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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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*.

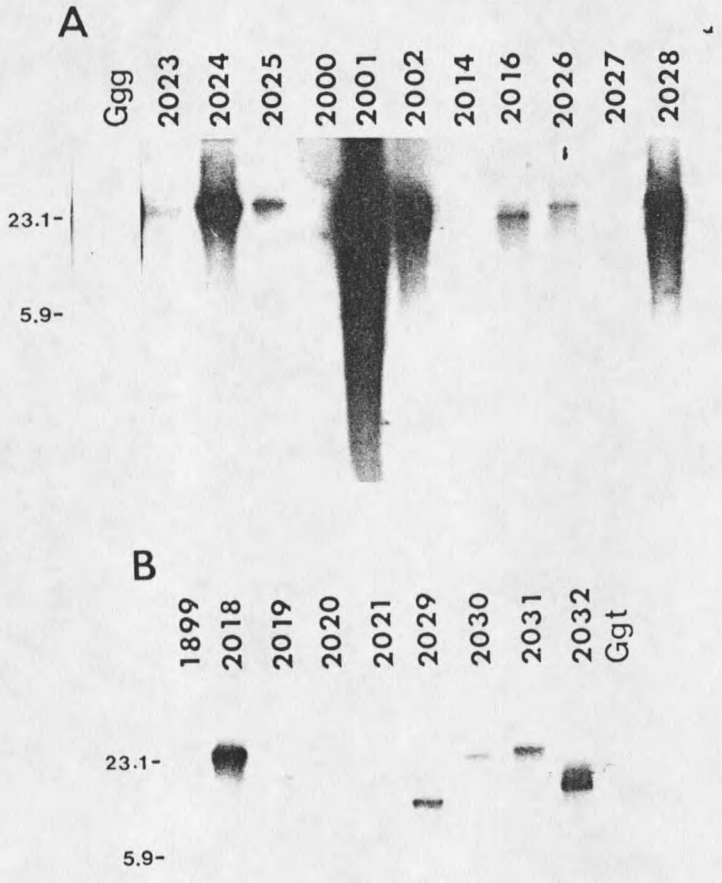

Fig. 1. Hybridization of labeled pAN8-1 with control *Gaeumannomyces graminis* and transformant genomic DNA, which was digested with *Hin*dIII, an enzyme which does not cut pAN8-1. **A.** *G. g. graminis* (*Ggg*) control and transformants. **B.** *G. g. tritici* (*Ggt*) control and transformants. Molecular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