*) strain (JH964) transformed with multiple copies of pBT3 and S1 progeny.

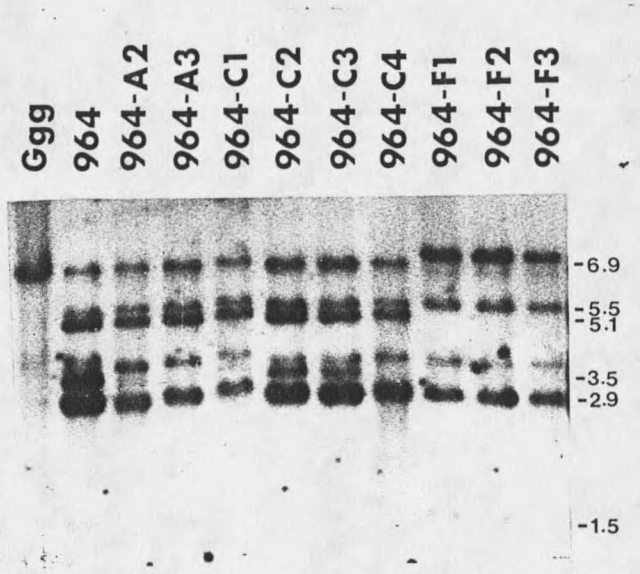

Fig. 14. Meiotic stability of the *TUB2* gene in a multi-copy transformant of *G. g. graminis* (*Ggg*) (strain JH964), restricted with *Hin*dIII and probed with ^{32}P-labeled pBT3.

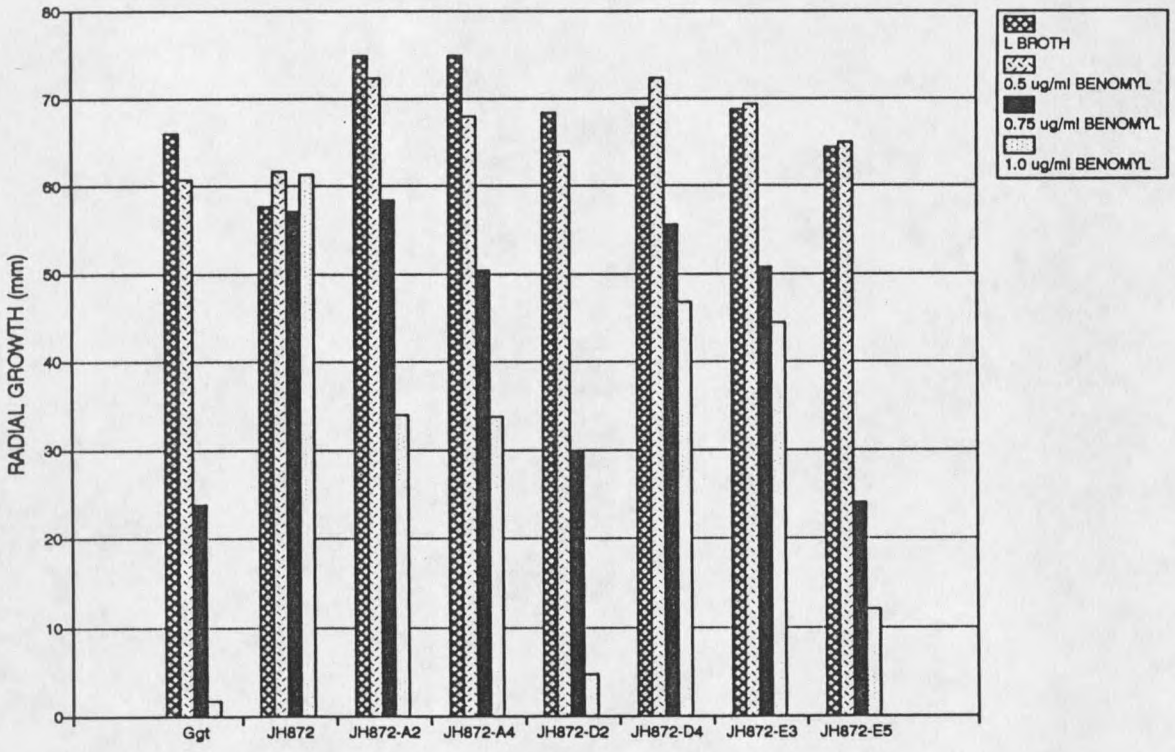

Fig. 15. Meiotic stability of transforming DNA in strain JH872, a *G. g. tritici* transformant with multiple copies of pBT3. Benomyl resistance of strain JH872 and first generation progeny. Radial growth was measured eight days after inoculation.

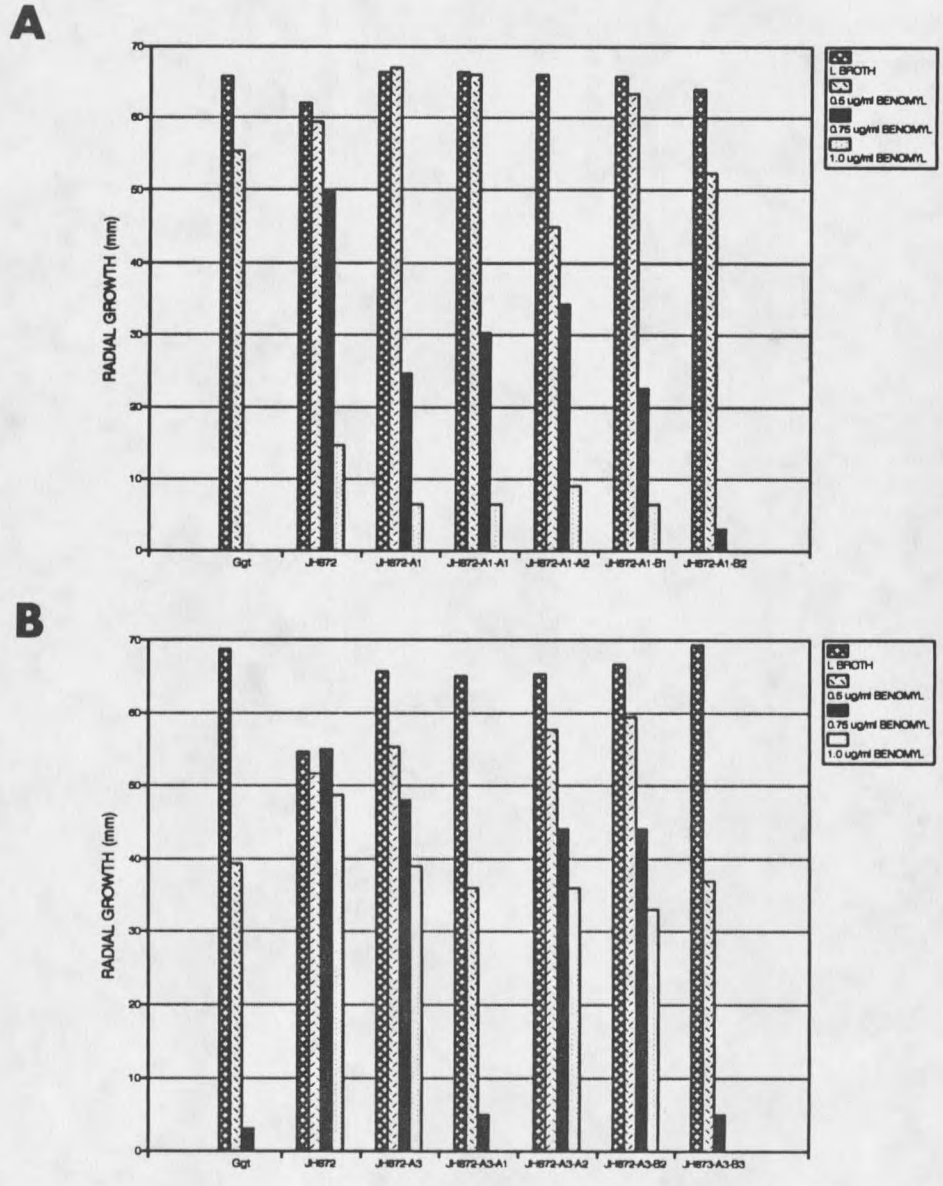

Fig. 16. Benomyl resistance of JH872, first generation and second generation ascospores. **A** JH872-A1 and progeny. **B** JH872-A3 and progeny. Radial growth was measured eight days after inoculation.

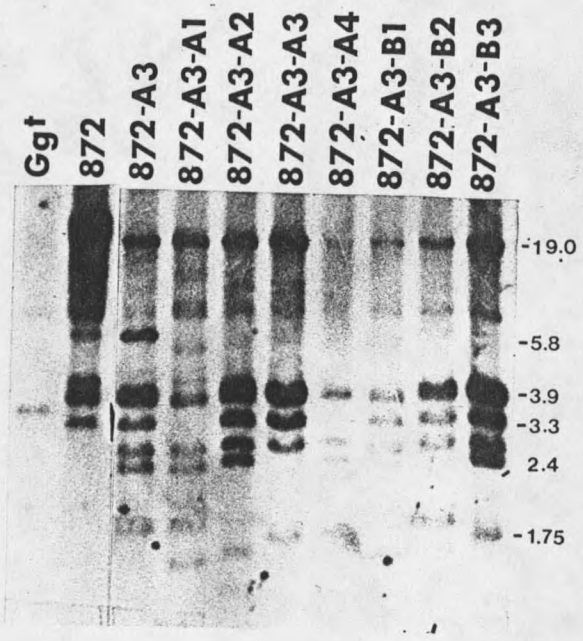

Fig. 17. Hybridization of ^{32}P-labeled pBT3 with genomic DNA isolated from strains JH872, JH872-A3 and JH872-A3 progeny and restricted with *Hin*dIII. Molecular weight markers are in kilobase pairs.

CHAPTER FIVE

SUMMARY

Molecular biology will contribute to our understanding of *G. graminis* and its interaction with host plants. Transformants of *G. graminis* can be differentiated from other strains (transformed or non-transformed) on the basis of phenotype (fungicide resistance) and genotype. Thus, the effect of a single isolate of *G. graminis* in the development of disease could be determined by inoculating a single plant in a field with a marked strain and following disease progression.

Furthermore, transformation is not limited to genes which confer fungicide resistance and may be helpful in isolating pathogenicity or host range genes. *G. g. avenae* is closely related to *G. g. tritici*, but is capable of attacking oats. *G. g. avenae* produces avenacinase, an enzyme which inactivates avenacin, an antifungal glucoside present in oat roots, enabling the pathogen to establish a persistent infection (Turner 1961). *G. g. tritici* transformed with the gene encoding avenacinase may also be pathogenic on oats.

Perithecia are produced on the remains of host plants under certain environmental conditions (reviewed in Skou 1981). Selfed and crossed perithecia, recovered from plants inoculated with transformants of *G. g. graminis* or *G. g. tritici*, could easily be differentiated using the transforming phenotype. Normal distributions of transforming DNA were observed in outcrossed progeny. Thus,

outcrossing and the subsequent rearrangement of genetic traits can occur in *G. graminis*.

Selfed progeny of *G. graminis* were less resistant to benomyl than their multiple copy parent. Therefore, the phenotype and genotype of an ascospore culture derived from a BenR transformant with multiple integrated copies of the *TUB2* would be different from that of the transformant or the wild-type *G. graminis*. New take-all patches can arise from the mechanical transfer of infected plant debris, but ascospores may also be a source of inoculum. If a patch resulted from the mechanical transfer of a BenR transformant from an established patch, the phenotype and genotype of the fungi isolated from each patch should be identical. If the patch originated from an ascospore from the BenR transformant, fungi isolated from the two patches will be phenotypically and genotypically different.

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