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EXPRESSION ANALYSIS OF AN AFLATOXIN NOR-1/UIDA REPORTER FUSION IN ASPERGILLUS PARASITICUS

By

David L. Wilson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

Department of Food Science and Human Nutrition

1999

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ABSTRACT

EXPRESSION ANALYSIS OF AN AFLATOXIN NOR-1/UIDA REPORTER FUSION IN ASPERGILLUS PARASITICUS

By

David L. Wilson

Aflatoxins are carcinogenic contaminants of the agricultural environment that are produced as a result of fungal infection of plant tissue. Reporter gene fusions are powerful tools that can be used to monitor the interaction between plant and fungus, and are being developed in our laboratory to aid in the analysis of aflatoxin production in *Aspergillus parasiticus*. This study evaluates the expression of a β-glucuronidase (GUS) reporter gene (*uidA*) which is fused to the *nor-1* aflatoxin gene. Under aflatoxin - inducing conditions, our data showed that the patterns of *nor-1* and *nor-1/uidA* transcript and protein accumulation were similar. The data also demonstrated that the timing of aflatoxin production and GUS activity were identical, while the pattern of aflatoxin accumulation was consistent with the levels of GUS activity. Transformation experiments were supportive of a position-dependent expression mechanism operating in the aflatoxin gene cluster (Liang *et al.*, 1997). Integration of the *nor-1/uidA* reporter construct outside of the aflatoxin gene cluster resulted in a GUS$^-$ phenotype. In order to avoid chromosomal position effects on reporter fusion expression, the 3' *nor-1* region of the chromosome was targeted for integration of the *nor-1/uidA* reporter construct. Transformants with the reporter integrated at this site will be used in future reporter expression studies. A qualitative GUS assay and a PCR assay were developed to screen for transformants with this type of integration event.

This thesis is dedicated to my family. They provided constant support and the occasional distraction.

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ACKNOWLEDGMENTS

I am grateful for the guidance of Dr. John Linz during the course of my research. His support and critical analysis of my designs and results not only shaped this work but also my development as a scientist. I would also like to thank Dr. James Pestka, Dr. Gale Strasburg, and Dr. Mark Uebersax for their valuable time and expertise as part of my guidance committee. I was fortunate to have all of these scholars as teachers during my degree program and each provided a unique perspective of the relationship between the food industry and food science. My friendships with Matt Rarick, Mike Miller, Tzong-Shoon Wu, Ludamila Roze, Frances Trail, Shun-Hsin Liang, Nibedita Mahanti, Richard Zhou, Li-Wei Lee, Ching-Hsun Chiou, Kristin Kneely, and Jaimie Oatley were important for the every day insight and support crucial for success in scientific endeavors. To all I owe a debt of friendship and the rights to hear my paradigm shifting ideas.

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LIST OF ABBREVIATIONS

AF	Aflatoxin
AFB_1	Aflatoxin B_1
CZ	Czapek-Dox
GAL	β-galactosidase
GUS	β-glucuronidase
SGC	SpoC1 gene cluster
YES	Yeast Extract Sucrose

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LITERATURE REVIEW

Overview

Aflatoxins comprise a family of toxic and carcinogenic secondary metabolites produced primarily by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Diener and Davis, 1969). The aflatoxins were first characterized in the early 1960's as feed contaminants and the causative agents of Turkey-X-Disease, an illness that decimated the domestic turkey population in the London region of England (Blount, 1961; Sargeant *et al.*, 1961). Thin layer chromatographic analysis on suspect feed led to the identification of the major aflatoxins (termed Aflatoxin B_1, B_2, G_1, and G_2) based on their R_f values and fluorescent color under ultraviolet light (Hartley *et al.*, 1963; Goldblatt, 1969; Pons and Goldblatt, 1969). Aflatoxin B_1 (AFB_1) is considered the most acutely toxic of the aflatoxins with lethal doses in test animals such as ducklings, rats, hamsters, guinea pigs, rabbits, dogs and trout generally resulting in gross liver necrosis (Wogan, 1966). Day old ducklings are among the most sensitive species with an LD_{50} of approximately 0.5 mg/kg after 48 hours (Asao *et al.*, 1963; Hartley *et al.*, 1963; Wogan, 1966).

Animal studies have also determined AFB_1 to be extremely hepatocarcinogenic. An early analysis on the carcinogenic effect of AFB_1 on ducklings, reported that eight of eleven birds developed hepatic tumors after being fed rations containing 30 ppb AFB_1 for fourteen months (Carnaghan *et al.*, 1965). A similar study using rainbow trout showed 24 of 30 fish developed hepatomas after consuming a diet consisting of 7.9 ppb AFB_1 for fifteen months (Sinnhuber *et al.*, 1968). In one of many experiments with rats, 25 of 25 animals fed rations containing 15 ppb AFB_1 for up to 80 weeks developed hepatocellular *e*

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carcinomas (Wogan and Newberne, 1967).

The initiation of the carcinogenesis process requires mutations in genomic DNA, and AFB_1 has mutagenic potential due to its *in vivo* oxidation to the highly reactive AFB_1-8,9-epoxide by hepatic cytochrome P450 (Eaton and Gallagher, 1994). This epoxide can form adducts with the N^7-guanine of DNA and consequently induce mutations. Numerous studies in animals have demonstrated that AFB_1-DNA adduct formation and cancer risk are linearly proportional (Bechtel, 1989; Eaton and Gallagher, 1994). With regard to the human health threat, the available evidence on the carcinogenicity of AFB_1 has been deemed sufficient by the International Agency for Research on Cancer to classify AFB_1 as a human carcinogen (IARC, 1993). Epidemiological analyses have indicated a role for the p53 tumor suppressor gene in human hepatocellular carcinomas from regions of the world with dietary aflatoxin exposure (Hsu *et al.*, 1991; Bressac *et al.*, 1991; Eaton and Gallagher, 1994). Codon-specific GC→TA transversions and GC→CG transitions in the p53 gene of these carcinoma cells have been identified, and the same types of mutations can be found in human cells mutagenized with AFB_1 and the AFB_1 epoxide (Loechler, 1994; Aguilar *et al.*, 1993). The formation of adducts between the AFB_1 epoxide and this p53 mutational hotspot region have also been demonstrated (Kobertz *et al.*, 1997).

Economic Impact

A. flavus and *A. parasiticus* occupy a broad niche in the agricultural environment and are capable of producing aflatoxins on commercially important substrates such as corn, peanuts, cottonseed and treenuts (Diener *et al.*, 1987; Ellis *et al.*, 1991). When drought-damaged or insect-damaged, these crops become susceptible to fungal colonization, which in turn can lead to aflatoxin biosynthesis and crop contamination

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(Diener *et al.*, 1987; Pier, 1992). The transportation and storage of these commodities at inappropriate temperatures and moisture levels also provides opportunities for fungal growth and toxin formation (Ellis *et al.*, 1991; Pier, 1992).

Aflatoxin contamination of the food supply occurs in many regions of the world (Jelinek *et al.*, 1989) jeopardizing both human and animal health. Acute aflatoxin poisoning can have a dramatic impact on animal production as illustrated by the high mortality rate of Turkey-X-Disease (Blount, 1961). However, the more common financial concern among the beef, poultry, swine and dairy industries is with chronic aflatoxin exposure, which results in inferior feed conversion, growth suppression, decreased reproductive potential, impaired immune responses and toxin carryover into meat, milk and eggs (Pier, 1992; Shane, 1994).

Because of the global threat of aflatoxins to health and the economy, many countries have regulatory limits on the amount of toxin permitted in food and feed. The United States Food and Drug Administration has set an action level of 20 ppb total aflatoxins in food. The allowable levels in different areas of the world range from zero ppb to 500 ppb total aflatoxins (Gourama and Bullerman, 1995; Rustom, 1997).

The restrictions on aflatoxins in the food supply leads to an additional economic burden on growers, who are unable to market contaminated commodities or are forced to sell them at reduced prices. Detoxification methods, which include treatment with ammonia, sodium bisulfite, calcium hydroxide or formaldehyde are options available to help eliminate post-harvest contamination. A more recent approach to maintaining animal performance while using contaminated feed is to include alumina, silica or aluminosilicates as toxin binding materials. In theory, these binding compounds inhibit toxin absorption in the intestinal tract, thus preventing AFB_1 metabolism by the animal

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(Piva *et al.*, 1995). Despite their apparent effectiveness, the detoxification methods raise concern over the safety, nutritional stability and functionality of treated food and feed, and ultimately offer only short term solutions while maintaining a long term financial onus on producers.

Hypothesis

A major objective in the aflatoxin control effort is the prevention of crop contamination prior to harvest. Two strategies based on the molecular biology of aflatoxin biosynthesis have been developed to help achieve this goal. One approach is to produce highly competitive, atoxigenic strains of *Aspergillus*, that when released into the farming environment, can exclude toxigenic fungi from colonizing crops (Cotty, 1990; Dorner *et al.*, 1992). The success of such a scheme would most likely depend on the ability to genetically engineer, and monitor in the field, fungal strains lacking key aflatoxin regulatory and/or anabolic genes. The second strategy involves the genetic engineering of crops. This approach is targeted to the identification of either endogenous plant genes or foreign genes which produce compounds that repress or inhibit aflatoxin formation and/or fungal growth (Burow *et al.*, 1997). Manipulating these genes to be constitutively expressed in plants or induced in response to external stimuli should help reduce aflatoxin levels in food and feed. In both strategies, fundamental knowledge about the biosynthesis of aflatoxin and its molecular regulation is requisite.

A proposed pathway for aflatoxin biosynthesis has been reported (Bhatnagar *et al.*, 1993), and the genes encoding proteins involved in aflatoxin production have been found to be clustered in the genomes of *A. flavus* and *A. parasiticus* (Figure 1) (Yu *et al.*, 1995; Trail *et al.*, 1995; Payne and Brown, 1998). Our laboratory has targeted the *nor-1* and *ver-1A* genes of *A. parasiticus*, which express two distinct enzymes in the aflatoxin

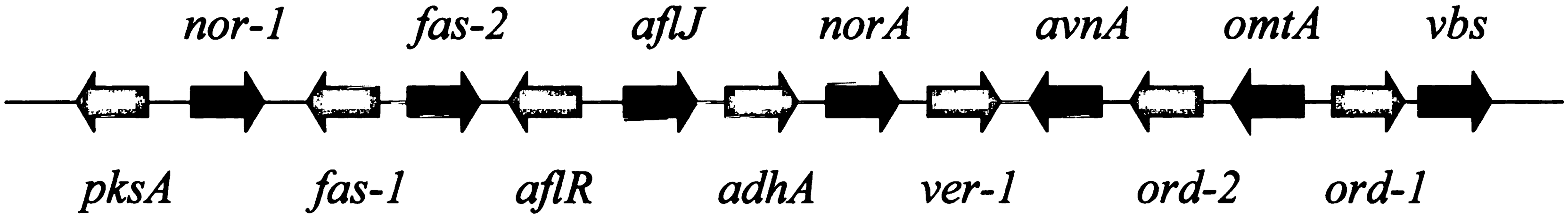

Figure 1. Aflatoxin Gene Cluster

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pathway, for regulatory studies. The *nor-1* gene encodes a reductase (NOR), catalyzing the conversion of norsolorinic acid to averantin early in the pathway (Trail *et al.*, 1994), while *ver-1A* is believed to express a reductase involved in a metabolic step converting versicolorin A to sterigmatocystin late in the pathway (Skory *et al.*, 1992). Translational fusions of the *nor-1* and *ver-1A* 5' regions with the *Escherichia coli* beta-glucuronidase reporter gene (*uidA*) (Jefferson *et al.*, 1986) were constructed in order to help identify cis-acting regions and trans-acting factors essential for the regulation of these genes. These aflatoxin gene fusions should also be useful for monitoring aflatoxin biosynthesis in response to environmental stimuli and as tools to evaluate compounds with a potential aflatoxin inhibiting nature. This study focuses on the *nor-1* gene, and is based on the hypothesis that the expression of the *nor-1/uidA* reporter fusion is similar to the expression of wild-type *nor-1* gene. If the two genes can be shown to be regulated in a similar manner, then the *nor-1/uidA* reporter construct can be used as a reliable indicator of wild-type gene expression and aflatoxin biosynthesis.

Reporter Fusions

Reporter genes express stable enzymes for which sensitive and efficient assays have been developed. By fusing the structural gene of a reporter enzyme to the promoter region of a gene of interest, the regulation of a variety of genes can be studied with the same reporter enzyme assays.

Early gene fusions were often characterized in strains of *Escherichia coli* where transposition and deletion events brought expression of lactose metabolizing genes under tryptophan operon control (Reznikoff *et al.*, 1969; Miller *et al.*, 1970; Bassford *et al.*, 1978). In these fusions, activity of beta-galactosidase (GAL), the product of the *E. coli* *lacZ* gene, was used to indicate tryptophan promoter control over the lactose genes. GAL

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hydrolyzes lactose to its constituent monomers glucose and galactose, but maintains specificity for substrates such as orthonitrophenyl-J-D-galactoside (ONPG) and 5-bromo-4chloro-3indolyl-J-D-galactoside (XGAL) which substitute an indicator moiety for the glucose. The cleavage of the ONPG and XGAL chromogenic substrates allows for sensitive assays of GAL expression (Beckwith, 1978). The ease in assaying for GAL spurred the development of *in vivo* methods for fusing *lac* genes to other *E. coli* promoters whose products were difficult to detect or unknown (Casadaban, 1976; Casadaban and Cohen, 1979).

The ability to manipulate DNA *in vitro*, with endonuclease digestion and ligation into plasmid vectors, increased the flexibility and efficiency for locating and examining promoters of both procaryotes and eucaryotes. In one general scheme, the chromosomal DNA of an organism was digested, and the fragments inserted into plasmid cloning sites upstream of a gene conferring antibiotic resistance. The selectable antibiotic gene lacked transcription control signals. Fusions containing transcription initiation signals could be identified by transforming *E. coli* with recombinant plasmids and then selecting transformants for antibiotic resistance (An and Friesen, 1979; Neve *et al*., 1979; West *et al*., 1979). Vectors containing *lac* genes were utilized to isolate and characterize promoters in a similar manner. After identifying promoter regions, assays for *lacZ* expression could be used to measure gene regulation on different types of media and to isolate mutations within a regulatory insert. Also, genes which express factors that act on a promoter of interest, could be isolated by introducing cloned DNA in trans of *lacZ* fusions, and then analyzing for the affect on GAL activity (Casadaban and Cohen, 1980).

The GAL enzyme has the additional reporter advantages of being able to tolerate both large additions of amino acids to its amino terminal end (Muller-Hill and

Kania,1974), and/or the elimination of at least its first 27 amino acid codons (Welply *et al.*, 1980) without the loss of enzyme activity. By replacing the amino terminal portion of GAL with the amino end of a protein of interest, a translational fusion can be constructed to study both transcriptional and translational initiation signals (Casadaban *et al.*, 1980; Casadaban *et al.*, 1983). Translational fusion expression results in the production of hybrid proteins. With *lacZ* fusion technology it is possible to create a series of hybrid proteins that can be useful in identifying amino terminal sequences involved in protein localization (Bassford *et al.*, 1978; Silhavy *et al.*, 1977).

Fungal Fusions

The 5' regions of the *Saccharomyces cerevisiae* orotidine-5'-monophosphate decarboxylase (*ura3*) (Rose *et al.*, 1981) and iso-1-cytochrome *c* (*cyc1*) (Guarente and Ptashne, 1981) genes were used to demonstrate the first fungal expression of reporter fusions. In both studies, translational *lacZ* fusions were created in vitro using vectors with *E. coli* and yeast origins of replication and selectable markers. The absence of endogenous GAL activity in *S. cerevisiae* allowed for assay of fusion expression without background interference. Since *ura3* is under uracil regulation and *cyc1* is repressed by glucose, expression of GAL was tested under appropriate media conditions to give data supporting the assertion that the fusions used *ura3* and *cyc1* expression control signals.

As was the case with *S. cerevisiae*, the application of fusion technology in filamentous fungi was preceded by the development of transformation methodologies. Transformation experiments with the pioneering organisms *Neurospora crassa* (Case *et al.*, 1979) and *Aspergillus nidulans* (Tilburn *et al.*, 1983; Yelton *et al.*, 1984; Wernars *et al.*, 1985) showed that chromosomal integration of transforming plasmid sequences is essentially as has been defined for yeast (Hinnen et al., 1978). Typically, the

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transforming DNA can be classified into one of three types of integrative events. Type I is characterized by a complete plasmid integration at a homologous location in the chromosome. Type II integration is identical to Type I with the exception that it occurs at a heterologous chromosomal locus. Type III involves gene replacement of the selectable marker with other vector sequences being excluded from integration. Studies with integrative vectors containing *A. nidulans trpC/lacZ* (van Gorcom *et al.*, 1985) and *gpd/lacZ* (Kolar *et al.*, 1988) fusions, established the feasibility of using reporter constructs to analyze gene expression in filamentous fungi.

Beta-Glucuronidase

Beta-Glucuronidase (GUS) is the product of the *uidA* gene of *E. coli*, and catalyzes the cleavage of β-O-glycosidic linkages to glucuronic acid. Detoxification processes in vertebrates often produce natural glucuronides. In these reactions, certain waste products (aglycones) are conjugated to glucuronic acid and excreted. *E. coli*, as part of the flora of the vertebrate gut, is uniquely able to metabolize these compounds (Jefferson, 1989).

The development of GUS as a reporter enzyme was in response to concerns regarding the high endogenous GAL activity in several biological systems. Alternative enzyme markers, such as chloramphenicol acetyltransferase and firefly luciferase, were relatively expensive, difficult to assay, and lacked applications for analyzing tissue specific expression in higher eucaryotes (Jefferson *et al.*, 1986; Jefferson *et al.*, 1987).

The ~73 kilodalton GUS protein has several characteristics that make it an excellent reporter enzyme. It is a stable protein that is resistant to thermal inactivation at 50°C, and has a broad pH optimum ranging from 5.0 to 7.5 (Jefferson *et al.*, 1986). It can also tolerate the addition of large amino terminal sequences without the loss of enzyme activity and can traverse membranes when signal sequences are attached to its amino terminus (Jefferson *et al.*, 1986; Jefferson *et al.*, 1987; Jefferson, 1989). A major advantage of GUS as a reporter enzyme, is the absence of endogenous GUS activity in many bacteria, fungi, and plants (Jefferson, 1989). With this lack of background interference and the commercial availability of sensitive and specific chromogenic and fluorogenic substrates, tissue and subcellular localization analyses can be performed with relative efficiency (Jefferson *et al.*, 1986; Jefferson *et al.*, 1987).

GUS Reporter Constructs

Filamentous fungi are of economic interest because they can produce homologous and heterologous proteins in industrial fermentations, they are successful plant pathogens, and because they produce food contaminating mycotoxins (Bennett, 1997). Research efforts into optimizing protein production in fermentations, and in understanding plant-fungus interactions have often focused on the analysis of gene expression in these fungi. Many laboratories have recognized the advantages of GUS fusions as potential tools for gene expression studies. In one such application, GUS expression vectors, lacking selectable markers, were successfully utilized in transient expression assays. These assays were used to confirm DNA uptake and the functionality of reporter constructs during development of a stable transformation system for the potato and tomato pathogen *Phytophthora infestans* (Judelson and Michelmore, 1991). A rapid transient GUS assay was also used to establish optimal transformation parameters, and to identify functional promoters in the corn pathogen *Cochliobolus heterostrophus* (Monke and Schafer, 1993). The expression of GUS activity in stably transformed strains of *C. heterostrophus* did not have an observable effect on their ability to infect the host.

The cotransformation of *A. nidulans, A. niger* and the tomato pathogen *Fulvia*

fulva, with a glyceraldehyde 3-phosphate dehydrogenase (*gpd*)/*uidA* fusion vector resulted in about 50% of the selected colonies expressing detectable GUS activity (Roberts *et al.*, 1989). This study indicated that GUS expression in *F. fulva* did not have a discernible effect on fungal pathogenicity and that the plant tissue harboring the pathogen could be efficiently monitored by GUS assay. In similar cotransformation studies with the same *gpd*/*uidA* fusion vector, 50% to 75% of selected *Fusarium oxysporum* colonies (Couteaudier *et al.*, 1993), 69% of selected *Claviceps purpurea* colonies (Smit and Tudzynski, 1992), and 38% to 92% of selected *Pseudocercosporella herpotrichoides* colonies (Bunkers, 1991) expressed GUS. Reporter activity in *P. herpotrichoides* cotransformants appeared to have no effect on fungal growth rate, morphology or pathogenicity; and *C. purpurea* cotransformants exhibited normal sclerotial formation and expressed GUS activity during the parasitic cycle. Because GUS expression in *F. oxysporum* cotransformants did not have an effect on their ability to cause disease, it was utilized to monitor plant-pathogen interaction in competition studies between pathogenic and nonpathogenic populations.

Bipolaris sorokiniana, a fungal pathogen of cereal crops, was also successfully transformed with a *gpd*/*uidA* fusion vector. Transformants were able to infect plants and express GUS activity prior to the development of obvious disease symptoms. It was suggested that GUS activity could be used for quantification of the fungus in plant tissue because of the high sensitivity of the fluorometric GUS assay, the lack of background activity in plants, and the positive correlation between GUS activity and ergosterol content (a frequently used indicator of fungal biomass) during infectious growth (Liljeroth *et al.*, 1993).

A *ver-1*/*uidA* fusion in *A. flavus* enabled the use of more reliable and sensitive

GUS assays to replace direct aflatoxin measurements in experiments designed to identify aflatoxin inducing compounds in plant extracts (Flaherty *et al.*, 1995). A GUS reporter construct that included the promoter region of the constitutively expressed beta-tubulin gene, was used in *A. flavus* as a marker to indicate fungal colonization of aflatoxin resistant and susceptible maize genotypes (Brown *et al.*, 1995). GUS activity was also used to demonstrate the regulation of *uidA* by the 5' region of the *Colletotrichum gloeosporioides cap5* gene. *Cap5* is involved in appressorium formation and GUS expression in this study was found to be located exclusively in conidia forming appressoria (Hwang and Kolattukundy, 1995).

In order to study the mechanisms of transcriptional induction of a β-amylase gene (*amyB*) in *Aspergillus oryzae*, an *amyB*/*uidA* transcriptional fusion was constructed, inserted into a vector with a selectable marker and transformed into an appropriate auxotrophic *A. oryzae* mutant. Data from expression studies with β-amylase inducing and non-inducing sugars, indicated that GUS activity was controlled by the amylase promoter (Tada *et al.*, 1991). *Rhizopus niveus*, because of its exceptional ability to secrete a variety of enzymes, is potentially useful for the production of commercially important proteins. The 5' regions of *R. niveus* 3-phosphoglycerate kinase genes (*pgk*), were used to construct *pgk*/*uidA* transcriptional fusions. The expression of GUS in *R. niveus* transformants was then used to indicate the ability of these high activity promoters to produce heterologous proteins (Takaya *et al.*, 1994). In efforts to understand the molecular regulation of penicillin biosynthesis, *ipnA*/*lacZ* and *acvA*/*uidA* fusions were both employed in the same *A. nidulans* strain. *IpnA* and *acvA* encode two enzymes involved in penicillin metabolism. GAL and GUS assays were able to be performed on the same fungal extracts to identify trans-acting mutations that affected both *ipnA* and *acvA* expression (Brakhage and Den Brulle, 1995).

Position-Dependent Expression

The integration of transforming DNA in filamentous fungi can occur at homologous or heterologous genomic sites, and the chromosomal environment can be a factor in the expression of the integrating genes (Timberlake and Marshall, 1989). In a study involving phosphoglycerate kinase/*lacZ* fusions in *A. nidulans*, transformants were characterized based on fusion integration position in the chromosome (Streatfield *et al.*, 1992). Transformants with single fusion integrations at a homologous site (selectable marker locus), and transformants with integrations at other undetermined sites (probably heterologous loci), were identified and their GAL activities compared. All homologous integrations maintained similar reporter activity levels. The heterologous integrations gave relatively higher or lower activity levels, and in certain cases these transformants completely lacked GAL activity. In other studies with *Phytophthora infestans* (Judelson *et al.*, 1993) and *Aspergillus oryzae* (Hata *et al.*, 1992), chromosomal position was believed to be a possible factor affecting expression of *uidA* reporter fusions. This assumption was based on the differences in GUS expression among individual transformants and the prevalence of non-homologous recombination in these experiments. An analysis of *Fusarium oxysporum* transformants containing single copies of an *A. nidulans gpd*/*uidA* reporter fusion, showed an approximately 80-fold difference in GUS activity between certain isolates (Couteaudier *et al.*, 1993). These transformants were characterized by unique chromosomal positions of fusion integration. Subsequent plasmid rescue experiments indicated that the putative elements responsible for higher GUS activity could be isolated from the *Fusarium* chromosome. In a study that examined the effect of chromosomal position on the cloned glutamate dehydrogenase gene (*am*) of *Neurospora crassa* (Kinsey and Rambosek, 1984), transformants with *am* integration in a non-normal chromosome expressed only 5%-20% of the dehydrogenase activity of the wild-type, while transformants with a normal *am* linkage relationship produced wild-type activity levels. It was hypothesized that the lower *am* expression levels were the result of the separation of an enhancer element from the *am* gene during cloning, which was not effectively replaced by the chromosomal copy in non-normal chromosome integrations. The phenomenon of position-dependent expression has been recognized as a potential hindrance to promoter activity studies, and it has been suggested that efforts be made in such studies to target gene integration to a specific locus in order to avoid position effects on gene transcription (van Gorcom et al., 1986; Hamer and Timberlake, 1987; Timberlake and Marshall, 1989).

Gene Clusters

The clustering, or close linking of genes involved in the same metabolic pathway appears to be an emerging theme in the filamentous fungi, particularly when secondary metabolic pathways and pathways expressing activity under a limited range of growth conditions are examined (Keller and Hohn, 1997). Physical and transcriptional maps of the proline catabolism gene cluster and the ethanol catabolism gene cluster in *Aspergillus nidulans* have been determined (Hull *et al.*, 1989; Fillinger and Felenbok, 1996). The proline cluster comprises five inducible genes, ranging over 14.2 kb of chromosome, and includes a gene expressing a positive acting, pathway regulator. A centrally located, cis-acting region in this cluster regulates an adjacent gene and also appears to have an enhancer-type effect on a non-adjacent cluster gene (Hull *et al.*, 1989; Sophianopoulou *et al.*, 1993). Since proline can be used as both a carbon and nitrogen source, this pathway is subject to regulation by the broad domain, carbon catabolite repressor CreA (Arst and

MacDonald, 1975), and the global nitrogen regulator AreA, which acts positively to mediate nitrogen metabolite repression (Arst and Cove, 1973; Sophianopoulou et al., 1993). The ethanol-utilization cluster consists of seven inducible genes, extending over approximately 15 kb of DNA. It is positively regulated by a cluster gene expressing the pathway-specific, trans-acting protein AlcR. This pathway is also controlled by carbon catabolite repression mediated by CreA. The genes in the cluster have been categorized based on their interactions with AlcR and CreA (Fillinger and Felenbok, 1996).

A quinic acid (QA) catabolism gene cluster has been defined in both *N. crassa* (Geever *et al.*, 1989) and *A. nidulans* (Lamb *et al.*, 1990). The 18 kb, QA-inducible cluster in *Neurospora* contains five structural genes and two pathway specific regulators. One of these regulators, a positive-acting protein, has been shown to bind the promoter regions of the QA genes, while the second regulator protein is believed to repress the pathway by binding and inhibiting the activator protein. Repressor activity is inhibited by QA, and transcription of the cluster is repressed by glucose (Geever *et al.*, 1989). Similarly, the 17 kb *Aspergillus* cluster is characterized by seven genetic loci, which are controlled by carbon catabolite repression and induced by QA. The cluster includes two regulatory genes that express a pathway activator protein and a repressor protein. Both clusters have three pairs of divergently transcribed genes, however, the order of genes in the two clusters is different. Southern hybridization data suggests that *A. fumigatus* and *Penicillium chrysogenum* also contain a QA cluster (Lamb *et al.*, 1990).

The three structural genes encoding the nitrate assimilation pathway in *A. nidulans* are localized in a cluster (Johnstone *et al.*, 1990). These genes are induced by nitrate via a pathway-specific, positive-acting factor, and are also subject to the activity of the AreA protein (Johnstone *et al.*, 1990; Unkles *et al.*, 1991). There are also data which indicate a regulatory role for the product of the *niaD* gene in the expression of the other two structural genes in this cluster (Cove, 1979; Unkles *et al.*, 1991). The nitrate reductase and nitrite reductase genes in *A. parasiticus*, *A. oryzae, A. niger*, *P. chrysogenum*, and *Leptosphaeria maculans* have also been found to be linked (Chang *et al.*, 1996).

The genes responsible for antibiotic production in procaryotes and eucaryotes are commonly associated in clusters (Smith *et al.*, 1990a; Hopwood *et al.*, 1995). Two well-characterized examples in the filamentous fungi are penicillin (PE) biosynthesis in *A. nidulans* and *P. chrysogenum* (MacCabe *et al.*, 1990; Smith *et al.*, 1990a; Smith *et al.*, 1990b). In each of these examples, the identified linkage group consists of three structural genes ranging over approximately 13 kb of chromosome (Smith et al., 1990a). Promoters in the *Penicillium* cluster appear to be controlled by carbon catabolite repression and nitrogen repression (Feng *et al.*, 1994). The *areA* homologue in *P. chrysogenum* has been designated *nre*, and it has been suggested that the *nre* gene product is the factor responsible for mediating nitrogen repression of PE biosynthesis in this fungus (Haas and Marzluf, 1995). Data from studies on *A. nidulans* have also defined a role for the PacC protein in the transcriptional regulation of the PE cluster (Espeso *et al.*, 1993). PacC is a transcriptional activator and repressor, which is part of a complex regulatory system, controlling gene expression in response to external pH (Tilburn *et al.*, 1995). The PE cluster in *Aspergillus* is also regulated by carbon source, and it has been argued that this regulation is CreA-independent (Espeso et al., 1993).

Other fungal secondary metabolites that have their biosynthetic genes clustered include the mycotoxins. The best characterized examples are the groups of genes expressing sterigmatocystin (ST) in *A. nidulans* (Brown *et a*l., 1996); and aflatoxin (AF) in *A. flavus* (Yu *et al.*, 1995) and *A. parasiticus* (Trail *et al.*, 1995; Yu *et al.*, 1995). The genes involved in AF biosynthesis are clustered in a 60 kb stretch of chromosome in both of the AF producing species (Yu *et al.*, 1995). Northern hybridization analysis in *A. parasiticus*, has identified more transcripts expressed from the AF cluster than there are known genes, indicating that the complete AF cluster is yet to be defined (Trail *et al.*, 1995). Examination of six AF genes in both *A. flavus* and *A. parasiticus*, has revealed a highly conserved gene sequence and organization between the two clusters (Yu *et al.*, 1995). Despite this conservation, the AF profile in these two organisms is inconsistent, and it has been suggested that the differences may be due to the subtleties in gene spacing within the two clusters and/or the duplication of part of the gene group in *A. parasiticus* (Liang *et al.*, 1996; Yu *et al.*, 1995). ST is an AF precursor in *A. flavus* and *A. parasiticus*, but it is expressed as an end product in *A. nidulans*. The ST biosynthetic genes in *A. nidulans* are located in a 60 kb linkage group, from which 25 transcripts are coordinately expressed. All of the genes identified in AF metabolism are homologues of ST cluster genes, however, there are differences in gene order and direction of transcription between the AF and ST clusters (Brown *et al.*, 1996). Both the AF (Payne *et al.*, 1993; Chang *et al.*, 1993; Woloshuk *et al.*, 1994) and ST (Yu *et al.*, 1996) gene clusters encode a homologous, pathway-specific regulator, that appears to act positively in the transcriptional regulation of these toxins (Payne *et al.*, 1993; Chang *et al.*, 1993; Chang *et al.*, 1995; Yu *et al.*, 1995).

Fumonisin, another mycotoxin, appears to have its biosynthetic genes clustered in *Gibberella fujikuroi* (Desjardins *et al.*, 1996); and a possible trichothecene gene cluster in *Fusarium sporotrichioides* has been identified through mutant complementation studies (Hohn *et al.*, 1993). *Alternaria alternata* has three genes required for biosynthesis of melanin clustered in a 30 kb region of chromosome, and the pattern of expression of these genes has indicated coordinated regulation of this secondary metabolism cluster (Kimura and Tsuge, 1993).

SpoC1 Cluster

Secondary metabolism produces compounds that are generally considered to be nonessential for growth. In a laboratory environment, secondary metabolites are usually expressed after log growth of a culture, and primary metabolism provides the building blocks used in their construction (Drew and Demain, 1977; Demain, 1992). The functions of many secondary metabolites are unknown, and this has contributed to their classification as secondary in importance, when considering the entire metabolic activities of an organism (Drew and Demain, 1977; Bennett and Christensen, 1983). Several possible functions of secondary metabolic activity have been proposed and these include roles in cellular differentiation, reproduction, and protection from competition or predation (Davies, 1992; Vining, 1992).

The SpoC1 gene cluster (SGC) was first isolated in experiments performed to identify genes involved in *A. nidulans* sporulation (Zimmermann *et al.*, 1980). This study provided evidence that developmentally regulated genes were nonrandomly dispersed in the *Aspergillus* genome, and many genes of this type were subsequently shown to be clustered (Orr and Timberlake, 1982). Characterization of the original 13.3 kb SGC fragment, confirmed that the expression of transcripts from this chromosomal region were temporally coordinated and that transcript accumulation was specific to conidia (Timberlake and Barnard, 1981). Further analysis of the cluster provided a transcript map that extended over 38 kb of DNA and included 19 coding regions that were preferentially expressed during conidiation. In addition, the cluster was found to be flanked by 1.1 kb direct repeats (Gwynne *et al.*, 1984; Miller *et al.*, 1987).

To investigate the possibility that the clustering of these spore-specific genes served a regulatory purpose, transformation experiments were performed in which a gene from the SGC was integrated at ectopic chromosomal locations, and conversely a constitutively expressed gene was integrated into the cluster. The expression of these genes was then monitored in hyphae and conidia (Miller *et al.*, 1987). The results indicated that chromosomal position was a factor in cell-specific expression of the cluster gene examined. In these experiments the wild-type strain did not show expression of the cluster gene in hyphae, while derivative strains, with the cluster gene placed at presumably random heterologous locations, expressed this gene in hyphae. The conclusion by the authors that a property of the SGC negatively regulates transcription of cluster genes in hyphae, was supported by experiments which placed the constitutively expressed gene in the SGC. A reduction in transcription of at least five-fold was observed when this housekeeping gene was integrated at different positions in the SGC.

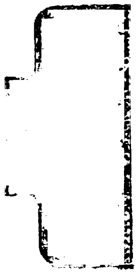
The data from the SGC experiments indicates that the clustering of genes can serve to regulate gene expression, although the mechanisms of this regulation remain to be defined. The physical linkage of aflatoxin biosynthesis genes may likewise have a regulatory role and studies designed to establish such a phenomenon and in turn to determine the nature of the regulation, may eventually lead to a broader understanding of the expression of secondary metabolism and the ability to manipulate its expression in microorganisms. With knowledge about aflatoxin gene regulation still being acquired, it seems prudent to validate aflatoxin reporter constructs as reliable indicators of wild-type gene expression, which of course this work attempts to do with the *nor-1/uidA* reporter fusion.

MATERIALS AND METHODS

Strains, Plasmids and Culture Conditions

Plasmid DNA was propagated in *E. coli* DH5a F'^{e} [F'/*endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) D*relA1* ($lacZYA\text{-}argF)_{u169}$:(m80 D*lacZ M15*)] (Gibco BRL, Life Technologies, Inc. Gaithersburg, MD). The *A. parasiticus* nitrate reductase (*niaD*) mutant strain NR1 (Horng Dissertation, 1990; Liang *et al.*, 1996), was derived from the wild-type aflatoxin producer NRRL 5862 (SU-1; ATCC 56775). NR1 was used as the recipient strain in all fungal transformation experiments. The plasmids pAPGUSNNA and pAPGUSNNB contain the same *nor-1*/*uidA* translational fusion, and were constructed for analysis of *uidA* expression in NR1. These vectors were created by ligating the 9.6-kb, *XhoI* digested fusion construct pAPGUSN, with the 7.4-kb *XhoI-SalI* fragment of pSL82 (Horng *et al.*, 1990) (Figure 2). The pSL82 fragment contains the *A. parasiticus niaD* gene as a selectable marker. The plasmid pGAPN2 (Liang *et al.*, 1997) was used in transformants as a control for constitutive expression of GUS. It contains an *A. flavus* β-tubulin promoter fused to the *uidA* gene, and the *A. parasiticus niaD* gene as a selectable marker. Bacterial strains harboring plasmids were stored in 20% glycerol at -80°C. Plasmids used for fungal transformation were purified by CsCl density gradient centrifugation (Ausubel *et al.*, 1987).

For the analysis of RNA, protein, and aflatoxin in time-course studies, the following culture conditions were used: 250 ml Erlenmeyer flasks, coated with Sigmacote (Sigma Chemical, St. Louis, MO), and containing 100 ml of YES broth (2% yeast extract [Difco Laboratories, Detroit, MI], 6% sucrose, pH 5.8) and five 6 mm glass beads (Fisher Scientific, Pittsburgh, PA), were inoculated with $2x10^6$ viable conidia



Figure

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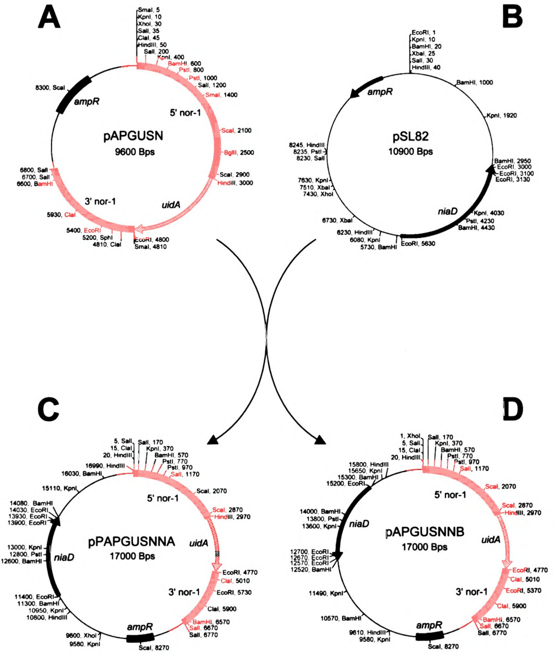
Figure 2. Construction of pAPGUSNNA and pAPGUSNNB reporter plasmids. (A) pAPGUSN was derived from pGLGUS-1 (Wu, 1995) and contains a *nor-1/uidA* translational fusion. The solid red boxes represent the 5' and 3' regions of the *nor-1* gene. The 5' region is approximately 3-kb and includes the sequence encoding the first 21 amino acids of the NOR protein. The 3' region is approximately 1.8-kb and contains sequence encoding the final 6 amino acids of the carboxyl terminus of the NOR protein, the translation termination codon and the predicted polyadenylation site of the *nor-1* transcript (Trail *et al.*, 1994). The red hashed region represents the *uidA* structural gene (Jefferson *et al.*, 1986) and begins with the sequence encoding the third amino acid (arginine) of GUS. The black box indicates an ampicillin-resistance gene (cloned from Promega's pGL2-Basic) for selection in *E. coli.* (B) pSL82 contains an 8.2-kb *SalI* fragment of *A. parasiticus* genomic DNA. The blue arrow represents the *A. parasiticus niaD* gene, which is used as a selectable marker in fungal transformations. The black arrow represents the ampicillin-resistance gene from pUC19. A sticky-end ligation was performed between the 7.4-kb *XhoI-SalI* fragment of pSL82 and the *XhoI* linearized pAPGUSN vector, to create the pAPGUSNN plasmids. (C) pAPGUSNNA is oriented with both *niaD* and the *uidA* fusion being transcribed off the same DNA strand, while (D) pAPGUSNNB has these two genes transcribed in opposite directions.


A
Smal, 5
Kpnl, 10
Xhol, 30
Sall, 35
Clal, 45
HindIII, 50
Sall, 200
Kpnl, 400
BamHI, 600
PstI, 800
PstI, 1000
Sall, 1200
Smal, 1400
Scal, 2100
BglII, 2500
Scal, 2900
HindIII, 3000
EcoRI, 4800
Smal, 4810
4810, Clal
5200, SphI
5400, EcoRI
5930, Clal
6600, BamHI
6700, Sall
6800, Sall
8300, Scal
ampR
5' nor-1
pAPGUSN
9600 Bps
3' nor-1
uidA
B
EcoRI, 1
Kpnl, 10
BamHI, 20
Xbal, 25
Sall, 30
HindIII, 40
BamHI, 1000
Kpnl, 1920
BamHI, 2950
EcoRI, 3000
EcoRI, 3100
EcoRI, 3130
Kpnl, 4030
PstI, 4230
BamHI, 4430
EcoRI, 5630
5730, BamHI
6080, Kpnl
6230, HindIII
6730, Xbal
7430, Xhol
7510, Xbal
7630, Kpnl
8230, Sall
8235, PstI
8245, HindIII
ampR
pSL82
10900 Bps
niaD
C
5, Sall
15, Clal
20, HindIII
Sall, 170
Kpnl, 370
BamHI, 570
PstI, 770
PstI, 970
Sall, 1170
16990, HindIII
16030, BamHI
15110, Kpnl
Scal, 2070
Scal, 2870
HindIII, 2970
EcoRI, 4770
Clal, 5010
EcoRI, 5730
Clal, 5900
BamHI, 6570
Sall, 6670
Sall, 6770
Scal, 8270
9580, Kpnl
9600, Xhol
10800, HindIII
10950, Kpnl
11300, BamHI
11400, EcoRI
12600, BamHI
12800, PstI
13000, Kpnl
13900, EcoRI
13930, EcoRI
14030, EcoRI
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5' nor-1
pPAPGUSNNA
17000 Bps
uidA
niaD
3' nor-1
ampR
D
1, Xhol
5, Sall
15, Clal
20, HindIII
Sall, 170
Kpnl, 370
BamHI, 570
PstI, 770
PstI, 970
Sall, 1170
15800, HindIII
15650, Kpnl
15300, BamHI
15200, EcoRI
Scal, 2070
Scal, 2870
HindIII, 2970
EcoRI, 4770
Clal, 5010
EcoRI, 5370
Clal, 5900
BamHI, 6570
Sall, 6670
Sall, 6770
Scal, 8270
9580, Kpnl
9610, HindIII
10570, BamHI
11490, Kpnl
12520, BamHI
12570, EcoRI
12670, EcoRI
12700, EcoRI
13600, Kpnl
13800, PstI
14000, BamHI
5' nor-1
niaD
pAPGUSNNB
17000 Bps
uidA
3' nor-1
ampR

Figure 2

($2x10^4$/ml). Sigmacote was applied to prevent fungal growth on flasks and glass beads were used to help maintain uniform culture conditions by interfering with mycelial aggregation (Skory *et al.*, 1993). Cultures were incubated in the dark at 29°C, with shaking at 150 rpm for 24, 48, or 72 hr. Mycelia were harvested at the appropriate time points by vacuum filtration on Miracloth (Calbiochem-Novabiochem, San Diego, CA), and 65% - 85% of mycelial mat was immediately frozen in liquid nitrogen. The remaining 15% - 35% of the mycelia were thoroughly dried at 70-80°C and then used for dry weight determination of the culture. The culture filtrate and frozen mycelia were stored at -80°C.

Fungal Transformation

A homologous transformation system for *A. parasiticus*, using the *niaD* gene as a selectable marker, has been developed (Oakley *et al.*, 1987; Horng *et al.*, 1990), and was used in this study with modifications. Approximately $2.5x10^8$ NR1 spores were inoculated into a 250 ml Erlenmeyer flask containing 100 ml Czapek-Dox (CZ) broth (Difco Laboratories) supplemented with 1% Bacto Peptone (Difco Laboratories). Cultures were incubated in the dark at 29°C with shaking at 150 rpm for 18 hr. Two flasks of mycelia were used for each transformation. Mycelia were harvested by vacuum filtration using Miracloth, and then mixed with 10 ml CZ broth. Ten ml (5 mg/ml) of Novozyme 234 (Interspex Products, Foster City, CA) in reaction buffer (1.1 M KCl, 100 mM NaCitrate, pH 5.8) was gently mixed with mycelia for 5 hr at 29°C in the dark. Protoplasts were separated from cell wall debris by centrifugation at 160 x g, 25°C for 60 sec (IEC PR-6000 Centrifuge), and the supernatant was filtered through 29 mm mesh. The filtrate was centrifuged at 3,000 x g, 4°C for 15 min (Sorvall SS-34 rotor). Protoplasts were kept on ice and washed 3X with 1 ml protobuffer (600 mM KCl, 50 mM

$CaCl_2$, 10 mM Tris-HCl [pH 8.0]) and finally suspended in 110 µl protobuffer. 1-10 µg of circular plasmid DNA in 10 µl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) was added to 100 µl of protoplast suspension. 50 µl of a filter-sterilized PEG solution (25% polyethylene glycol 3350, 600 mM KCl, 50 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.5]) was gently mixed with the protoplast/DNA suspension and then placed on ice for 20 min. An additional 850 µl of PEG solution was added and the transformation mixture incubated at 25°C for 30 min. Transformants were selected on CZ agar supplemented with either 600 mM KCl or 20% sucrose as an osmotic stabilizer. Agar plates were incubated in the dark at 29°C for 3-5 days.

Spore Preparation

Transformants selected for further analysis were subjected to three cycles of single spore isolation. One cycle consisted of the following: A single transformant was allowed to sporulate on CZ agar for 1-2 wk in the dark at 29°C. Asexual conidiospores were dislodged from the colony with a sterile spatula and water. The crude spore suspension was filtered through sterile 8 µm fiberglass (Corning, Corning, NY) in a 10 cc syringe (Becton Dickinson, Sparks, MD). The spore concentration was determined with an improved Neubauer hemacytometer. The spore suspension was then appropriately diluted for the plating of 1-10 spores per CZ plate. Plates were incubated as described above for approximately 3 days. Mycelia from a single colony were transferred to a fresh CZ plate to complete the cycle. After the completion of three cycles, large scale preparations of spores from certain transformants were performed. This entailed inoculating a 150 cm^2 tissue culture flask (Corning) containing 100 ml of Potato Dextrose Agar (Difco Laboratories) with the appropriate spores. Flasks were incubated as above for 2-3 wks. Spores were dislodged by spinning a sterile stir bar, in approximately 50 ml of water, over the colony. The spore suspension was filtered through 8 µm fiber glass in a 60 cc syringe (Becton Dickinson). Spores were pelleted by centrifugation at 2,600 x g, 25°C for 10 min (Sorvall GSA rotor). Three to five ml of 20% glycerol was used to resuspend spores. The spore suspension was aliquoted, frozen in an ethanol/dry ice bath, and stored at -80°C. Spore viability counts were performed on frozen stocks by serial dilutions of a spore sample and plating in triplicate on CZ agar. Plates were incubated as above and colony counts performed after approximately 3 days of growth.

Fungal DNA Isolation and Southern Blotting

250 ml Erlenmeyer flasks containing 100 ml YES broth were inoculated with spore stocks and incubated in the dark at 29°C with shaking at 150 rpm for approximately 48 hr. Mycelia were collected by vacuum filtration on Miracloth. A fraction of the mycelia was exposed to liquid nitrogen and macerated with a mortar and pestle. DNA was extracted according to a modified procedure based on a phenol-chloroform protocol for mammalian DNA isolation (Ausubel *et al.*, 1987). Mycelial powder was mixed with an extraction buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 25 mM EDTA, 0.5% lauryl sulfate, 100 µg/ml Proteinase K [Boehringer Mannheim]) at 50°C with gentle agitation for approximately 20 hr. The crude sample was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and then precipitated with ammonium acetate and ethanol at -20°C. DNA was pelleted by centrifugation, dissolved in TE, and treated with DNase-free RNase A (Boehringer Mannheim, Indianapolis, IN). Further purification was achieved with a series of two phenol-cloroform-isoamylalcohol (25:24:1) extractions, followed by precipitation, redissolving in TE, and treatment with RNase A. The appropriate dilutions were then performed on the sample and the DNA concentration

determined with a Genequant RNA/DNA calculator (Pharmacia Biotech, Bridgewater, NJ).

For Southern blotting, approximately 8 µg of each DNA sample was digested with the appropriate endonuclease (Boehringer Mannheim, Gibco/BRL) and loaded into a 0.8% agarose gel (1xTBE buffer [89 mM Tris, 89 mM Boric Acid, 2 mM EDTA], 0.5 µg/ml ethidium bromide). Electrophoresis was performed for approximately 15 hr at 50 volts and the gel was subsequently treated with 5 volumes of 250 mM HCl in order to partially depurinate DNA fragments to facilitate DNA transfer. Further gel treatment and blotting to Nytran membrane (Schleicher & Schuell, Keene, NH) was performed according to standard methods (Maniatis *et al.*, 1989). DNA transfer was allowed to continue for 20-24 hr, and then ultraviolet irradiation was used to crosslink DNA to the membrane (Stratagene UV Stratalinker 1800, La Jolla, CA).

Fungal RNA Isolation and Northern Blot Analysis

Mycelial samples stored at -80°C were macerated with a mortar and pestle in liquid nitrogen. RNA was extracted from 100 mg of mycelial powder with one ml of TRIzol reagent (Gibco/BRL) using the recommended manufacturer procedures. RNA concentrations were determined using a Genequant RNA/DNA calculator. Northern blot analysis was performed according to standard procedures (Ausubel *et al.*, 1987; Maniatis *et al.*, 1989). 30 µg of total RNA from each sample was loaded into a denaturing, 1.2% agarose gel (1xMOPS [40 mM 3-(N-morpholino)-propanesulfonicacid, [pH 7.0], 10 mM sodium acetate, 1 mM EDTA], 800 mM formaldehyde). RNA was separated by electrophoresis at 70 volts for 3 hr and then transferred to Nytran membrane by capillary action. RNA transfer was allowed to continue for 20-24 hr and the nucleic acid was subsequently crosslinked to the membrane with the UV Stratalinker 1800 at standard crosslink settings.

Nucleic Acid Hybridization

For prehybridization, membranes were incubated in hybridization solution (50% formamide, 5xSSC [750 mM NaCl, 75 mM sodium citrate, pH 7.0], 1xPE [50 mM Tris-HCl [pH 7.5], 0.1% sodium pyrophosphate, 1% lauryl sulfate, 0.2% polyvinylpyrolidone 40,000, 0.2% ficoll 400,000, 5 mM EDTA, 0.2% bovine serum albumin], 150 µg/ml salmon sperm DNA) at 42°C for 3-5 hr in rotating vessel. Probe DNA was gel purified with GELase (Epicentre Technologies, Madison, WI) and labeled with α-dCTP ^{32}P (NEN Life Science, Boston, MA) using a random primed DNA labeling kit (Boehringer Mannheim). Radioactive probes were separated from free nucleotides using a Nuc Trap Push Column (Stratagene). Denatured probe was added to hybridization solution and incubated with the membrane, in a rotating vessel, at 42°C for 10-15 hr. Membranes were washed under high stringency conditions (0.1% lauryl sulfate, 0.1xSSC, 65°C), and exposed to X-Ray film (Kodak XAR-5) at -80°C.

Qualitative and Quantitative GUS assays

To rapidly screen for the ability of a transformant to express GUS activity, a qualitative GUS assay (Kolar *et al.*, 1991; Verdoes *et al.*, 1994) was used. Transformants were transferred from selective medium to plates containing an aflatoxin-inducing, solid growth medium (YES), and then overlaid with circular 82 mm Nytran Plus membranes (Schleicher & Schuell). Plates were incubated in the dark at 29°C for 36-48 hr. Membranes, with attached fungal colonies, were removed from plates and exposed to liquid nitrogen for 30 sec. Membranes were allowed to thaw and then incubated in a GUS substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.27% β-mercaptoethanol, 0.04% X-GLUC [Gold Biotechnology St. Louis, MO]) for up to 24 hr.

For quantitative GUS analysis, samples of mycelial stocks stored at -80°C, were macerated with a mortar and pestle in liquid nitrogen. Approximately 100 mg of crushed tissue was mixed with 500 µl of chilled GUS lysis buffer (50 mM NaH_2PO_4 [pH 7.0], 10 mM EDTA, 0.1% Triton-X-100, 0.1% lauryl sulfate, 10mM β-mercaptoethanol, 25 µg/ml phenylmethanesulfonyl fluoride [PMSF]) and centrifuged at 12,000 x g at 4°C for 10 min. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a protein standard and Protein Assay Dye Reagent (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's instructions. One µg of total protein from each extract was then incubated in a 200 µl volume of 2 mM 4-methylumbelliferyl-β-D-glucuronide, at 37°C, for 0, 3, 5, 6, 9, 10, 12, 15, and/or 30 min as necessary. GUS reactions were terminated with the addition of 800 µl of 200 mM Na_2CO_3. To determine GUS activity, the fluorescence of each reaction mixture was measured at an excitation wavelength of 365 nm and an emission wavelength of 455 nm, with an SLM-4000 fluorometer (SLM Instruments, Champaign, IL).

Western Blot Analysis

Frozen mycelial samples were macerated with a mortar and pestle in liquid nitrogen. Approximately 400 mg of powdered tissue was mixed with 300-400 µl of chilled GUS lysis buffer (described above with the addition of 1 mM PMSF, 1 µg/ml leupeptin, and 2 µg/ml peptain A). Cell material was incubated on ice for at least 30 min. The extract was then centrifuged at 12,000 x g at 4°C for 30 min. The protein concentration of the supernatant was determined as described above. Discontinuous SDS-PAGE and Western blot analysis were performed with the Mini-Protean II electrophoresis apparatus and the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories) according to manufacturer's procedures. 60-75 µg of total protein from each sample was loaded into a 4% acrylamide stacking gel (125 mM Tris-HCl [pH 6.8], 0.5% SDS) and electrophoresed through a 12% acrylamide separating gel (375mM Tris-HCl [pH 8.8], 0.5% SDS). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (NEN Life Sciences), and then incubated for 1 hr at 25°C in blocking solution (150 mM NaCl, 0.05% Tween 20, 1% blocking powder [Schleicher & Schuell]). Primary antibody incubations were performed in blocking solution at 25°C for 45 min, with either rabbit α-NOR (Zhou, 1997) at a concentration of 2 µg/ml or rabbit α-GUS (Clontech Laboratories, Palo Alto, CA), at a dilution of 1:1600. A goat α-rabbit, alkaline phophatase conjugate (Schleicher & Schuell) at a dilution of 1:10,000, was used for all secondary antibody incubations (30 min, 25°C, in blocking solution). Washes were performed in 150 mM NaCl, 0.05% Tween 20, after each antibody incubation. Secondary antibody was detected by incubation in chromogenic substrate solution (30 ml water, one BCIP/NBT Substrate Tablet [Schleicher & Schuell]).

Polymerase Chain Reaction Analysis

A PCR assay was developed for the screening of fungal genomic DNA in order to identify transformants with specific vector integration events. The PCR reactions included 200 ng of genomic DNA, primer (1 pmole/µl), 3.5 mM $MgCl_2$, 800 mM dNTPs, and 0.05 units/µl Taq polymerase (Perkin Elmer). The reaction volume was 50 µl. Samples were amplified in the GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT) as follows: 1 min annealing at 68°C, polymerization for 4 min at 72°C, and denaturation for 1 min at 95°C. 40 cycles were used per reaction. The initial denaturation was for 2 min at 95°C, and the final polymerization was extended for 10 min. Primers JL 99 (5'TTT CAC GGG TTG GGG TTT CTA CAG G3') and JL 100 (5'GAC GGG GAA CCT CTT TAC AAA CAT C3') were used for the identification of 5' *nor-1* pAPGUSNNB integration events. Primers JL 102 (5'CGC AAG GTG AGG GTT CGA ACC GAG G 3') and JL 103 (5'CCG CAG CAG GGA GGC AAA CAA TGA A3') were used for the identification of 3' *nor-1* pAPGUSNNB integration events.

Aflatoxin Analysis

The concentration of aflatoxin in culture filtrates was determined by direct competitive ELISA (Ram *et. al.*, 1986). The rabbit polyclonal AFB_1 antibody and AFB_1-horseradish peroxidase conjugate (AFB_1-HRP) were kindly provided by J. Pestka, Michigan State University. 125 µl of a 1:900 dilution of α-AFB_1 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) was applied to Immulon 4 microtiter plates (Dynatech Laboratories, Chantilly, VA), and the plates then dried for 18 hr at 42°C in a forced air oven (Fisher Isotemp Oven 338F). Plates were washed eight times (four times in each orientation) in a Skatron A/S Microwash II instrument (Skatron, Lier, Norway) using 0.02% Tween 20 in PBS. Plates were incubated with 300 µl of blocking solution (1% bovine serum albumin in PBS [pH 7.2]) at 37°C for 30 min to eliminate nonspecific binding. Plates were washed as described above. A 1:300 dilution of AFB_1-HRP was prepared in blocking solution, and then mixed with culture filtrates in a 1:1 ratio (a total volume of 100 µl). The mixture was prepared in the microtiter plates and then incubated in the dark at 37°C for 1 hr. After incubation, plates were washed with the PBS, Tween 20 solution eight times in each orientation using the A/S Microwash II instrument. 100 µl of chromogenic substrate solution (42 mM citric acid [pH 4.0], 0.03% H_2O_2, 420 mM 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) was used as an indicator of peroxidase activity and was incubated in plates at 25°C for 4 min. Color reactions were terminated with 100 µl of 300 mM citric acid. Color intensity was analyzed using a Vmax spectrophotometer at 405 nm (Molecular Devices, Sunnyvale, CA).

RESULTS

Transformation and screening of *A. parasiticus*

Three separate transformation experiments of NR-1 were performed using the pAPGUSNNA and pAPGUSNNB reporter vectors. Because both vectors contain the *A. parasiticus niaD* gene as a selectable marker, transformation mixtures were plated on CZ agar with nitrate as the sole nitrogen source. The transformation frequencies in each experiment are presented in Table 1. All transformants from the second and third experiments were selected on CZ agar supplemented with sucrose as an osmotic stabilizer. These colonies had a morphology that was similar to the NR-1 parent strain. However, in the first transformation experiment, KCl was used as an alternative osmotic stabilizer and this experiment resulted in transformants with two colony morphology types. The first morphology type was characterized by green pigmentation of the conidiospores and was similar to the phenotype of the parent strain. The second morphology type of the first transformation experiment was distinguished by yellow spores, highly concentrated in the center of the colony. A plate from the first transformation experiment would either contain all green-spored or all yellow-spored colonies, which suggested that the unique environment in an individual plate was a factor

Table 1. NR-1 transformation frequencies from three separate experiments.

Transformation	pAPGUSNNA	pAPGUSNNB
First	287 cfus/6.1 µg DNA/1.0x10^7 protoplasts	322 cfus/6.5 µg DNA/8.0x10^6 protoplasts
Second	355 cfus/6.1 µg DNA/5.9x10^6 protoplasts	492 cfus/6.5 µg DNA/9.6x10^6 protoplasts
Third	891 cfus/6.1 µg DNA/1.1x10^7 protoplasts	706 cfus/3.3 µg DNA/1.4x10^7 protoplasts

in morphology determination. In most cases, when a yellow-spored colony was transferred and reincubated, it assumed the typical green-spored phenotype.

In each of the three transformation experiments, a number of the *niaD*$^+$ colonies were chosen in order to screen for their ability to express GUS activity in a qualitative assay (Figure 3 and Table 2). This was a colorimetric assay where the substrate X-Gluc was cleaved by the GUS protein *in situ*. Cleavage of X-Gluc by GUS resulted in a blue color that was indicative of reporter gene expression. None of the pAPGUSNNA transformants examined expressed GUS qualitatively. Although this result was interesting, our major goal was to develop a feasible reporter enzyme system in *A. parasiticus*, and therefore the pAPGUSNNA transformants were not further analyzed. Since DNA sequence analysis of the reporter vectors was not performed, nor was the expression of GUS activity in *E. coli* examined, the lack of activity from pAPGUSNNA transformants may have been due to DNA mutation in the plasmid. Another possible explanation for nonexpression in pAPGUSNNA transformants, is that the proximity of the *nor-1* promoter to unknown cis-acting factors in this particular orientation of the reporter vector, repressed *uidA* transcription in the fungus.

Table 2. The ratios of GUS$^+$ transformants from three separate transformation experiments. The plasmids pAPGUSNNA and pAPGUSNNB were used to transform NR-1. A qualitative GUS assay was used to screen *niaD*$^+$ colonies from these transformations, for their ability to express GUS activity on an aflatoxin inducing media (YES agar).

Transformation	NR-1/pAPGUSNNA GUS$^+$	NR-1/pAPGUSNNB GUS$^+$
First	0/15	4/15
Second	0/32	9/32
Third	0/81	22/102

Qualitative GUS analysis of transformants from three pAPGUSNNB

Figure 3. Qualitative GUS assay of fungal colonies transformed with pAPGUSNNB. A sterile toothpick was used to transfer fungal transformants onto an aflatoxin inducing medium (YES agar). The inoculated agar was overlaid with a nylon filter and then incubated at 29°C for 36-48 h in the dark. The filter with the accompanying fungal growth, was subjected to a freeze thaw cycle, using liquid nitrogen, in order to break down cell walls and membranes. After a thawing period of five minutes, the nylon membrane was then incubated for 30 min in a GUS substrate solution. The freeze-thaw cycle enhances the opportunity for substrate-enzyme interaction by eliminating physical barriers to ligand binding. The blue colonies resulted from cleavage of the colorimetric substrate X-Gluc by β-glucuronidase, and were indicative of transformants with pAPGUSNNB integrated into the *nor-1* chromosomal region. The non-blue or GUS$^-$ strains were characterized by fusion integration into the *niaD* region of the NR-1 chromosome.

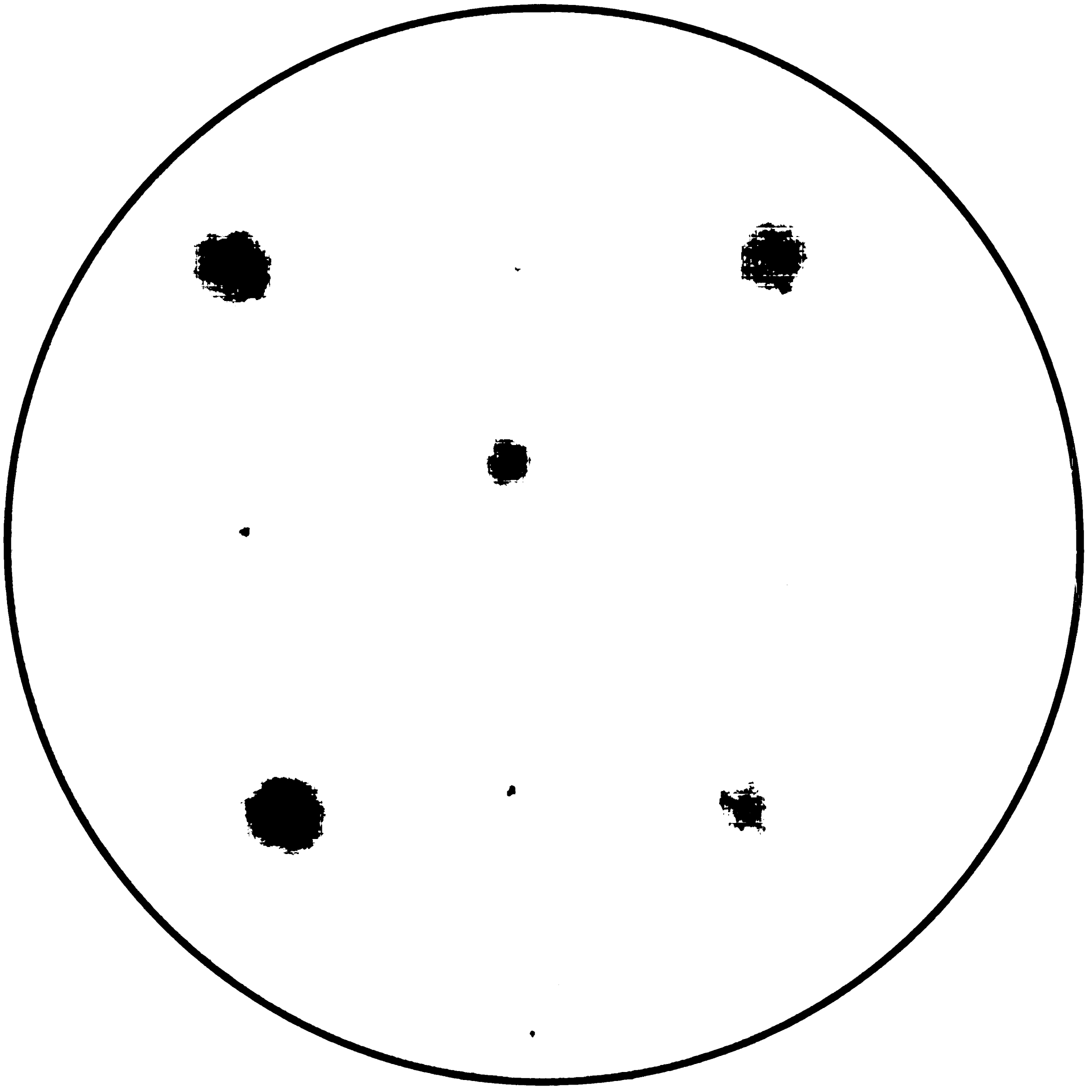

Figure 3

transformations suggested that 27%, 28%, and 22% of the screened colonies expressed GUS. In order to help explain the low ratio of GUS expressors in the pAPGUSNNB transformant population, a number of GUS^+ and GUS^- transformants were chosen for genetic analysis.

Southern Hybridization Analysis

The integration of transforming DNA can occur at either homologous or heterologous locations in the genomes of filamentous fungi. Restriction enzyme analyses of the *nor-1* and *niaD* regions of the *A. parasiticus* chromosome were performed in order to develop Southern hybridization schemes used to identify transformants with pAPGUSNNB single crossover integrations into either the chromosomal *nor-1* or *niaD* genes (Figure 4). GUS^+ and GUS^- transformants were selected from previous experiments and evaluated according to these schemes.

Initial experiments identified three types of transformants (Figure 5), each characterized by a different integration event and each event predicted by the schemes displayed in Figure 4. Transformants D8D1 and D8D6 were GUS^+ and represent single crossover integration of pAPGUSNNB into the 5' region of *nor-1*. The intensity of the hybridization signals from the D8D1 samples in Figures 5A, 5B, 6A, and 6B suggested that multiple integration of the vector occurred in this transformant, while the D8D6 transformant appeared to carry a single copy of the vector. Transformant D8D3 was GUS^+ and represented single crossover integration of pAPGUSNNB into the 3' region of *nor-1*. Transformant D8D4 was GUS^- and represented single crossover integration of pAPGUSNNB into the *niaD* chromosomal region. It is likely that the GUS^- transformant D8E5 was also a *niaD* integrant, but the data presented in Figure 5 did not completely confirm this characterization.

Figure 4

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ScaI frag

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Figure 4A. Schematics for Southern hybridization and PCR analyses of *niaD*$^{+}$ transformants. To determine whether the pAPGUSNNB vector integrated into the 5' region of *nor-1*, a Southern blot of a *XhoI* chromosomal digest was probed with a 750-bp *ScaI* fragment from the 5' region of *nor-1*. This probe is common to both vector and chromosome. A single crossover of pAPGUSNNB into the 5' region of *nor-1* was predicted to produce two Southern hybridization signals of 19-kb and 3.4-kb, with the disappearance of the 5.8-kb wild-type hybridization signal.

The analysis of the 5' *nor-1* pAPGUSNNB transformants by PCR was performed using primers represented in this figure by golden arrows. One primer was designed to be unique to the vector and to anneal to the 5' region of the *uidA* gene. The second primer is unique to chromosomal sequence and anneals to DNA in the 5' region of *nor-1*. A 3.1-kb amplicon should result only when DNA from a 5' *nor-1* pAPGUSNNB integrant is used as template.

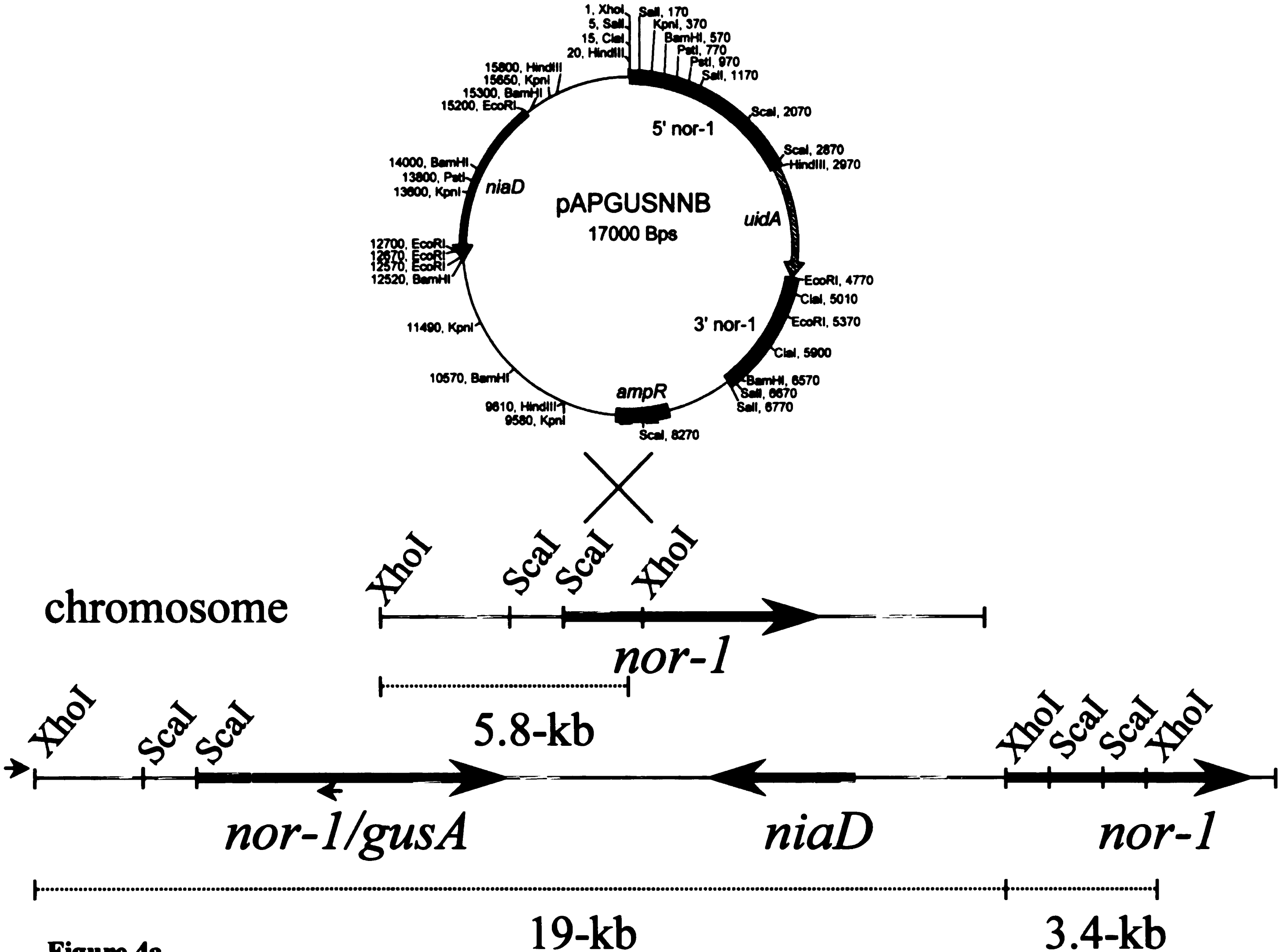

1, XhoI
5, SalI
15, ClaI
20, HindIII
SalI, 170
KpnI, 370
BamHI, 570
PstI, 770
PstI, 970
SalI, 1170
ScaI, 2070
ScaI, 2870
HindIII, 2970
EcoRI, 4770
ClaI, 5010
EcoRI, 5370
ClaI, 5900
BamHI, 6570
SalI, 6670
SalI, 6770
ScaI, 8270
9610, HindIII
9580, KpnI
10570, BamHI
11490, KpnI
12700, EcoRI
12670, EcoRI
12570, EcoRI
12520, BamHI
14000, BamHI
13800, PstI
13600, KpnI
15800, HindIII
15650, KpnI
15300, BamHI
15200, EcoRI
5' nor-1
uidA
3' nor-1
ampR
niaD
pAPGUSNNB
17000 Bps
chromosome
XhoI
ScaI
ScaI
XhoI
nor-1
5.8-kb
XhoI
ScaI
ScaI
nor-1/gusA
niaD
XhoI
ScaI
ScaI
XhoI
nor-1
19-kb
3.4-kb

Figure 4a

Figure 4B. Schematics for Southern hybridization and PCR analyses of *niaD*$^+$ transformants. To determine whether the pAPGUSNNB vector integrated into the 3' region of *nor-1*, a Southern blot of *ScaI* chromosomal digests was probed with a 900-bp *ClaI* fragment from the 3' region of *nor-1*. This probe is common to both vector and chromosome. A single crossover of pAPGUSNNB into the 3' region of *nor-1* was predicted to result in two Southern hybridization signals of 4.0-kb and 4.4-kb, with the disappearance of the 3.0-kb wild-type hybridization signal.

The analysis of the 3' *nor-1* pAPGUSNNB transformants by PCR was performed using primers represented in this figure by golden arrows. One primer was designed to be unique to the vector and to anneal to the 3' region of the *uidA* gene. The second primer is unique to chromosomal sequence and anneals to DNA in the 3' region of *nor-1*. A 2.1-kb amplicon should result only when DNA from a 3' *nor-1* pAPGUSNNB integrant is used as template.

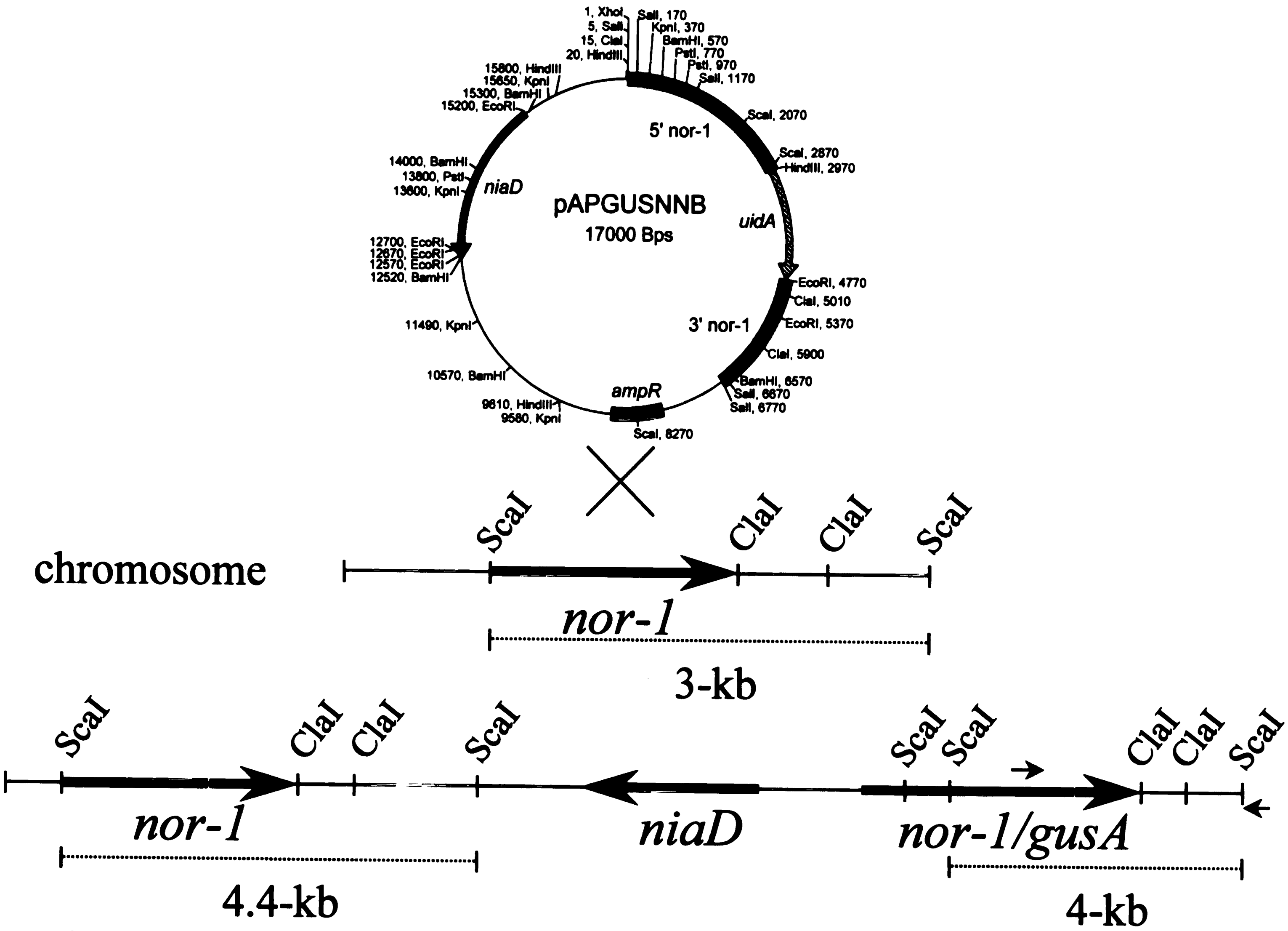

1, XhoI
5, SalI
15, ClaI
20, HindIII
SalI, 170
KpnI, 370
BamHI, 570
PstI, 770
PstI, 970
SalI, 1170
ScaI, 2070
ScaI, 2870
HindIII, 2970
EcoRI, 4770
ClaI, 5010
EcoRI, 5370
ClaI, 5900
BamHI, 6570
SalI, 6670
SalI, 6770
ScaI, 8270
9610, HindIII
9580, KpnI
10570, BamHI
11490, KpnI
12700, EcoRI
12670, EcoRI
12570, EcoRI
12520, BamHI
14000, BamHI
13800, PstI
13600, KpnI
15800, HindIII
15650, KpnI
15300, BamHI
15200, EcoRI
5' nor-1
uidA
3' nor-1
ampR
niaD
pAPGUSNNB
17000 Bps
chromosome
ScaI
ClaI
ClaI
ScaI
nor-1
3-kb
ScaI
ClaI
ClaI
ScaI
ScaI
ScaI
ClaI
ClaI
ScaI
nor-1
niaD
nor-1/gusA
4.4-kb
4-kb

Figure 4b

Figure 4C. Schematics for Southern hybridization analysis of *niaD*$^+$ transformants. To determine whether the pAPGUSNNB vector integrated into the *niaD* region of the NR-1 chromosome, a Southern blot of *SalI* chromosomal digests was probed with an 800-bp *SalI-XhoI* fragment from the *niaD* chromosomal region. This probe is unique to the chromosome. A single crossover of pAPGUSNNB at *niaD* was predicted to yield an 11-kb Southern hybridization signal, with the disappearance of the 8.2-kb wild-type hybridization signal.

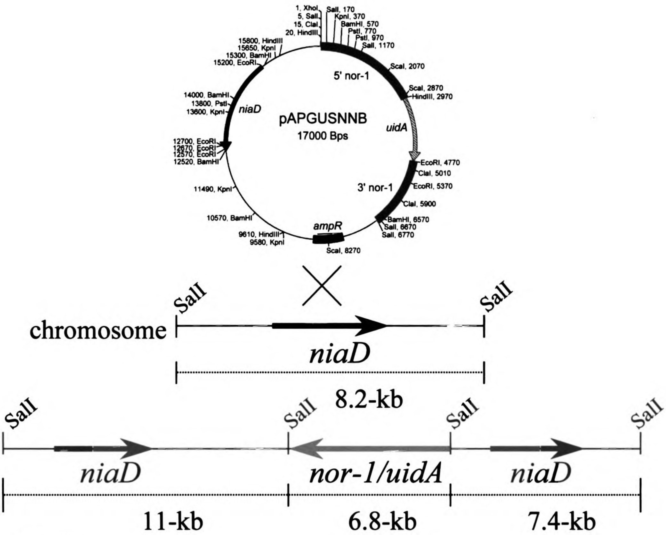

1, XhoI
5, SalI
15, ClaI
20, HindIII
SalI, 170
KpnI, 370
BamHI, 570
PstI, 770
PstI, 970
SalI, 1170
ScaI, 2070
ScaI, 2870
HindIII, 2970
EcoRI, 4770
ClaI, 5010
EcoRI, 5370
ClaI, 5900
BamHI, 6570
SalI, 6670
SalI, 6770
ScaI, 8270
9610, HindIII
9580, KpnI
10570, BamHI
11490, KpnI
12700, EcoRI
12670, EcoRI
12570, EcoRI
12520, BamHI
14000, BamHI
13800, PstI
13600, KpnI
15800, HindIII
15650, KpnI
15300, BamHI
15200, EcoRI
5' nor-1
uidA
3' nor-1
ampR
niaD
pAPGUSNNB
17000 Bps
chromosome
SalI
SalI
niaD
8.2-kb
SalI
SalI
SalI
SalI
niaD
nor-1/uidA
niaD
11-kb
6.8-kb
7.4-kb

Figure 4c

Figure 5A. Southern hybridization analysis of NR-1 and NR-1/pAPGUSNNB transformants. To determine whether the pAPGUSNNB vector integrated into the 5' region of *nor-1*, a Southern blot of *XhoI* chromosomal digests was probed with a 750-bp *ScaI* fragment from the 5' region of *nor-1*. This probe is common to both vector and chromosome. A single crossover of pAPGUSNNB into the 5' region of *nor-1* was predicted to produce two Southern hybridization signals of 19-kb and 3.4-kb, with the disappearance of the 5.8-kb parental strain hybridization signal. The sizes of the DNA fragments are shown on the left and the DNA marker sizes are indicated on the far right. NR1 served as the parental strain control. These data indicate that transformants D8D1 and D8D6 are 5' *nor-1* integrants.

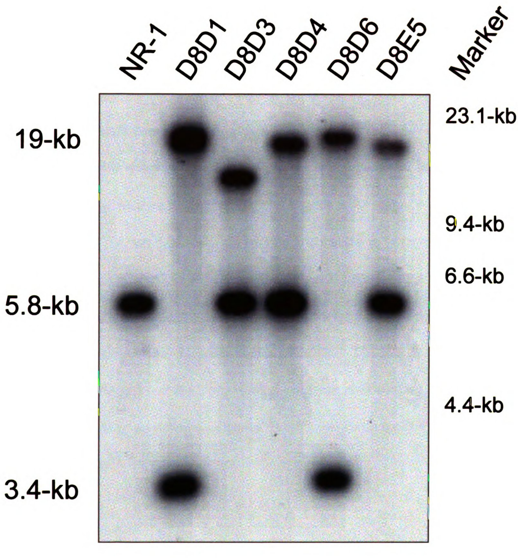

NR-1
D8D1
D8D3
D8D4
D8D6
D8E5
Marker
19-kb
5.8-kb
3.4-kb
23.1-kb
9.4-kb
6.6-kb
4.4-kb

Figure 5a

Figure 5B. Southern hybridization analysis of NR-1 and NR-1/pAPGUSNNB transformants. To determine whether the pAPGUSNNB vector integrated into the 3' region of *nor-1*, a Southern blot of *ScaI* chromosomal digests was probed with a 900-bp *ClaI* fragment from the 3' region of *nor-1*. This probe is common to both vector and chromosome. A single crossover of pAPGUSNNB into the 3' region of *nor-1* was predicted to result in two Southern hybridization signals of 4.0-kb and 4.4-kb, with the disappearance of the 3.0-kb parental strain hybridization signal. The sizes of the DNA fragments are shown on the left and the DNA marker sizes are indicated on the far right. NR1 served as the parental strain control. These data indicate that transformant D8D3 is a 3' *nor-1* integrant.

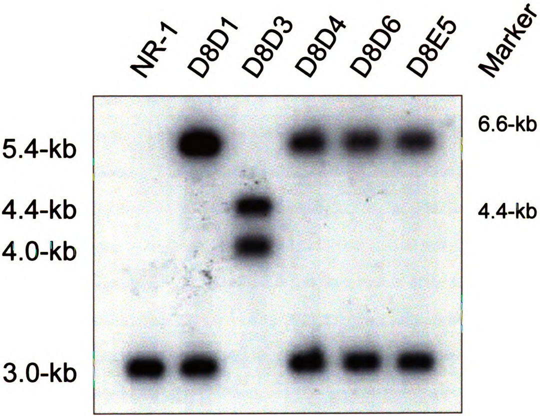

NR-1
D8D1
D8D3
D8D4
D8D6
D8E5
Marker
5.4-kb
4.4-kb
4.0-kb
3.0-kb
6.6-kb
4.4-kb

Figure 5b

Figure 5C. Southern hybridization analysis of NR-1 and NR-1/pAPGUSNNB transformants. To determine whether the pAPGUSNNB vector integrated into the *niaD* region of NR-1 chromosome, a Southern blot of *SalI* chromosomal digests was probed with an 800-bp *SalI-XhoI* fragment from the *niaD* chromosomal region. This probe is unique to the chromosome. A single crossover of pAPGUSNNB at *niaD* was predicted to yield an 11-kb Southern hybridization signal, with the disappearance of the 8.2-kb parental strain hybridization signal. The sizes of the DNA fragments are shown on the left and the DNA marker sizes are indicated on the far right. NR1 served as the parental strain control. These data indicate that transformant D8D4 is a *niaD* integrant.

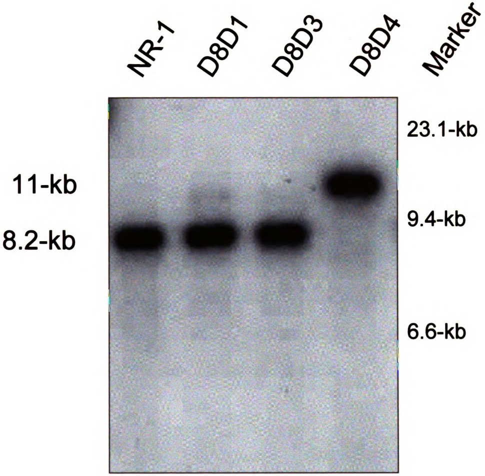


Figure 5c

Figure 6. Southern hybridization analysis of NR-1 and NR-1/pAPGUSNNB transformants. To confirm the presence of the of the *uidA* reporter gene in the *niaD* selected transformants, *ScaI* (A) and *XhoI* (B) chromosomal digests were probed with a 670-bp *MluI* fragment from the *uidA* gene. The sizes of the DNA fragments are shown on the left and the DNA marker sizes are indicated on the far right. NR1 served as the parental strain control. These data illustrate that each of the transformants contains the reporter gene, and that the NR-1 parent strain is negative for *uidA*.

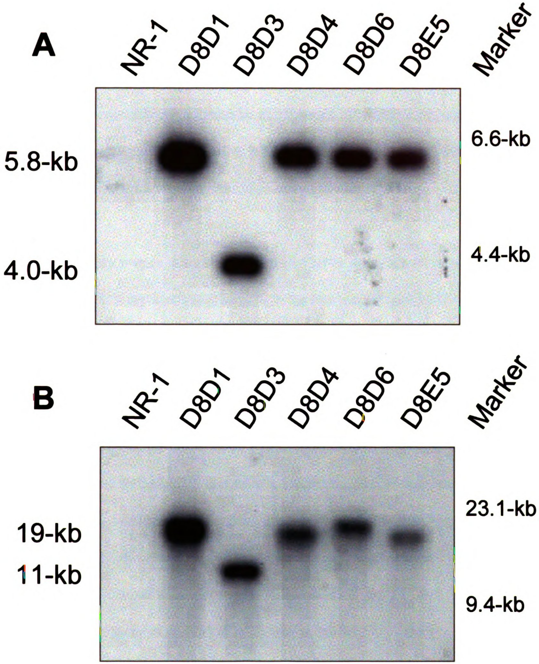

A
NR-1
D8D1
D8D3
D8D4
D8D6
D8E5
Marker
5.8-kb
4.0-kb
6.6-kb
4.4-kb
B
NR-1
D8D1
D8D3
D8D4
D8D6
D8E5
Marker
19-kb
11-kb
23.1-kb
9.4-kb

Figure 6

Once the experimental schemes were tested, a larger group of GUS$^+$ and GUS$^-$ pAPGUSNNB transformants was assayed to identify the integration events (Figures 7 and 8). Ten of eleven (91%) GUS$^+$ transformants examined were either 5' (four) or 3' (six) *nor-1* integrants. The exception is transformant 15, and although its hybridization patterns could not confirm *nor-1* integration of the vector, this possibility still remains. Six GUS$^-$ transformants were shown to be either single crossover integrations at *niaD* (two) or double crossover integrations of the selectable marker (four). The hybridization pattern of transformant 15 was not indicative of integration at *niaD*.

PCR Analysis

A PCR assay was developed to efficiently screen a population of transformants for those isolates with reporter genes located in the *nor-1* region of the chromosome. The assay requires one primer unique to the vector and one primer unique to the chromosomal DNA. When the primers anneal to the template in close proximity to each other, an amplicon can be produced. If the sequence in the region of interest is known, then the site of vector integration can be surmised by the size of the amplicon produced under stringent conditions. In the assay, one set of primers was used to determine a 5' *nor-1* integration event (3.1-kb amplicon) and another set was used to determine a 3' *nor-1* integration event (2.1-kb amplicon). The assay scheme is illustrated in Figures 4A and 4B. Several transformants were screened with this assay (Figure 9) and the interpretation of the data from the assay is supported by Southern hybridization analyses (Figures 7 and 8).

As indicated in Figure 9, transformants 50, 59, 61, 68, 85, and 96 produced a 2.1-kb amplicon with primers JL 102 and JL103. The production of this specific amplicon suggests that these transformants have pAPGUSNNB integrated into the 3' region of *nor-*

1 (refer to Figure 4B for PCR schematics). Transformants 8, 34, 67, and 73 produced a 3.1-kb amplicon with primers JL 99 and JL 100. The production of this specific amplicon indicates that these transformants have pAPGUSNNB integrated into the 5' region of *nor-1* (refer to Figure 4A for PCR schematics).

Time Course Analysis of NR-1/D8D3

Transformant D8D3 was grown in an aflatoxin-inducing medium (YES broth) and evaluated for *nor-1* and *uidA* expression at 24, 48, and 72 h after inoculation. Mycelia dry weight (Table 3A, Figure 10), *nor-1* and *uidA* RNA (Figure 11), NOR and GUS protein (Figure 12), and GUS activity and aflatoxin production (Tables 3B, 3C and Figure 13) were compared in this study. NR-1, NR-1/pSL82B, and NR-1/pGAPN2B served as controls in the experiment. The pGAPN2B vector contains a constitutively expressed *A. flavus* b-tubulin promoter fused to the *uidA* gene, and the *A. parasiticus niaD* gene as a selectable marker. The NR-1/pGAPN2B transformant had vector integration at the *niaD* locus (data not shown). The location of integration of the pSL82 plasmid in transformant NR-1/pSL82B was not identified. Growth of the four strains, as determined by dry weight analysis (Figure 10), was similar during the course of the study. Between 24 and

Table 3A. Time course analysis of transformant D8D3. The dry weights of D8D3 and control cultures were determined at 24, 48, and 72 h after inoculation in aflatoxin inducing medium (YES broth). D8D3 was tested in triplicate while only single cultures of the control strains were harvested for analysis. The data were reported in mg of mycelia.

Strain	24 hours	48 hours	72 hours
NR-1	205	1,150	1,510
NR-1/pSL82	207	1,010	1,660
NR-1/pGAPN2	279	1,230	1,470
NR-1/D8D3A	227	1,080	1,690
NR-1/D8D3B	238	1,230	1,700
NR-1/D8D3C	251	1,210	1,660

48 hours of incubation, each of the cultures was in log phase growth. A transition from log phase to stationary phase was observed for each culture between 48 and 72 hours. Previous studies have shown that it is during this transition that *nor-1* expression and aflatoxin production are first detected in culture (Skory *et al.*, 1993; Trail *et al.*, 1995).

Time Course Analysis of NR-1/D8D3 Transcripts

The accumulation of *nor-1* transcript was detected by Northern blot analysis at 48 and 72 h after inoculation in all strains (Figure 11A). These data indicate that NR-1 and D8D3 express a relatively high level of *nor-1* transcript when compared to the pSL82 and pGAPN2 transformants. NR-1/pGAPN2 is clearly the weakest expressor of *nor-1* transcript.

The timing of *uidA* transcript accumulation in D8D3 (Figure 11B) mimicked that of *nor-1*, with detection of transcript only at 48 and 72 h after inoculation. NR-1 and NR-1/pSL82 were negative for *uidA* transcript at all time points. The NR-1/pGAPN2 transformant is positive for *uidA* transcript at 24, 48, and 72 h. Figures 11C and 11D demonstrate the consistency of D8D3 transcription from replicate cultures. The Northern blot data suggest that *nor-1* transcription is more efficient than *nor-1/uidA* transcription in D8D3, despite the use of the same promoter to regulate the expression of these two genes. The specific activities of the *nor-1* and *uidA* probes in these experiments were roughly equivalent.

Time Course Analysis of NR-1/D8D3 Proteins

Nor-1 and GUS proteins were detected by Western blot analysis in mycelial extracts of D8D3 at 48 and 72 h after inoculation (Figures 12A and 12B). This pattern of protein accumulation was consistent with the accumulation of *nor-1* and *uidA* transcripts from the same mycelia samples. Western blot analysis failed to detect GUS protein in

Figure 7. Southern hybridization analysis of NR-1 and NR-1/pAPGUSNNB GUS$^+$ transformants. Eleven GUS$^+$ transformants were analyzed. A) Six (50, 59, 61, 68, 85, and 96) demonstrating the characteristic hybridization pattern of 3' *nor-1* integrants. B) Four transformants (8, 34, 67, and 73) exhibited a hybridization pattern consistent with pAPGUSNNB integration into the 5' region of *nor-1*. The probes used in these hybridization analyses were the same as those described in Figures 5A and 5B. The sizes of the DNA fragments are shown on the left. NR1 served as the parental strain control. These data do not allow for definite conclusions to be drawn about the location of pAPGUSNNB in the chromosome of transformant 15.

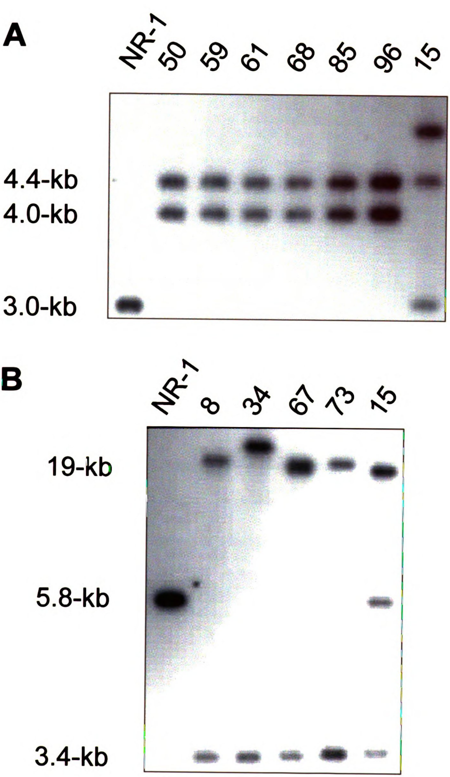

A
NR-1
50
59
61
68
85
96
15
4.4-kb
4.0-kb
3.0-kb
B
NR-1
8
34
67
73
15
19-kb
5.8-kb
3.4-kb

Figure 7

Figure 8. Southern hybridization analysis of NR-1 and NR-1/pAPGUSNNB transformants. Six GUS$^-$ transformants were analyzed. A) Two (10 and 20) produced the characteristic hybridization pattern of pAPGUSNNB integration into the *niaD* region of the chromosome. Transformant 15 (GUS$^+$) does not have a hybridization pattern indicative of *niaD* integration. The probe used in this hybridization analysis was the same as that described in Figure 5C. The sizes of the DNA fragments are shown on the left. NR1 served as the parental strain control. B) A 670-bp MluI fragment of *uidA* was used as a probe to confirm the presence of the reporter gene in the two *niaD* integrants. NR1 served as the parental strain control. The absence of signal from the four remaining GUS$^-$ transformants (25, 35, 40, and 45), suggests that these transformants have double crossover integration of the selectable marker.

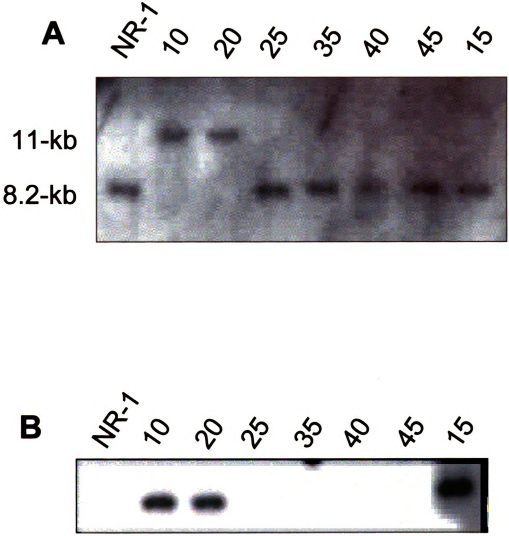


Figure 8

Figure 9. PCR assay for identifying transformants with reporter constructs integrated into the *nor-1* region of the NR-1 chromosome. A) Primers JL 102 and JL103 were used in this assay to produce a 2.1-kb amplicon, specific for pAPGUSNNB integration into the 3' region of *nor-1*. B) Primers JL 99 and JL 100 were used in this assay to produce a 3.1-kb amplicon, specific for pAPGUSNNB integration into the 5' region of *nor-1*. Amplicon size is indicated on the left, and the DNA marker sizes are shown on the far right. NR-1 and transformants 10 (single pAPGUSNNB crossover integration at *niaD*) and 15 (integration site not defined) were used as negative controls in this experiment.

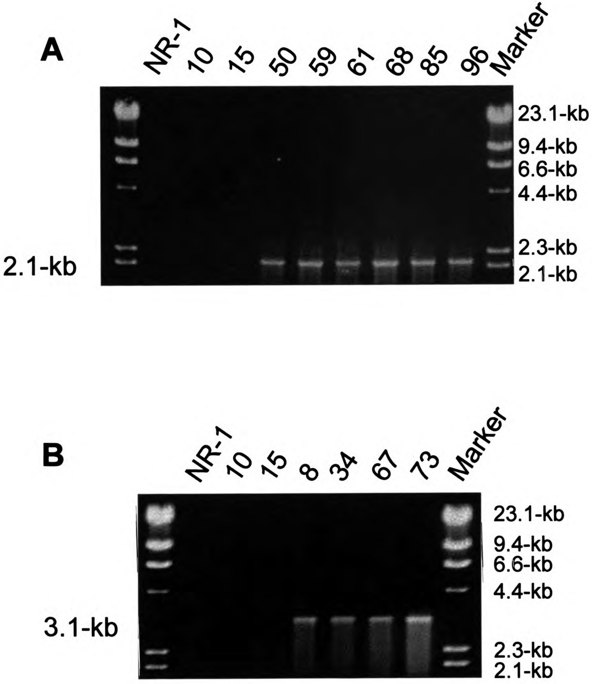
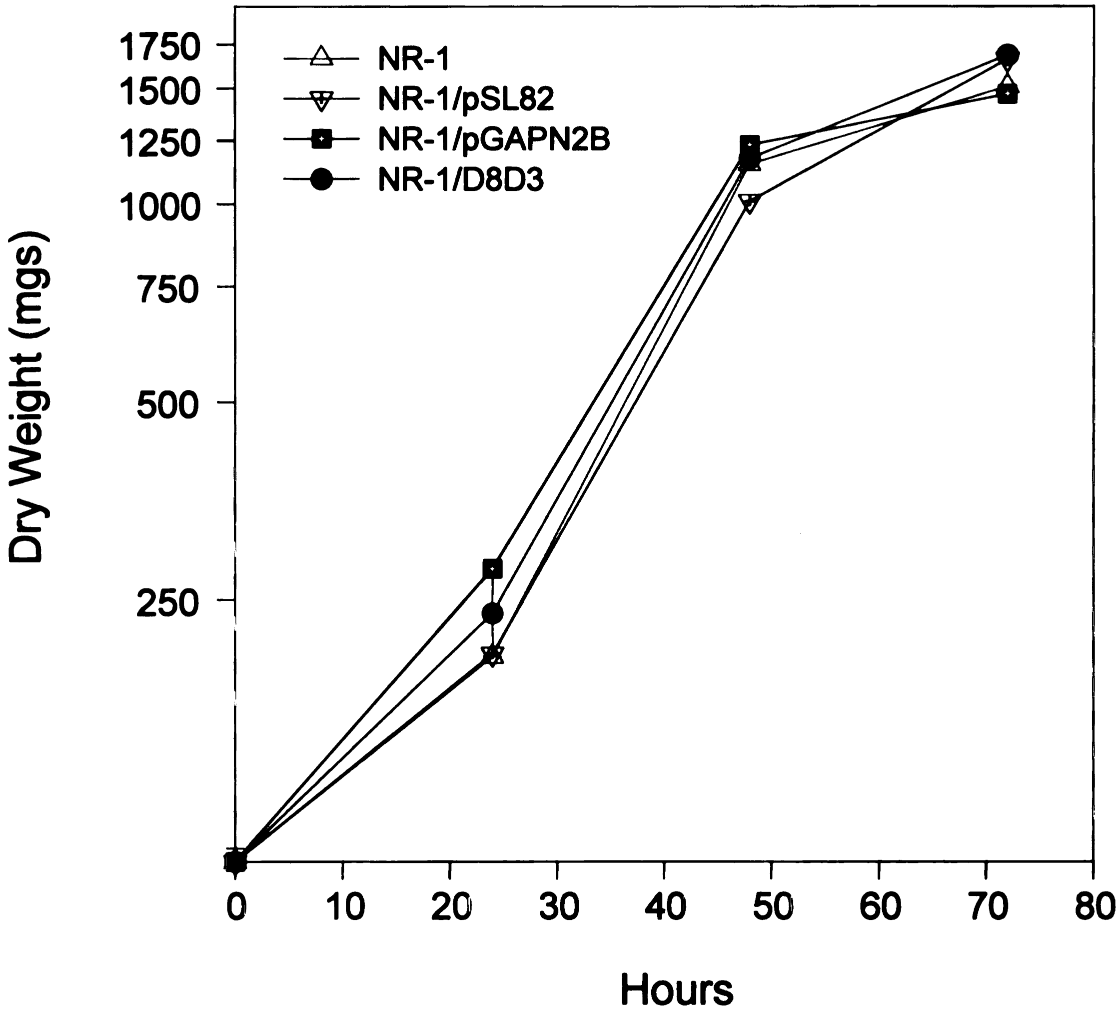

A
NR-1
10
15
50
59
61
68
85
96
Marker
23.1-kb
9.4-kb
6.6-kb
4.4-kb
2.3-kb
2.1-kb
2.1-kb
B
NR-1
10
15
8
34
67
73
Marker
23.1-kb
9.4-kb
6.6-kb
4.4-kb
3.1-kb
2.3-kb
2.1-kb

Figure 9

Figure 10. Dry weight analysis of transformant D8D3 and control strains. Mycelia were harvested (see Methods) at 24, 48, and 72 h after inoculation in aflatoxin-inducing medium (YES broth). A portion of each sample was dried for weight determination. For the control strains, the points on the graph represent data from a single culture. D8D3 was grown in triplicate and the error bars indicate the standard error.



Dry Weight (mgs)
1750
1500
1250
1000
750
500
250
0
10
20
30
40
50
60
70
80
Hours
NR-1
NR-1/pSL82
NR-1/pGAPN2B
NR-1/D8D3

Figure 10.

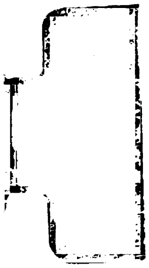
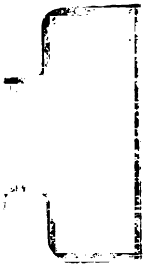


Figure 11. Northern hybridization analysis of *nor-1* and *uidA* transcripts in transformant D8D3 and control strains. 30 µg of total RNA was loaded into each gel lane. Each autoradiogram represents RNA isolated from mycelia after growth for 24, 48, and 72 h in aflatoxin-inducing medium. A and C) Blotted RNA was hybridized with a PCR amplified 780 bp *nor-1* probe. The target DNA for this amplification was *nor-1* cDNA. The *nor-1* transcript is approximately 1.3 kb. B and D) Blotted RNA was hybridized with an approximately 1.7 kb amplification product of the *uidA* gene. The predicted *nor-1/uidA* fusion transcript is approximately 2.2 kb. This estimate is based on the size of the *uidA* gene in the fusion and the *nor-1* transcription initiation site and polyadenylation site which are also included in the fusion (Trail *et al.*, 1994). Transcript sizes are indicated on the left and RNA marker sizes are indicated on the far right. NR-1 was used as the parental control strain. pGAPN2 was used as a control for constitutive *uidA* expression. pSL82 was used as a transformation control and was *uidA*$^-$. D8D3 A, B, and C (in 11 C, D) are replicate samples.



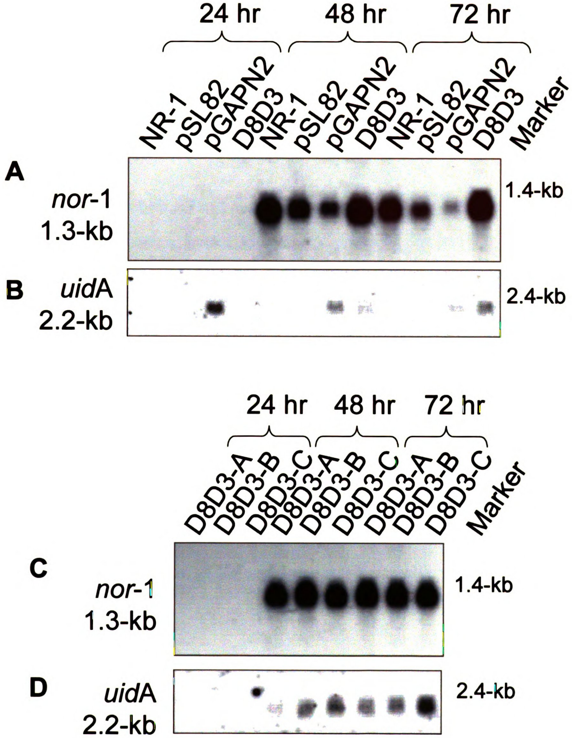

24 hr
48 hr
72 hr
NR-1
pSL82
pGAPN2
D8D3
NR-1
pSL82
pGAPN2
D8D3
NR-1
pSL82
pGAPN2
D8D3
Marker
A
nor-1
1.3-kb
1.4-kb
B
uidA
2.2-kb
2.4-kb
24 hr
48 hr
72 hr
D8D3-A
D8D3-B
D8D3-C
D8D3-A
D8D3-B
D8D3-C
D8D3-A
D8D3-B
D8D3-C
Marker
C
nor-1
1.3-kb
1.4-kb
D
uidA
2.2-kb
2.4-kb

Figure 11

Figure 12. Western blot analysis of Nor-1 and GUS proteins in transformant D8D3 and control strains. Protein was electrophoresed through a 12% separating gel. 60 – 75 µg of extracted protein was loaded into each gel lane. Each blot represents protein isolated from mycelia after growth for 24, 48, and 72 h in aflatoxin inducing-medium. Only the sections of the blot containing proteins of relevant sizes are included in this figure. A and C) Blotted protein was probed with α-NOR antibody. The Nor-1 protein is approximately 31 kDa (Zhou, 1997). B and D) Blotted protein was probed with α-GUS antibody (Clontech Laboratories). The size of the Nor-1/GUS fusion protein, as determined by these data, is approximately 73 kDa. The estimated size of this fusion protein is similar to the wildtype *E. coli* GUS protein which is also approximately 73 kDa (Jefferson *et al.*, 1986). Protein sizes are indicated on the far left. D8D3 A, B, and C (in 12 C, D) are replicate samples.

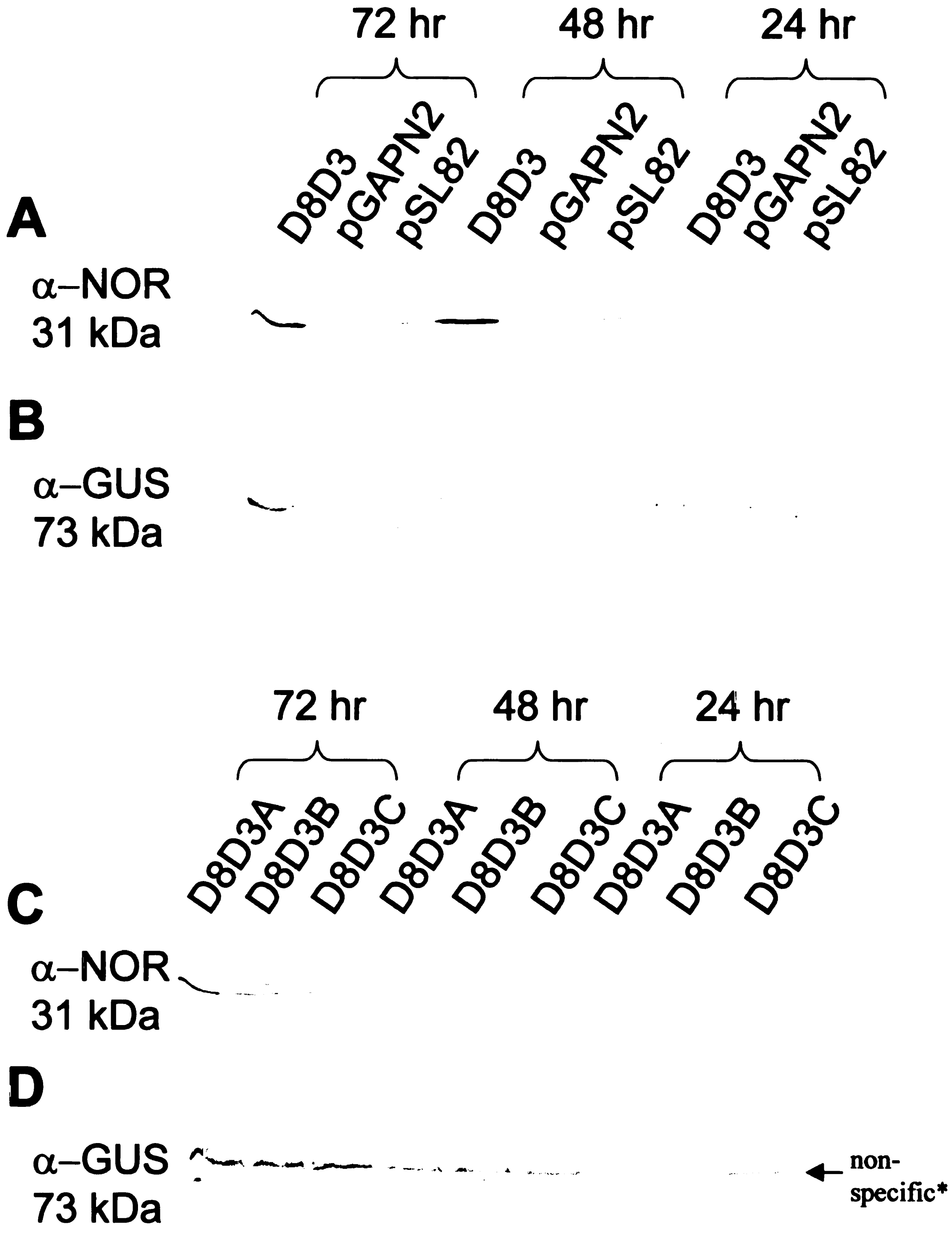


Figure 12
*The signals at 24 hours are non-specific, however those at 48 and 72 hours are GUS specific.

Figure 13. Analysis of GUS activity and aflatoxin production in transformant D8D3 and control strains. Mycelia were harvested (see methods) at 24, 48, and 72 h after inoculation in aflatoxin-inducing medium (YES broth). A) One μg of protein from each mycelia sample was used to determine GUS activity. The substrate for GUS analysis was 4-methylumbelliferyl-β–D-glucuronide. B) Culture filtrate was used for the determination of aflatoxin levels. For the control strains, the points on the graph represent data from a single culture. D8D3 was grown in triplicate and the error bars indicate the standard error.

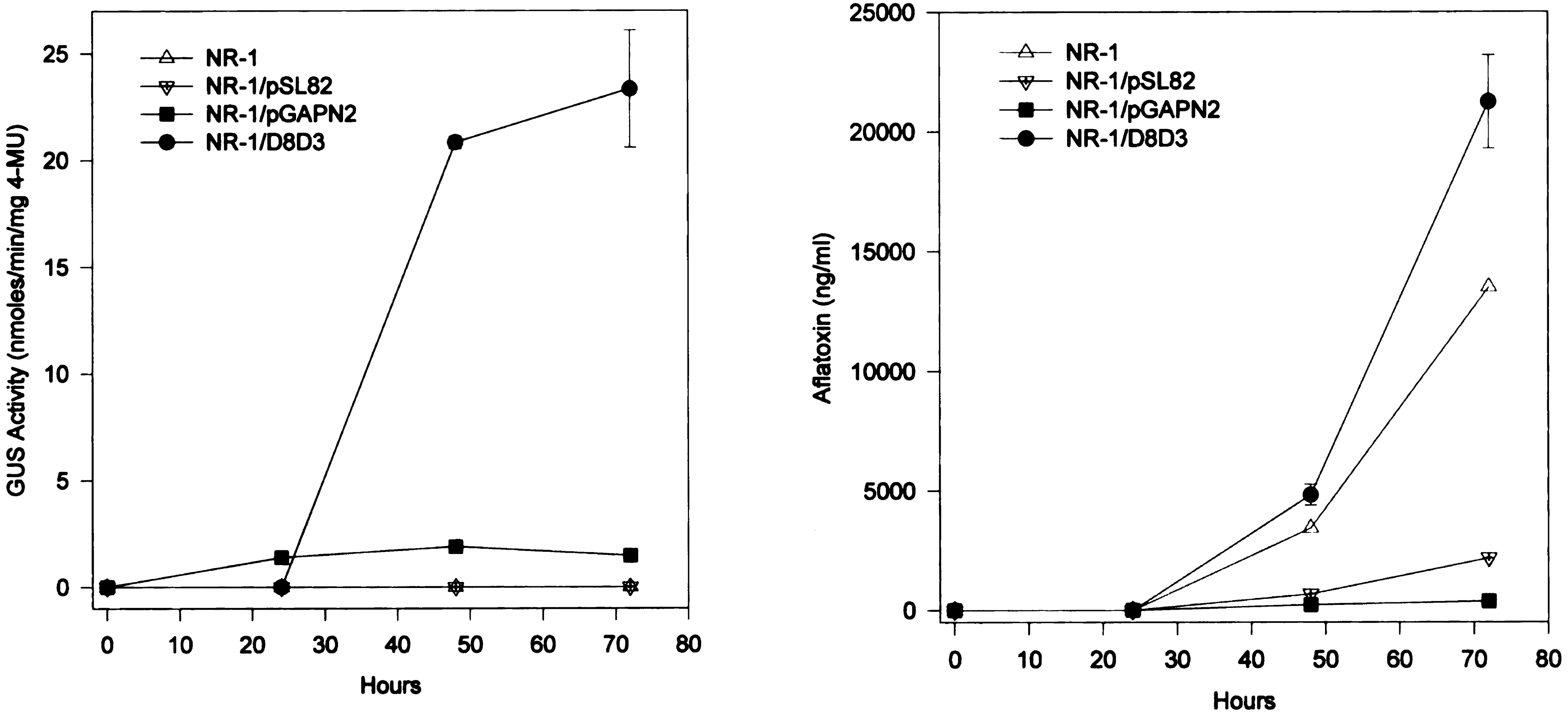

NR-1
NR-1/pSL82
NR-1/pGAPN2
NR-1/D8D3
GUS Activity (nmoles/min/mg 4-MU)
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Hours
Aflatoxin (ng/ml)
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
60
70
80
Hours

Figure 13

NR-1/pGAPN2 extracts at any time point although the presence of *uidA* transcript and GUS activity were established in these cultures. Northern hybridization analysis indicated that the level of *uidA* transcript and GUS activity were relatively low in the pGAPN2 transformant during the course of the study, and apparently the Western technique lacks the sensitivity to detect this low yield of GUS protein.

The GUS protein was only identified in D8D3 at the 48 and 72 h time points. A protein with similar size, but slightly larger than GUS, displayed cross-reactivity with α-GUS and was seen with the 24 h samples in Figure 12B and 12D. The lack of *uidA* transcript and GUS activity in D8D3 at these timepoints supports the interpretation of this signal as nonspecific protein.

The Nor-1 protein was identified in all samples at 48 and 72 h after inoculation. The expression of Nor-1 was weakest in the NR-1/pGAPN2 strain, and although the representation of the data in Figure 12A failed to show this weak expression, the Nor-1 protein in NR-1/pGAPN2 could be identified in the original blot. The low level of Nor-1 in NR-1/pGAPN2 is probably due to the weak transcription of *nor-1* demonstrated in Figure 11A. Figures 12C and 12D demonstrate the consistency of D8D3 translation from replicate cultures.

Time Course Analysis of NR-1/D8D3 Protein Activity

Nor-1 protein activity was indirectly assayed by measuring aflatoxin concentrations. Both aflatoxin (Table 3C and Figure 13B) and GUS activity (Table 3B and Figure 13A) were first detected in D8D3 after 48 h of fungal growth. This is consistent with the accumulation of *nor-1* and *uidA* transcripts (Figure 11) and Nor-1 and GUS proteins (Figure 12) from D8D3 mycelia samples. Although GUS activity in D8D3 increased slightly from 48 h to 72 h, a greater increase in aflatoxin levels during this

Table 3B. Time course analysis of transformant D8D3. The GUS activities from the mycelia of D8D3 and control strains were determined at 24, 48, and 72 h. D8D3 was tested in triplicate while only single cultures of the control strains were analyzed. The data were reported in nanomoles 4-methylumbelliferone/min/mg of protein.

Strain	24 hours	48 hours	72 hours
NR-1	No detection	No detection	No detection
NR-1/pSL82	No detection	No detection	No detection
NR-1/pGAPN2	1.39	1.89	1.47
NR-1/D8D3A	No detection	21.4	21.1
NR-1/D8D3B	No detection	20.4	20.1
NR-1/D8D3C	No detection	20.7	28.8

Table 3C. Time course analysis of transformant D8D3. The aflatoxin concentrations in the filtrates of cultures of D8D3 and control strains were determined at 24, 48, and 72 h. D8D3 was tested in triplicate while only single cultures of the control strains were analyzed. The data were reported in ng/ml as determined by direct competitive ELISA.

Strain	24 hours	48 hours	72 hours
NR-1	< 50	3,440	13,512
NR-1/pSL82	< 50	680.3	2,186
NR-1/pGAPN2	< 50	233.8	369
NR-1/D8D3A	< 50	5,367	25,015
NR-1/D8D3B	< 50	5,141	20,256
NR-1/D8D3C	< 50	3,974	18,474

period was observed. This apparent discrepancy in proportion is likely due to the stability of aflatoxin and its continuous production from active enzymes, and would probably be absent if Nor-1 activity was measured directly. The pSL82 and pGAPN2 transformants were relatively weak producers of aflatoxin, as might be expected when comparing the *nor-1* transcripts (Figure11) and Nor-1 proteins (Figure 12) of these strains with those of D8D3 and NR-1.

GUS activity was detected in NR-1/pGAPN2 at all three time points. At 48 h after inoculation, D8D3 expressed 11 times more GUS activity than GAPN2 and at 72 h, D8D3 expressed 48 times more activity. Since *uidA* transcript levels were similar in the

D8D3 and GAPN2 transformants (Figure 11B), the lower level of GUS activity in GAPN2 is likely the result of relatively inefficient translation of the β-tubulin fusion protein (Figure 12B). GUS activity was absent in NR-1 and NR-1/pSL82 at all time points.

Table 4. GUS phenotypes of pAPGUSNNB transformants on media supportive (YES) and non-supportive (CZ) of aflatoxin production. Qualitative GUS assays (see Methods) were performed on fungal colonies after 48 h of growth on both types of media. Site of vector integration is indicated in parenthesis.

Strain	**YES**	**CZ**
D8D1 (*nor-1*)	GUS^+	GUS^-
D8D3 (*nor-1*)	GUS^+	GUS^-
D8D4 (*niaD*)	GUS^-	GUS^-
D8D6 (*nor-1*)	GUS^+	GUS^-
D8E5 (*niaD**)	GUS^-	GUS^-

Table 5. GUS activities in NR-1 transformants. Mycelia from each of the strains were harvested at 48 hrs after growth in aflatoxin inducing medium (YES broth). One mg of protein from each sample was used to determine GUS activity. The data were reported in nanomoles 4-methylumbelliferone/min/mg of protein. The GUS activities for the transformants that are presumably genetically identical to D8D3 were reported in an experiment separate from the analysis that produced the GUS activity data for the D8D4 and D8D6 strains. Transformant D8D6 has the GUS reporter fusion located in the 5' region of *nor-1* whereas D8D4 has the reporter integrated at *niaD* (Figure 5).

Strain	**Sample A**	**Sample B**	**Mean**
D8D6	78.6	65.2	71.9
D8D4	No detection	No detection	NA
D8D3 (50)	19.6	19.0	19.3
D8D3 (59)	20.4	16.6	18.5
D8D3 (61)	16.0	19.2	17.6
D8D3 (68)	15.7	14.6	15.2
D8D3 (85)	11.0	12.5	11.8

DISCUSSION

Biological strategies for eliminating pre-harvest aflatoxin contamination of crops are directed toward the development of atoxigenic fungal strains or toxin-inhibiting plants, which will be successful in the agricultural environment. The long-term success of this type of strategy requires an understanding of the plant-fungal relationship. The interaction between plant and fungus can be efficiently monitored with reporter gene fusions as has been demonstrated in other plant-pathogen relationships (Couteaudier *et al.*, 1993; Maor *et al.*, 1998; Olivain and Alabouvette, 1997; Spellig *et al.*, 1996). With regard to aflatoxin elimination, the ability to assay for aflatoxin reporter enzymes in plant tissue inoculated with fungal spores should allow for rapid screening of compounds and/or plant strains with the ability to impair toxin production. Experiments involving mutagenesis of the promoter region of reporter constructs should also aid in identifying cis-acting regions and trans-acting factors essential for the regulation of a gene of interest. Knowledge of aflatoxin regulatory control mechanisms should prove valuable in the development or identification of anti-toxin compounds. A prerequisite of such reporter studies, is the analysis of fusion expression to confirm that the wild-type gene of interest and the derived reporter construct have a similar pattern of regulation.

Our experiments have demonstrated that the *nor-1/uidA* reporter construct and the wild-type *nor-1* gene are regulated in a similar manner in strain D8D3. Under aflatoxin-inducing conditions, the timing and pattern of transcript and protein accumulation from the expression of these two genes were similar (Figure 11 & 12). Data from the same time course experiment also showed that the timing of aflatoxin production and GUS activity was identical (Figure 13), while the pattern of aflatoxin accumulation was consistent with the levels of GUS activity at 48 and 72 hrs (i.e. relatively high GUS activity precedes high levels of aflatoxin production). Furthermore, when qualitative GUS assays were performed on a medium not supportive of aflatoxin production (CZ), GUS activity in D8D3 was not detected (Table 4). To demonstrate the reproducibility of GUS expression from transformants that are presumably genotypically identical to D8D3 (Figure 7), selected isolates were cultured under identical growth conditions and then were assayed for GUS activity (Table 5). The difference between strains did not exceed 65% GUS activity (strains 50 & 85). The overall data support the use of the *nor-1/uidA* fusion as a reliable reporter for *nor-1* expression and for *nor-1* promoter analysis.

Our data have also suggested the presence of a position-dependent expression mechanism operating in the *nor-1* region of the *A. parasiticus* chromosome. The absence of detectable GUS activity in 77% of pAPGUSNNB transformants (Table 2) had indicated the possibility of such a mechanism. Subsequent genetic characterization of GUS^+ transformants (thirteen) showed all had reporter fusion integration into the *nor-1* locus (Figures 5 and 7), while GUS^- transformants were found either to be double crossover integrations of the selectable marker (four), or pAPGUSNNB integrations into the *niaD* chromosomal region (three) (Figures 5 and 8). The lack of GUS activity in transformants with the reporter construct located outside the *nor-1* region supports the existence of a position-dependent expression mechanism. Studies with a *ver-1/uidA* fusion (Liang *et al.*, 1997) have also demonstrated position-dependent expression of an aflatoxin promoter, and together the data indicate that the phenomenon may apply to a substantial area of the aflatoxin gene cluster. However, in both of these studies the GUS^- phenotype in single crossover recombinations, was the result of vector integration in the *niaD* chromosomal region. This locus has been shown to be both positively and negatively regulated in *A. parasiticus* (Chang *et al.*, 1996), thus lack of GUS expression in this region could be the result of local regulatory factors. These results indicate, that it is critical for future *nor-1/uidA* expression experiments to have integration of the pAPGUSNNB vector be targeted to the *nor-1* region of the NR-1 chromosome.

The targeting of reporter constructs to specific chromosomal locations for promoter analysis has been advised by other investigators wary of position-dependent expression of fungal genes (van Gorcom *et al.*, 1986; Hamer and Timberlake, 1987; Timberlake and Marshall, 1989), but the mechanisms responsible for the phenomenon have not been elucidated. It has been hypothesized that enhancer elements may be responsible for the position effect (Kinsey and Rambosek, 1984). The presence of this type of positive cis-acting factor having regional control over transcription of the aflatoxin gene cluster is plausible, since removal of aflatoxin reporter fusions from the cluster results in the GUS$^-$ phenotype. However, experiments involving the SpoC1 gene cluster (SGC) in *A. nidulans* (Miller *et al.*, 1987) suggest that position dependent expression of fungal genes may not be so simply explained.

In this work, removal of a cluster gene to novel chromosomal positions resulted in elevated cluster gene transcription in certain cell types. The authors hypothesized that a repressive cis-acting element with activity over large distances was a possible explanation for their data. This argument was supported when a constitutively expressed gene was positioned at different locations within the SGC and a reduction in transcript levels was detected. The possibility of a negative regulatory mechanism, similar to the SGC model, operating in the aflatoxin gene cluster is unlikely, since our experiments have shown an opposite effect (i.e. reporter expression occurs when the fusions are localized to the cluster, and the selectable marker (*niaD*), when placed in the cluster, is expressed without

any detectable difference in morphology between transformants with different fusion integration sites).

The study by Miller *et al.* (1987) also postulated that chromatin conformation was a factor in the repressive effect on cluster genes. This was based on the observation that genes at the border of the SGC were transcribed more efficiently than centrally located genes under repressive conditions. If such a conformational control mechanism exists in the aflatoxin gene cluster, it has to be reconciled with experiments by Flaherty and Payne (1997) that showed that by constitutively expressing *aflR* (positive aflatoxin regulatory gene) under aflatoxin inducing and non-inducing conditions, aflatoxin biosynthetic genes were transcriptionally activated. In the Flaherty and Payne (1997) experiments, an *aflR* structural gene was fused to a constitutively expressed promoter and then introduced into *A. flavus* strains. The chromosomal position of the *aflR* construct was not reported in this study.

The placement of an extra copy of *aflR* (Chang *et al.*, 1995) and the constitutive expression of *aflR* (Flaherty and Payne, 1997) in the *A. flavus* chromosome both appear to overcome the repressive effect of nitrate on aflatoxin gene transcription. However Flaherty and Payne demonstrated that under these derepressing conditions, aflatoxin production in nitrate containing media was still dramatically decreased. Again, the position of the integrating *aflR* vectors was not reported in these studies. When considering a chromatin conformation control mechanism for the aflatoxin gene cluster, it is interesting to note the central position of the *aflR* regulatory gene in the cluster. A non-coordinately expressed cluster gene (L8B) (Miller *et al.*, 1987) can also be found in a central location in the SGC, and its pattern of expression indicates it may too serve a regulatory role in cluster transcription. Additional studies placing aflatoxin reporter fusions at noncluster sites other than *niaD* are being performed in our laboratory to confirm the position-dependent expression of aflatoxin genes. Also, the construction of an *aflR/uidA* fusion and analysis of its expression at different chromosomal locations under aflatoxin inducing and repressing conditions is being planned, and should aid in elucidating aflatoxin regulatory mechanisms.

The targeting of the *nor-1/uidA* reporter fusion to the 3' *nor-1* region, as opposed to the 5' *nor-1* region, will also be important to future reporter expression analysis. Transformants with 3' *nor-1* integrations of pAPGUSNNB will more likely maintain the integrity of any *in vitro* modifications of the *nor-1/uidA* promoter during recombination. For example, if a mutation in the *nor-1/uidA* promoter is to be evaluated in the fungus, the position of a 5' *nor-1* integration of the vector could result in the mutated promoter regulating *nor-1* expression instead of *uidA* expression. A 3' *nor-1* integration would avoid this potential outcome. Two screening assays were developed to aid in the identification of transformants with 3' *nor-1* pAPGUSNNB integrations. The first is a qualitative GUS assay (Figure 3) adapted from the literature (Kolar *et al.*, 1991; Verdoes *et al.*, 1994) and can be used as an initial screening assay to identify transformants with integrations in the *nor-1* locus. This assay takes advantage of the position-dependent expression of the *nor-1/uidA* fusion in the NR-1 chromosome and should eliminate 75% (Table 2) of potential transformants from further analysis.

The remaining transformants will likely have pAPGUSNNB integrated in either the 5' *nor-1* or 3' *nor-1* chromosomal regions. A novel PCR assay (Figures 4A & 4B) was developed to identify transformants of both types and is designed with one primer specific to plasmid DNA and one primer specific to chromosomal DNA. In this study, all transformant types identified by PCR (Figure 9) were confirmed by Southern hybridization analyses (Figures 5 and 7).

We conclude that the expression of the *nor-1/uidA* reporter fusion is similar to the expression of the wild-type *nor-1* gene in *A. parasiticus*, and can therefore be used as a reliable indicator of *nor-1* expression and aflatoxin biosynthesis. In addition, the qualitative GUS assay and PCR assay developed in the course of this research can be adapted for further use in the study of position-dependent expression of fungal genes.

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