A GENETIC APPROACH TO DEVELOP A DETAILED UNDERSTANDING OF THE REGULATION OF AFLATOXIN BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*

BY

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ABSTRACT

A GENETIC APPROACH TO DEVELOP A DETAILED UNDERSTANDING OF THE REGULATION OF AFLATOXIN BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*

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Aflatoxins impose a potential threat to human health and cause a great economic loss annually. Understanding the aflatoxin biosynthetic pathway and its regulation at the molecular level may play a role in controlling aflatoxin contamination. A genetic approach to achieve these purposes was established in this report.

A mutant ß-tubulin gene (*ben*^{r}) potentially involved in conferring the resistance to the fungicide benomyl was isolated from a benomyl resistant strain of *Aspergillus parasiticus*. The *ben*^{r} gene was tested for its use as a dominant selectable marker for transformation of *A. parasiticus*. The resulting transformation efficiency (5 to 10 transformants per µg of DNA) indicates that *ben*^{r} is not suitable for gene isolation by complementation but does provide a valuable tool to study the aflatoxin biosynthetic genes by gene disruption in a wild type genetic background.

Recombinational inactivation (gene disruption) techniques were developed in a model system using the gene encoding nitrate reductase (*nia*D) in *A. parasiticus*. Functional disruption of *nia*D was successfully conducted by one-step gene replacement and integrative disruption. These gene disruption strategies were subsequently used to disrupt a gene potentially involved in polyketide biosynthesis/cell

development in *A. parasiticus* and in disruption of several genes involved in aflatoxin biosynthesis.

Data from previous studies suggest that aflatoxin production is regulated in part at the transcriptional level. To confirm this observation, analysis of transcription of a gene involved in aflatoxin biosynthesis was performed using vectors containing the promoter and transcription termination signal for *ver*-1 in the *uid*A gene (GUS, a reporter gene) which encodes for β-glucuronidase in *Escherichia coli*. The temporal expression pattern and level of expression of the *ver*-1 transcript and *ver*-1 protein were correlated to the level of GUS transcript, protein, and activity. The results confirmed that: (1) aflatoxin production is regulated in part at the transcriptional level; (2) GUS can be used as a reporter gene in the analysis of transcription of genes involved in aflatoxin biosynthesis. The tools developed here will allow future studies aimed at determining the cis-acting sites and trans-acting regulatory factors involved in regulation of aflatoxin synthesis.

Dedicated to my father, brother, and sister for their support and understanding as well my mother, to whom I will never again have the chance to say "thank you" because she passed away seven months before my graduate study in the United States.

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RATIONALE

Aflatoxins are well known mutagenic and carcinogenic secondary metabolites produced by certain strains of *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. Contamination by aflatoxins causes a great annual loss in food commodities and animal feeds in the U.S. and imposes a threat to human health in certain areas around the world (Busby and Wogan, 1984; Jelinek et al., 1989). In this regard, there is an urgent need for elimination of aflatoxins from the food chain.

The long term goal of my research is to provide technology allowing development of biocontrol agents to reduce or eliminate aflatoxins from the food chain. Strategies to eliminate aflatoxins from food have been grouped into two broad categories; preharvest and postharvest strategies. Among the preharvest strategies, the use of nontoxigenic biocontrol agents has been shown to be an effective approach in reducing aflatoxin contamination. The effectiveness of using biocontrol agents is mainly attributed to the reduction in the toxin production before or during fungal infection on plants as well as inhibition in the interaction between toxin producers and plants. Understanding of the aflatoxin biosynthetic pathway at the molecular level and/or the regulatory mechanisms governing toxin production can provide targets for interruption of aflatoxin production and generation of potential biocontrol agents. A

detailed understanding of the aflatoxin biosynthetic pathway and/or the regulatory mechanisms at the molecular level and disruption of the function of these genes require the application of modern genetic engineering technologies such as genetic transformation systems, recombinant DNA technologies, and gene disruption technologies. Therefore, three short term goals requisite for the accomplishment of the long term goal are: (1) development of a transformation system for *A. parasiticus* using a dominant selectable marker; (2) disruption of genes involved in aflatoxin biosynthesis to generate nontoxigenic biocontrol agents; (3) generation of an efficient system to study the regulation of genes involved in aflatoxin biosynthesis.

Three hypotheses are associated with these short term goals: (1) an efficient transformation system can be developed using a β-tubulin gene conferring benomyl resistance; (2) disruption of aflatoxin biosynthetic genes can be achieved through homologous recombination in *A. parasiticus*; (3) an efficient system to study regulation of AFB1 gene expression can be generated using a reporter gene (*uid*A, GUS) isolated from *Escherichia coli*. My research objectives were designed to test these hypotheses. The first objective was the development of a DNA-mediated transformation system using a dominant selectable marker (*ben*^{r}) isolated from a benomyl resistant strain of *A. parasiticus*. This selectable marker has now been successfully used to establish the functional role of the *ver*-1A gene (personal communication, Liang). The second objective was the development of a gene disruption model system in *A. parasiticus* through recombinational inactivation of the nitrate reductase structural gene (*nia*D). This model system has been used to

knockout several aflatoxin biosynthetic genes (Liang and Linz, 1994; Trail et al., 1994; Mahanti et al., 1992). The third objective was the development of a system to analyze the transcriptional regulation of aflatoxin pathway genes using the *ver*-1 gene promoter and the GUS reporter gene as the model. The experimental data confirm that GUS can be used as a reporter gene in the transcriptional analyses of genes involved in aflatoxin biosynthesis and in future transcriptional studies to identify crucial regulatory elements. This GUS gene reporter gene also has been used to study the interaction between the host plant and toxigenic fungi.

CHAPTER I

LITERATURE REVIEW

I.Mycotoxins

Characteristics of mycotoxins

Mycotoxins are a group of structurally diverse fungal metabolites that evoke toxic responses when introduced into vertebrates in low concentrations. Approximately 350 to 400 mycotoxins have be reported and newly discovered metabolites are frequently added to the list (Cole and Cox, 1981; Chu, 1991). Mycotoxins are commonly identified on agricultural commodities such as corn, peanuts, cottonseed, oats, wheat, barley, and rye (Jelinek et al., 1989; Pohland and Wood, 1991; Pestka, 1995). Mycotoxins have also been identified occasionally as carryover residues in foods of animal origin such as animal tissues, milk, and eggs (Mirocha et al., 1981; Groopman et al., 1988; Škrinjar et al., 1992; Dorner et al., 1994).

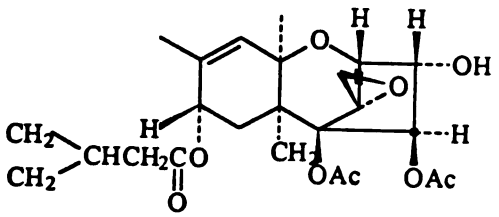
Mycotoxins became a major concern to the public and scientific community for two reasons; economic loss and the threat to human health. Consumption of mycotoxin-containing feeds or foods by animals or humans has been shown to cause a wide variety of acute or chronic diseases (Cole and Cox, 1981). Therefore, the identification of mycotoxin contamination in animal feeds or food products derived from mycotoxin-intoxicated animals usually leads to the condemnation and destruction of the crops and animal tissues, contributing to a great economic loss. However, the annual loss to the livestock and poultry industries due to mycotoxicosis is mainly attributed to the depression of live weight, decrease in feed conversion rate, lower reproduction efficiency, and elevation in mortality. The contamination of these natural microbial toxins in agricultural products and subsequently in milk, meat, or poultry

for human consumption is also considered a potential threat to human health. With regard to human health, a great deal of economic loss is derived from the cost of regulation, detection, and decontamination to prevent mycotoxins from entering the food chain. Monitoring, diagnosis, and treating malignancies which may result from ingestion of mycotoxins also contributes to a great economic loss. For example, in the United States, the estimated cost per life saved (cost-effectiveness ratio) increases from $56,000 at an aflatoxin tolerance level of 15 ppb to $1.7 million at 10 ppb (Shane, 1991).

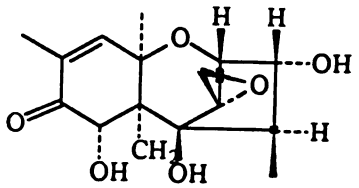
Major mycotoxins

The most significantly occurring, therefore well-studied, mycotoxins are aflatoxins, trichothecenes, zearalenone, ochratoxin, patulin, penicillic acid, and fumonisin (Northolt and Bullerman, 1982) (Figure 1). In addition, sterigmatocystin and cyclopiazonic acid are also major concerns in terms of their frequency of occurrence and toxicity (Gelderblom et al., 1988; Thiel et al., 1991; Wang et al., 1991; Dorner et al., 1994). The major feed- or food-borne mycotoxins are produced by common isolates of *Aspergillus*, *Fusarium*, and *Penicillium* sp. Aflatoxins, ochratoxins, sterigmatocystin, cyclopiazonic acid, penicillic acid, and patulin are grouped as *Aspergillus* and *Penicillium* toxins, whereas trichothecenes, zearalenone, and fumonisins are grouped as *Fusarium* toxins (Benita, 1984).

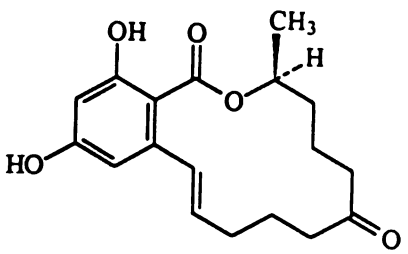
Trichothecenes are a group of structurally related sesquiterpenoids (ApSimon, 1994) produced by the imperfect fungi (no sexual stage) *Trichoderma*, *Trichothecium*,



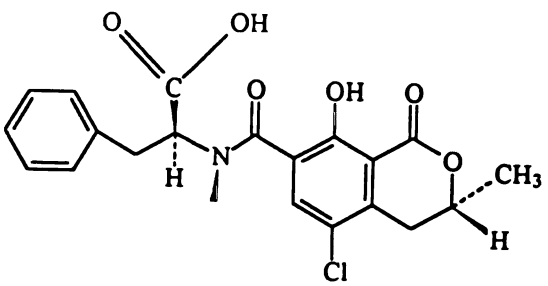
a. T-2 toxin



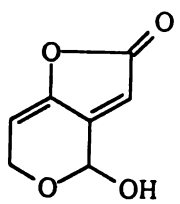
b. deoxynivalenol



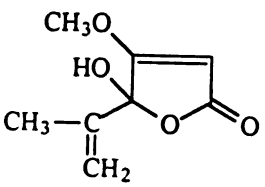
c. zearalenone



d.ochratoxin A



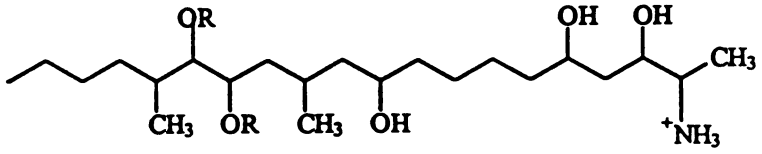
e. patulin

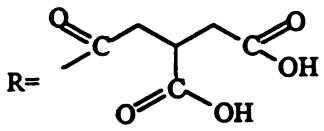


f. penicillic acid

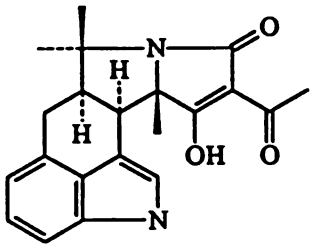
Figure 1. Structures of the major mycotoxins

Figure 1. (cont'd)

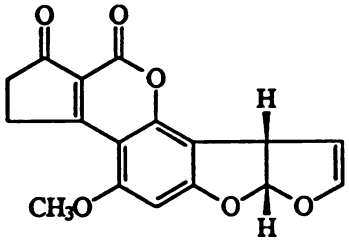




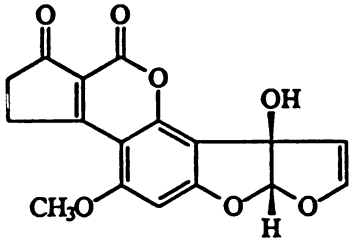
g. fumonisin B1



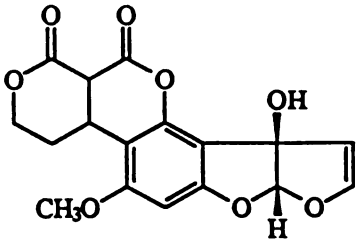
h. cyclopiazonic acid



i. aflatoxin B1



j. aflatoxin M1



k. aflatoxin G1

Myrothecium, and *Fusarium* sp. (Tamm and Tori, 1984). Among the more than 50 known trichothecenes, T-2 toxin (Figure 1. a) and deoxynivalenol (DON, vomitoxin) (Figure 1. b) are the most common.

In addition to aflatoxins, trichothecenes constitute the greatest threat to animal and human health (Joffe, 1978; Pestka and Casale, 1990). Several severe outbreaks of trichothecene contamination were reported during the years 1940 to 1970. Alimentary toxic aleukia (ATA) in Russia, red-mold disease in Japan, and moldy-corn toxicosis in the United States resulted in 6% to 20% mortality of affected humans and animals (Joffe, 1978). Recently, a survey conducted between 1970 to 1985 by several countries revealed that trichothecenes above the level of 1000 μg/kg in agricultural products were not uncommon (Jelinek et al., 1989). The frequent occurrence of trichothecenes in grains together with the finding that trichothecenes remain stable during food processing impose a great potential threat to human health.

The biological mode of action of trichothecences occurs via inhibition of protein synthesis (Pestka, 1995). Trichothecenes exert their toxicity especially on epithelial cells resulting in inflammation, desquamation, and general necrosis of the mouth and gastrointestinal mucosa. The general symptoms are indicated as vomiting, diarrhea, and food or feed refusal (Pestka and Casale, 1990). The most susceptible tissues to trichothecenes are actively dividing bone marrow, spleen, thymus, lymph nodes, and intestinal mucosa. The progression of the disease may result in leukopenia, agranulocytosis, sepsis, hemorrhaging, and exhaustion of the bone marrow. This differential susceptibility of tissues to trichothecenes was implicated in

the potential immunotoxicity of those toxins (Pestka, 1994). Several trichothecenes have been shown to cause both suppression and stimulation of lymphocyte proliferation in a dose-dependent manner in mice, rats, and humans. (Pestka and Bondy, 1994). Dietary DON caused an elevation of serum IgA in mouse and subsequently an increase in polymeric IgA, IgA immune complex, kidney mesangial IgA accumulation and hematuria, symptom analogous to IgA nephropathy in humans (Dong and Pestka, 1993). *In vitro* exposure of murine immunocompetent cells to vomitoxin was also shown to have effects on apoptosis of those cells and thereafter to cause immunosuppression (Pestka et al., 1994).

Zearalenones are a group of resorcyclic lactones (Figure 1. c) produced by *F. graminearum*, a pathogen on many small grains such as barley, oats, wheat, and corn. *F. graminearum* is also a producer of trichothecenes, indicating the potential of coproduction of zearalenone and trichothecenes (Jelinek et al., 1989). Zearalenone has also been identified in milk from dairy cows fed experimentally with zearalenone contaminated crops (Mirocha et al., 1981).

Zearalenone's major biological effect is hyperestrogenism in farm animals such as swine, cattle, and poultry. This includes vulval swelling, enlargement of the uterus, testicular atrophy, and mammary gland hyperplasia (Betina, 1984; Pestka and Casale, 1990). Zearalenone has also been implicated in infertility and abortion in farm animals.

Ochratoxins constitute a group of seven related metabolites with a dihydroisocoumarin moiety linked to phenylalanine via an amide bond (Figure 1. d).

Ochratoxin A, produced by certain strains of *A. ochraceus* and *P. viridicatum* (Steyn, 1984), is the most commonly occurring toxin in the group. Ochratoxin A is frequently detected in cereal grains in Scandinavian and Balkan countries and was implicated in endemic kidney disease in livestock, poultry, and human (Jelinek et al., 1989; Steyn, 1984). Ochratoxin A is only occasionally detected in crops in the United States. Of the most concern to human health, ochratoxin A can accumulate to a significant level in meat and poultry tissues used for human consumption (Pestka, 1995).

Ochratoxin A is a potential nephratoxin in farm animals as well as a renal and hepatic carcinogen in rats (Steyn, 1984; Kuiper-Goodman and Scott, 1989). The toxin possesses genetic activity indicated in DNA-adduct formation, DNA breakage and chromosomal aberration (Pfohl-Leszkowicz et al., 1993; Stark, 1980).

Patulin and Penicillic acid are two unsaturated lactones produced by *Aspergillus* and *Penicillium* (Figure 1. e, f). Patulin is commonly detected in fruit products, particularly in apple juice (Engel and Teuber, 1984). Therefore there is a potential health risk for humans exposed to this toxin via consumption of the juice made from the contaminated fruits. Both toxins are highly reactive and easily metabolized and detoxified once bound to the sulfhydryl group of the conjugation agent glutathione (GSH). Both patulin and penicillic acid are considered to be carcinogenic, antitumoral, and have antibiotic activity (Krivobok et al., 1994). Patulin is considered a mutagen, but the genotoxicity of penicillic acid is still under debate (Obana et al., 1994).

Fumonisins (Figure 1. g) are produced mainly by certain strains of *F. moniliforme* (Gelderblom et al., 1988). Fumonisins are synthesized through a polyketide pathway with inclusion of the amino acids methionine, glutamic acid, and serine. *F. moniliforme* is a prevalent fungus on corn, grain, and other agricultural commodities in the United States and throughout the world (Yoshizawa et al., 1994). Coexistence of AFB1 and FB1 was reported on field corn (Chamberlain et al., 1993). The concentrations of aflatoxin and fumonisin in the kernels are independent, in spite of the fact that infection by *A. flavus* and production of aflatoxin on corn was reported to be inhibited by preinoculated *F. moniliforme* (Zummo and Scott, 1992). This indicates that an additive or synergistic toxic response is possible.

Fumonisin B1 has been demonstrated to be the causative agent of equine leukoencephalomalacia. Ingestion of feeds or foods contaminated with *F. moniliforme* was also shown to cause porcine pulmonary edema and correlated with esophageal cancer in humans (Yoshizawa et al., 1994; Thiel et al., 1991). Fumonisin B1 is likely to exert its carcinogenic effect by inhibiting enzyme activity in the synthesis and turnover of sphingolipids, which may play important roles in the structural and regulatory integrity of cells (Wang et al., 1991; Chamberlain et al., 1993).

Cyclopiazonic acid (CPA), an indole tetramic acid (Figure 1. h), is produced by certain species of *Aspergillus* and *Penicillium* including *A. flavus*, *A. oryzae*, *A. versicolor* and *P. patulum*. Cyclopiazonic acid is mainly produced by fungi colonizing corn and peanuts. Recently, a concern was raised about the potential of human exposure to CPA through consumption not only of contaminated corn and

peanuts, but also animal tissue, eggs, and milk from CPA intoxicated animals (Le Bars, 1979; Dorner et al., 1994; Pestka, 1995).

Toxicity studies of CPA were conducted thoroughly by Voss (Voss, 1990). CPA was shown to cause degenerative, necrotic or inflammatory lesions of the liver, kidney, spleen, alimentary tract, lymphoid tissue, and skeletal muscle in rats, chickens, dogs, guinea pigs, swine, and monkeys. CPA exerts its toxic effects mainly through the inhibition of cellular Ca^{++} transport systems and protein synthesis (Bryden, 1991).

Though most mycotoxins are considered carcinogenic, they are not all mutagenic. T-2 toxin, zearalenone, vomitoxin, cyclopiazonic acid, and penicillic acid are not mutagenic in the Ames test (Stark, 1980); however, zearalenone and penicillic acid have been implicated in genotoxicity in the Rec assay using *Bacillus subtilis* as the tester strain (Ueno and Kubota, 1976).

Mycotoxins are secondary metabolites

Mycotoxins are toxic secondary metabolites produced by fungi. Secondary metabolites are structurally diverse organic compounds produced by some plants, fungi, algae, and bacteria during late growth phase (idiophase) (Campbell, 1984). In comparison with primary metabolites, secondary metabolites are characterized by their restricted distribution in nature (not commonly produced) and their lack of turnover (no catabolic processes). They also are not essential for the growth of the producing organisms. Alkaloids, antibiotics (Martín and Liras, 1989), mycotoxins, spore pigments (Wheeler and Bell, 1992), though largely derived from different biosynthetic

pathways, are all secondary metabolites.

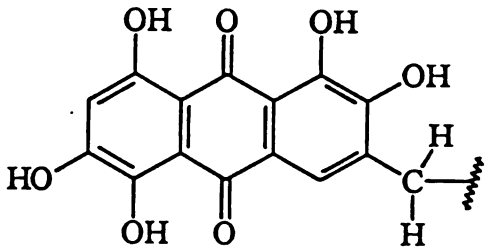
In regard to their ecological and evolutionary roles, it has been proposed that the production of secondary metabolites may play a role as an "escape valve" to release the accumulation of metabolites built up from primary metabolism (Decan, 1984). In spite of the proposition described above, it was also proposed that microbial secondary metabolites provide an advantage (competitive benefit) to the producing organisms (Lillehoj, 1982; Deacon, 1984; Maplestone et al., 1992). Maplestone (1992) proposed several aspects to support the beneficial roles of secondary metabolites: (1) secondary metabolites are usually produced by organisms (bacteria, algae, plants, and fungi) which lack immune systems, indicating the potential of secondary metabolites being alternative defense mechanisms; (2) numerous secondary metabolites have physiological functions such as antibiotic activity, antifungal activity, or are components of the fungal cell wall (melanin), indicating the contribution of secondary metabolites in microbial survival; (3) the genetic complexity of the biosynthetic pathways of secondary metabolites indicates that they might have evolved in favor of the producing organisms; (4) the clustering of the genes involved in secondary metabolism indicates that these genes may have evolved as a unit allowing coordinate regulation to facilitate their production. This in turn would confer a selective advantage to the producing organism.

Secondary metabolites are related to fungal development, morphorgenesis, and survival

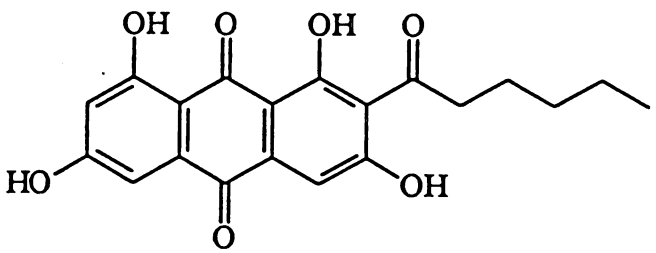
Secondary metabolites, including mycotoxins, may be related to the development, morphogenesis, and survival of the producing organisms (Campbell, 1984). Evidence to support the relationship between secondary metabolism and fungal/bacterial development may be observed in a morphological differentiation phase leading fungi or bacteria from vegetative growth to asexual reproduction. This transition phase is generally known as sporulation (Hopwood, 1988; Cannon et al., 1994; Timberlake, 1990). The overall processes of sporulation are different between distinct organisms. In *Aspergillus*, sporulation consists of formation of foot cells, aerial hyphae, conidiophores, metula, philalides, and asexual spores (conidia). Sporulation is generally considered as a response to depletion of nutritional supply (Timberlake, 1990; Springer, 1993) or a programmed part of the life cycle (Adams et al., 1992). The genetic mechanisms governing sporulation were partially revealed in *N. crassa* (Springer, 1993), *A. nidulans* (Timberlake, 1980; Boylan et al., 1987; Timberlake, 1990; Timberlake, 1993; Andrianopoulos and Timberlake, 1994), and *Streptomyces* (Hopwood, 1988). Most of the sporulation associated genes which have been identified are developmentally regulated and expressed at the onset of or during idiophase, a characteristic of secondary metabolism associated genes (Boylan et al., 1987; Miller et al., 1991; Prade and Timberlake, 1993). In this regard, sporulation *per se* links fungal/bacterial secondary metabolism to fungal/bacterial morphological differentiation.

Other indications of potential cross-connections between secondary metabolite production and fungal/bacterial morphogenesis are mainly derived from genetic studies. Both *afs*B and *bldA* are genes associated with sporulation in *S. coelicolor* A3(2). Loss of function mutations in the *bld*A gene abolish both aerial hyphae formation and biosynthesis of secondary metabolites such as actinorhodin, methylenomycin, and undecylprodigiosin. A similar effect was observed in *S. griseus* in which disruption in an A-factor associated gene (*afs*A) resulted in inhibition of both sporulation and streptomycin production (Miyake et al., 1990). These pleiotropic effects observed in *S. coelicolor* A3(2) and *S. griseus* indicate the close relationship between secondary metabolite production and morphogenesis (Hopwood, 1988). In a review by Campbell (1984), several uncharacterized secondary metabolites were also implicated in induction of aerial hyphae formation or sporulation in various bacterial and fungal species.

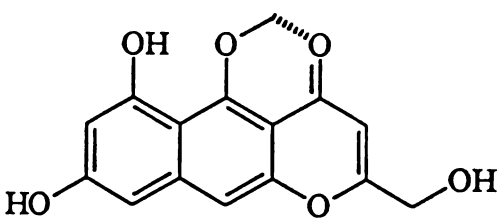
The implicit relationship between secondary metabolite production and fungal morphogenesis is also demonstrated by spore pigment formation. A striking structural similarity was observed between fungal spore pigments and polyketide-derived secondary metabolites. The structural similarity between ascoquinone A (Figure 2), a sexual spore pigment of *A. nidulans*, and norsolorinic acid, the first stable intermediate in the aflatoxin pathway, indicates that ascoquinone A and norsolorinic acid are likely to originate from similar polyketide-derived pathways (Brown and Salvo, 1994). An orange conidial pigment, parasperone A (Figure 2), which is considered a precursor of the final green spore pigment in *A. parasiticus*, was isolated



ascoquinone A



norsolorinic acid



parasperone A

Figure 2. Structures of the pigments ascoquinone A, parasperone A, and norsolorinic acid.

from a ρ-diphenol oxidase deficient strain of *A. parasiticus* (Brown et al., 1993). This pigment is also proposed to be derived from a PKS-generated heptaketide.

The close relationship between secondary metabolism and fungal/bacterial morphogenesis is also supported by several studies which compared nucleotide sequences or polypeptide sequences of genes or gene products involved in these processes (Blanco et al., 1993). The nucleotide sequence of the developmentally regulated spore-specific genes *wA* in *A. nidulans* and *whiE* in *S. coelicolor* A3(2) have been reported (Mayorga and Timberlake, 1992; Davis and Chater, 1990). Comparison of the deduced amino acid sequences of the *whiE*-encoded polypeptide with other reported polypeptides revealed significant similarity between *whiE* encoded polypeptide and polypeptides involved in synthesis of the antibiotics tetracenomycin and granaticin. Two extended regions of similarity were observed between the predicted *wA* polypeptide, the *eryA* polypeptide, and MSAS. The *eryA* gene from *Saccharopolyspora erythraea* encodes a polyketide synthase (PKS) involved in the production of the antibiotic erythromycin and the MSAS gene from *P. patulum* encodes a PKS involved in the synthesis of the mycotoxin patulin. High degrees of similarity (80%) and identity (64%) were also observed between a 100 amino acid stretch in the ACP domain (acyl carrier protein) of the *wA* polypeptide and the gene-1 (*pksA*) protein in *A. parasiticus* (Trail et al., 1995a). Gene-1 encodes a putative PKS involved in aflatoxin biosynthesis (described below).

Aflatoxin synthesis and fungal morphogenesis may be associated in certain toxigenic strains of *A. flavus* and *A. parasiticus*. A survey conducted by Torres et al.

(1980) indicates a correlation between diminution of aflatoxin production in *A. flavus* and *A. parasiticus* and decrease in the production of sclerotia and conidia as well as an increase in vegetative hyphae formation after successive subculturing (Torres et al., 1980). A similar observation for *A. parasiticus* was also reported by Bennett (Bennett, 1985). In a survey conducted on 70 *A. flavus* isolates from the Arizona desert, Cotty (1989) observed that strains producing more sclerotia have the tendency to produce more aflatoxins in culture. A recent survey performed in our laboratory indicates that disruption of *nor*-1 and early aflatoxin pathway genes, i.e. gene-1 and *uvm*8 (described in the following section), results either in accumulation of norsorlorinic acid or no intermediates, respectively. These strains which accumulate early pathway intermediates, NA, or no pathway intermediate (*pks*A and *uvm*8 disruptants) had wild-type level of sclerotia production. Accumulation of the intermediates between norsorlorinic acid (NA) and versicolorin A (VA) in *A. parasiticus* occurs concurrently with inhibition of sclerotium development (Trail et al., 1995a; N. Mahanti. unpublished data). Production of the mycotoxin zearalenone has been reported to have an effect on the formation of perithecia in *Gibberella zeae* (the sexual stage of *F. graminearum*) (Wolf and Mirocha, 1977). However, a study performed by Wang points out that there is no linear correlation between the amount of aflatoxin production and the number of sclerotia produced in certain strains of *A. flavus* (Wang et al., 1993).

Fungal secondary metabolites may play a role not only in morphogenesis but also survival of the producing organisms. Spore pigments, such as ascoquinone A and

parasperone A as described, are all polyketide derived phenolic melanins. Melanins, extracellular or intracellular, play numerous roles in fungal growth and survival (Wheller and Bell, 1992). Melanins can inhibit hydrolytic enzymes secreted by soil microorganisms, provide resistance against environmental stress, and absorb U.V light to prevent damage. Some fungal melanins are also implicated in the pathogenicity and virulence in plants (for a review, see Wheeler and Bell, 1992).

Characteristics of aflatoxins

Aflatoxins are difuranocoumarin derivatives (Figure 1. i, j, k) produced by certain strains of *Aspergillus flavus* and *A. parasiticus* (Maggon et al., 1977; Calvert et al., 1978). *A. nomius* is another *Aspergillus* capable of producing aflatoxins (Kurtzman, 1987). *A. nomius* is morphologically similar to *A. flavus* but produces both aflatoxin G and B. Occasionally, reports indicated that other strains of fungi also produced aflatoxins, although these reports were never confirmed (Kulik and Holaday, 1967; Leitao, et al., 1989).

Aflatoxins are mostly found in agricultural products such as peanuts, cottonseed, corn, and cassava (Busby and Wogan, 1984). Occasionally, contamination of aflatoxins on almonds, walnuts, rye, wheat, and sorghum was also reported (Pohland and Wood, 1991). The contamination of aflatoxins often occurs after severe stress to the crops. Stress conditions include insect infection, severe drought before harvest (preharvest), high humidity during storage (postharvest), as well as physical damage via inappropriate agronomic practices during harvesting (Cole, et al., 1982;

Wicklow et al., 1988; Bowen and Mack, 1991; Cotty, 1991; Wicklow et al., 1991).

Of the mycotoxins, aflatoxins earn the most concern because they are the most potent naturally occurring carcinogens studied so far (described below). The first severe outbreak of aflatoxin contamination dated back to the early 1960s when one hundred thousand turkey poults died from consumption of aflatoxin contaminated corn (Buchi and Rae, 1969). Several other outbreaks of severe aflatoxin contamination were reported following the 1960 incidence. An outbreak of human acute aflatoxicosis in 1972 caused 106 deaths in India. In 1983, a year when the midwest regions of the U.S. were confronted with severe drought weather, the average aflatoxin level in corn was elevated to 80 ppb (Jelinek et al., 1989), significantly higher than the tolerance level set at 20 ppb. These observations suggest that aflatoxins, like the other mycotoxins described above, remain as a continuous potential threat to human health and are an economic problem throughout the world. However, aflatoxin contamination in agricultural products and subsequently in the food chain is primarily an economic concern in the United States because of the strict regulations enforced by FDA.

Factors affecting aflatoxin biosynthesis

The production of aflatoxins is influenced by a variety of factors including chemical, biological, and environmental factors (Zaika and Buchanan, 1987). Understanding of the relationship between those factors and aflatoxin production can help generate strategies to control the occurrence of aflatoxins.

Chemical factors which support or suppress aflatoxin synthesis have been studied in laboratory conditions. Two critical chemical factors that contribute to high yields of aflatoxins are: (1) elevated levels of available carbon relative to nitrogen, and (2) requisite levels of certain trace elements. Generally, carbon sources catabolized through the glycolytic pathway and pentose-phosphate shunt are good for aflatoxin production (Buchanan and Lewis, 1984; Abdollahi and Buchanan, 1981a; Buchanan et al., 1987). Saturated fatty acids, amino acids (except aspartic acid, asparagine, alanine, and glutamine), terminal compounds of glycolysis (pyruvate and acetate), as well as TCA cycle intermediates are not supportive substrates (Davis and Diener, 1968, Abdollahi and Buchanan, 1981b; Niehaus and Jiang, 1989; Luchese and Harrigan, 1993). In the case of the nitrogen source, nitrate has been shown to repress aflatoxin production whereas ammonium is an aflatoxin supportive nitrogen source (Kachholz and Demain, 1983; Niehaus and Jiang, 1989).

Organic acids such as propionic acid and sorbic acid were reported to inhibit fungal growth and aflatoxin production (Beuchat and Gloden, 1989; Ghosh and Haggblom, 1985). Ammonium bicarbonate also has an inhibitory effect against aflatoxin production (DePasquale and Montville, 1990; Montville and Shih, 1991), as does caffeine, an antifungal agent which occurs naturally in foods such as cocoa and coffee beans (Northolt and Bullerman, 1982; Buchanan et al., 1983). As for the trace elements, specific concentrations of Cd^{2+}, Mg^{2+}, and Zn^{2+} have been found to enhance aflatoxin production (Niehaus and Jiang, 1989).

The biological factors which affect aflatoxin synthesis, include the host plants,

strain variability, and inoculum size of the competing microflora (Ellis et al., 1991). The effects of competing microflora on aflatoxin production will be discussed in the section "use of nontoxigenic biocontrol agents".

The environmental factors which affect aflatoxin synthesis include temperature, water activity (A_w), atmospheric gases, light, and pH (Jarvis, 1971; Moreno et al., 1987; Ellis et al., 1991). Of those factors described above, temperature and A_w are the most important factors affecting aflatoxin production. Generally, the optimal temperatures for aflatoxin production range from 25 to 30°C at a relative moisture of 88% to 99% (Northolt and Bullerman, 1982).

Aflatoxin is synthesized via a polyketide biosynthetic pathway

Aflatoxins are secondary metabolites synthesized through a polyketide biosynthetic pathway. An early intermediate in the aflatoxin biosynthesis is believed to be an anthrone decaketide (Figure 3), which is synthesized from an acetyl starter unit with successive addition of 9 malonyl extenders (Dutton, 1988).

Polyketide-derived secondary metabolites are a large, structurally diverse class of natural products produced by some bacteria (mostly actinomycete), fungi, and plants (for a review, see Martín and Liras, 1989; Hopwood and Sherman, 1990; Hopwood and Khosla, 1992; Katz and Donadio, 1993). A number of bacterial and fungal spore pigments (Davis and Chater, 1990; Mayorga and Timberlake, 1993), as well as plant-derived flavonoids (flower pigments and phytoalexins; Stoessl, 1982) are examples of polyketide derived secondary metabolites. Antitumor agents or

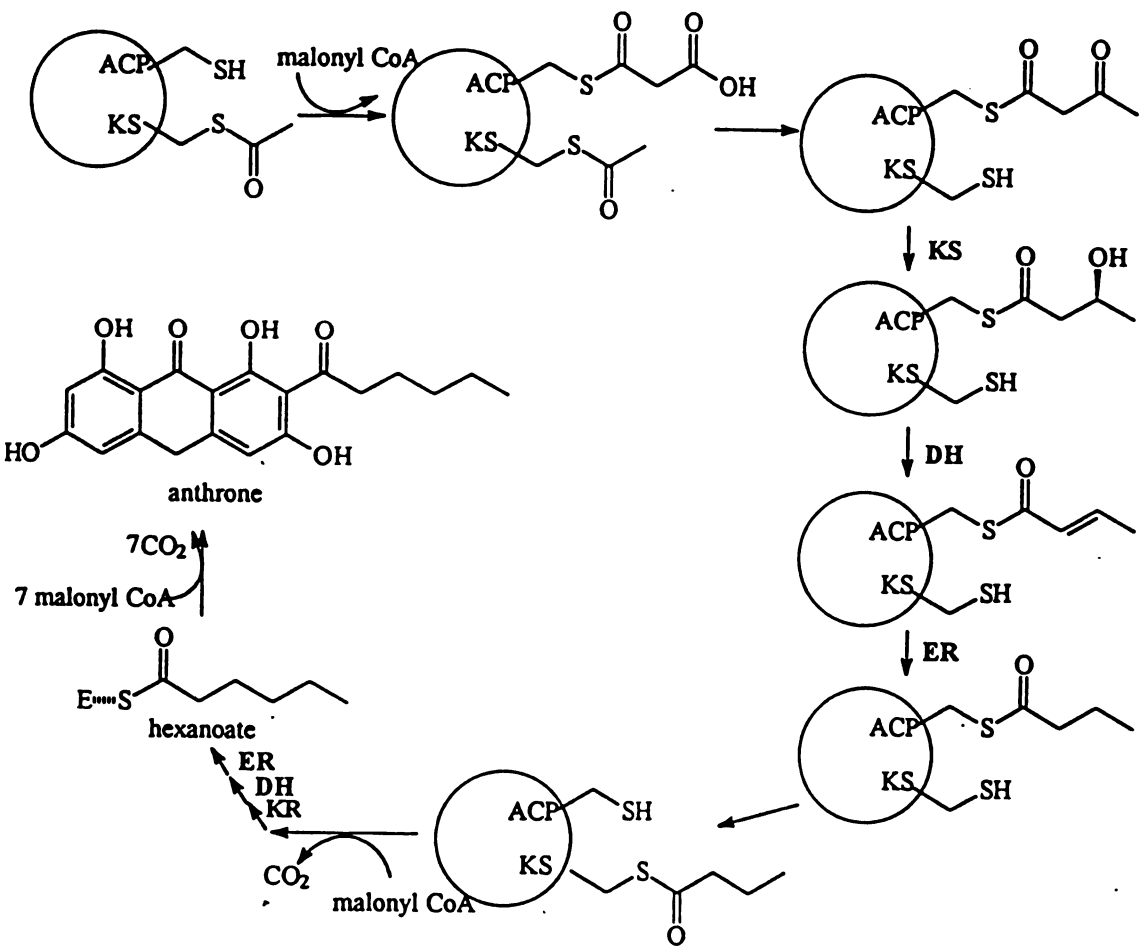


Figure 3. Schematic representation for the assembly of the decaketide noranthrone by a polyketide biosynthetic pathway. ACP, acyl carrier protein; KS, β-ketoacyl synthase; DH, dehydrase; ER, ketoreductase; KR, ketoreductase.

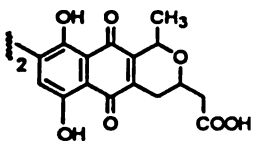
antibiotics, which have great value in the pharmaceutical industry and veterinary medicine, such as erythromycin (Cortes et al., 1990; Donadio et al., 1991; Bevitt et al., 1992), tetracenomycin (Motamedi and Hutchinson, 1987), and streptomycin (Figure 4) (Ohnuki et al., 1985) are polyketides produced by *Streptomyces*. In addition, mycotoxins such as zearalenone, ochratoxin, patulin, penicillic acid and aflatoxins are also polyketide derived secondary metabolites. The polyketide nature of numerous antibiotics and mycotoxins makes polyketides one of the most important classes of naturally occurring secondary metabolites.

Polyketides arise from self-condensation of the starter unit and extender units in a head-to-tail fashion (Figure 3). The organisms producing polyketides generally utilize acetate, propionate, or butyrate as the starter or extender units. The successive addition of carboxylic acid from the extenders and the alternate occurrence of the keto groups in the polyketide carbon chain give rise to the name "polyketide".

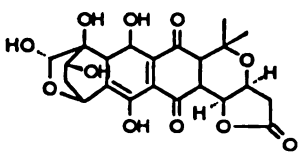
Polyketide biosynthetic pathway and fatty acid biosynthesis

Polyketide synthase (PKS) systems are similar to fatty acid synthase (FAS) systems in many features. FAS is a multivalent system consisting of eight functional units; acetyl, malonyl, and palmityl transferase, ACP (acyl carrier protein), ketoacyl synthase (condensing enzyme), ketoacyl reductase, dehydratase and enoyl reductase. In most bacteria and plants, these eight functional units occur as eight separate polypeptides which form a multienzyme complex (type II FAS) (Figure 5) (Cortes et al., 1990; Sherman et al., 1989). The genes encoding these polypeptides are usually clustered

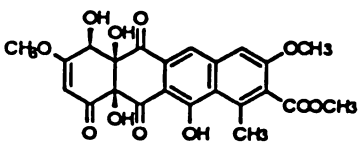
S. coelicolor
actinorhodin



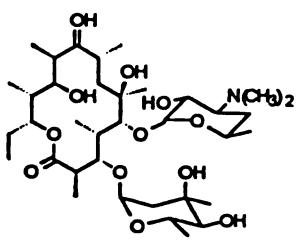
S. violaceoruber
granaticin



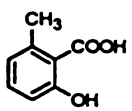
S. glaucescens
tetracenomycin



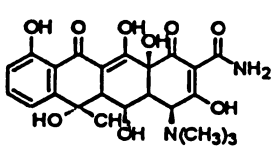
Saccharopolyspora erythraea
erythromycin D



P. patulum
6-methylsallicylic acid



S. rimosus
oxytetracycline



S. roseofulvus
frenolicin

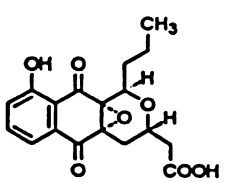


Figure 4. Structures of polyketide-derived secondary metabolites. The producing organisms and corresponding antibiotics are indicated. For details see text.

together in the genome to form one or several repeated motifs. In vertebrates, a single, multifunctional polypeptide carries the same eight functions (type I FAS). Yeast represents an intermediate case in which two unlinked FAS genes, FAS1 and FAS2, encode α-subunit and β-subunit polypeptides, respectively. These polypeptides aggregate to form a $\alpha_6\beta_6$ FAS. PKS systems share common structural and functional motifs but differ from FAS systems in at least two aspects: (1) the cycle in the step of ketoreduction-dehydration-enoyl reduction that follows every two-carbon addition in fatty acid biosynthesis is omitted, or curtailed, at some or all points in the polyketide chain; (2) many PKS select starter unit and successive extender units from a range of possibilities, whereas the FAS is constrained to use an acetate unit as starter and a malonate unit as extender.

Genetic organization and programming of polyketide biosynthetic pathways

The structural and functional diversity of the polyketides is mainly determined in the genetic organization of the PKS genes and programming of the biosynthetic pathway (Cane, 1994). The gene organization for numerous PKS in actinomycetes and fungi has been partially elucidated. Among numerous structurally determined aromatic polyketides, actinorhodin (Malpartida and Hopwood, 1986; Caballero et al., 1991), granaticin (Sherman et al., 1989), tetracenomycin (Motamedi and Hutchinson. 1987), oxytetracycline (Butler et al., 1989), and frenolicin (Figure 5) showed significant similarity in polypeptide sequences between the PKS counterparts and in the sequential order of the PKS genes in the genome (Figure 5). These aromatic

Figure 5. Organization of genes encoding several polyketide synthases. Arrows indicate the transcriptional directions in the corresponding genome. For details see text (adapted from Hopwood and Khosla, 1992 and Katz and Donadio, 1993). Abbreviations: ACP, acyl carrier protein; KS, ß-ketoacyl synthase; DH, dehydrase; ER, enoyl reductase; KR; ketoreductase; OMT, O-methyltransferase.

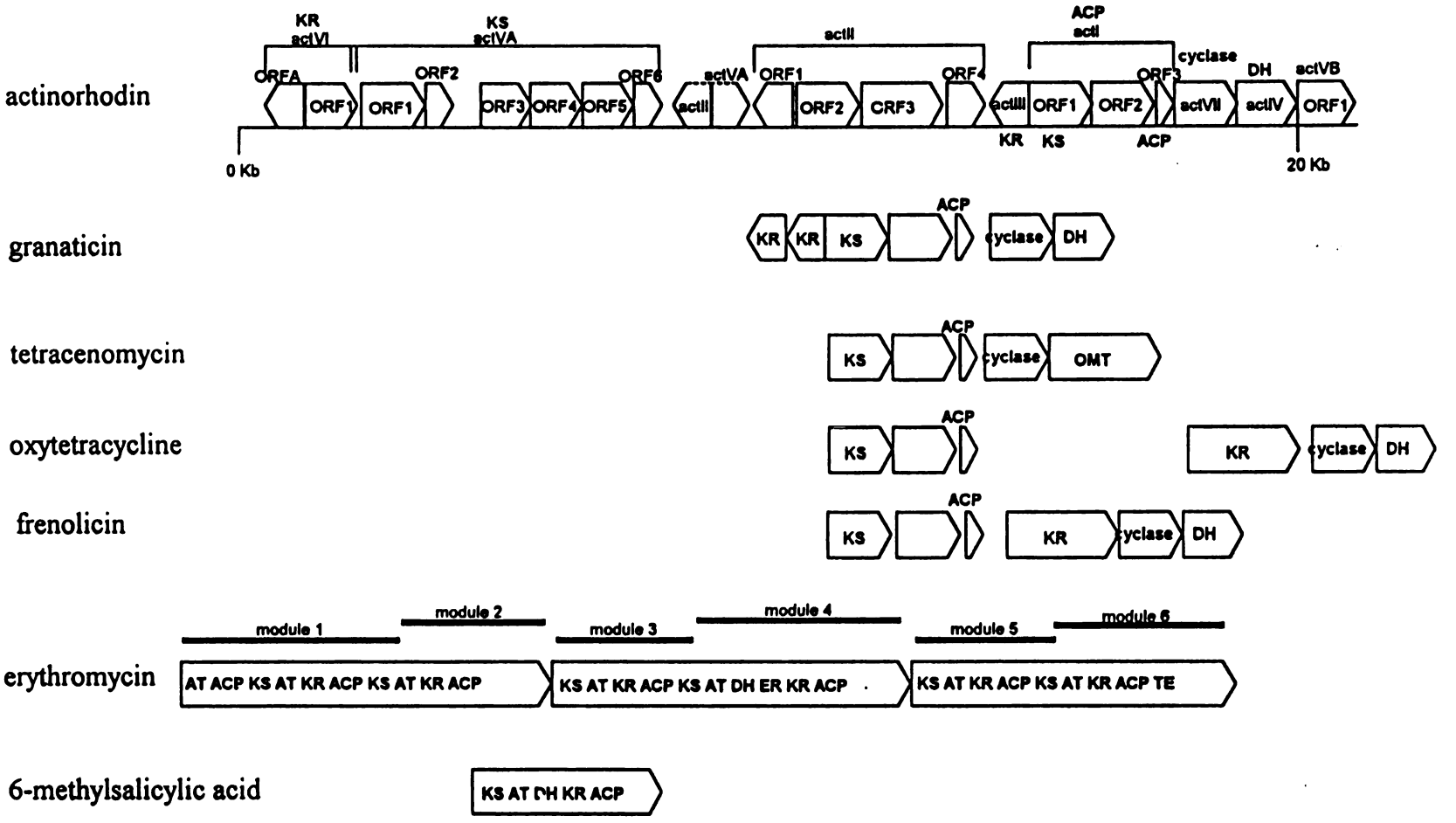

actinorhodin
KR actVI
KS actVA
actII
ACP actI
cyclase
DH
actVB
ORFA
ORF1
ORF1
ORF2
ORF3
ORF4
ORF5
ORF6
actVA
ORF1
ORF2
CRF3
ORF4
actIII
ORF1
ORF2
ORF3
actVII
actIV
ORF1
KR
KS
ACP
0 Kb
20 Kb
granaticin
KR
KR
KS
ACP
cyclase
DH
tetracenomycin
KS
ACP
cyclase
OMT
oxytetracycline
KS
ACP
KR
cyclase
DH
frenolicin
KS
ACP
KR
cyclase
DH
module 1
module 2
module 3
module 4
module 5
module 6
erythromycin
AT ACP KS AT KR ACP KS AT KR ACP
KS AT KR ACP KS AT DH ER KR ACP
KS AT KR ACP KS AT KR ACP TE
6-methylsalicylic acid
KS AT DH KR ACP

Figure 5.

polyketide encoding enzymes are classified as type II PKS (multiple subunits). On the other hand, the genes encoding enzymes leading to the production of complex macrolide polyketides such as erythromycin (Cortes et al., 1990; Donadio et al., 1991) and avermectin (MacNeil et al., 1992), as well as fungal 6-methylsalicylic acid (Beck et al., 1990; Wang et al., 1990) are grouped into one to four open reading frames (Figure 5). These multifunctional polypeptides are classified as type I PKS. Each of the open reading frames contains two modules which are responsible for completing one condensation and reduction cycle per module. The programming of the PKS determines the final carbon chain length, the choice of the extender and the processing of each β-carbonyl carbon. To make the final polyketide, type II PKS enzymes with a single set of functional domain are used iteratively. Although evidence showed that the chain length of actinorhodin, granaticin, and tetracenomycin is in part determined by the functionality of ORF2 (McDaniel et al., 1993), the rules of programming of type II PKS remain obscure. The strategy governing programming of the complex PKS (type I PKS) is more thoroughly understood. Although different models have been proposed (Bevitt et al., 1992), Donadia et al. (1991) proposed a more or less widely accepted concept based on the colinerity between the order of the modules and the synthetic steps that they govern. In this concept, each condensation cycle and the extent of β-keto reduction is governed by the functionalities in the corresponding module.

Recently, the PKS gene potentially involved in the production of aflatoxins has been identified in *A. parasiticus*. This gene was later designated as *pks*A (gene-1)

(Trail et al, 1995b; Chang et al., submitted). High degrees of similarity (80%) and identity (64%) were observed between a 100 amino acid stretch in the *pks*A protein and the ACP domain of *A. nidulans wA*, a PKS involved in spore pigment biosynthesis. In addition, a distinct region in the *pks*A protein showed 20% to 32% identity with the acyltransferase domain in *A. nidulans wA*. A recent study showed that *pks*A is closely linked to numerous aflatoxin biosynthetic genes in the genome of *A. parasiticus* (Trail et al, 1995b). Recombinational inactivation of *pks*A through homologous recombination resulted in a strain which did not produce any known aflatoxin pathway intermediates. This genetic evidence indicates that *pks*A is a PKS gene which is involved in the formation of an anthrone decaketide intermediate in the aflatoxin biosynthetic pathway. This evidence also supports the hypothesis that aflatoxins are synthesized through polyketide biosynthetic pathway which is followed by postpolyketide modifications (Dutton, 1988). The postpolyketide modifications in aflatoxin biosynthesis are described below.

Aflatoxin biosynthetic pathway

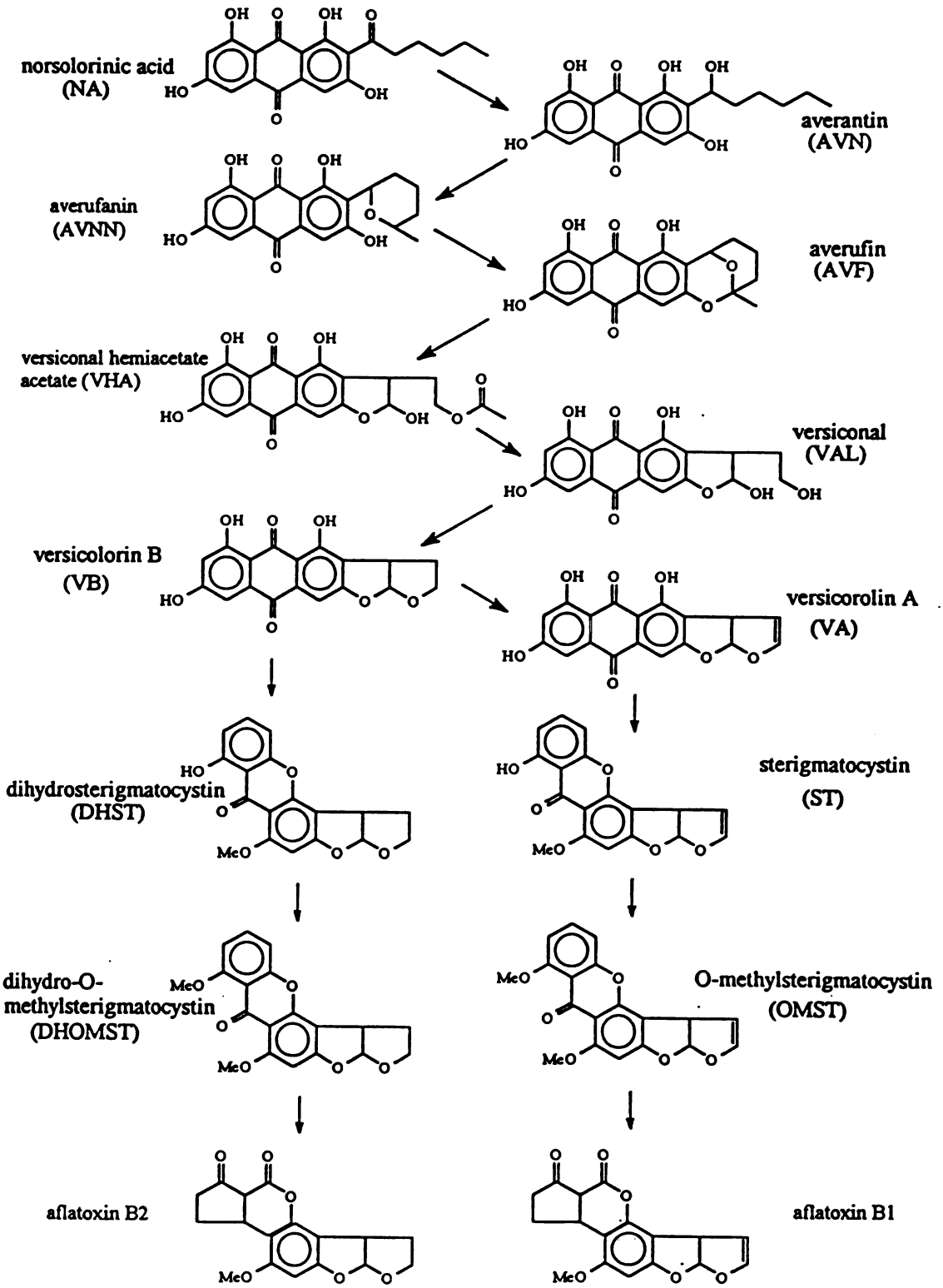
The aflatoxin biosynthetic pathway has been partially elucidated in *A. flavus* and *A. parasiticus* by using a combination of blocked mutants (mutant strains accumulating aflatoxin pathway intermediates), metabolic inhibitors, and radioactive metabolite feeding studies (for a review, see Bhatnagar and Cleveland, 1991). The commonly accepted metabolic scheme of the pathway is: C20 polyketide → noranthrone → norsolorinic acid (NA) → averantin (AVN) → averufanin (AVNN) → averufin (AVF)

→ hydroxyversicolorone → versiconol hemiacetate acetate (VHA) → versiconol (VAL) → versicolorin B (VB) → versicolorin A (VA) → demethylsterigmatocystin (DMST) → sterigmatocystin (ST) → O-methylsterigmatocystin (OMST) → aflatoxin B_1 (AFB1) (Figure 6). Aflatoxin B_2 (AFB2) was proposed to be synthesized via a branch in the AFB1 pathway beginning with VB which is converted to hydroxydihydro-sterigmatocystin (DHST) and dihydro-O-methylsterigmatocystin (DHOMST) and AFB2 (Dutton et al., 1985; Cleveland et al., 1987).

Various aflatoxin-blocked mutant strains have been generated and most of the aflatoxin biosynthetic pathway intermediates have been identified. In addition, several enzymes involved in the bioconversion of norsolorinic acid (NA) to aflatoxins were purified (Bhatnagar et al., 1992; Bhatnagar et al., 1988; Chuturgoon and Dutton, 1991; Hsieh et al., 1989; Keller et al., 1993; Lin and Anderson, 1992; Yabe et al., 1991a; Yabe et al., 1989; Yabe et al., 1991b). Among these enzymes, only a few were purified to homogenecity due to their low abundance and short-lived nature (Bhatnagar et al., 1988; Bhatnagar and Cleveland, 1990; Lin and Anderson, 1992; Keller et al., 1993). Although the aflatoxin metabolic pathway has been elucidated, only recently several genes involved in the aflatoxin biosynthesis have been cloned and characterized in toxigenic strains of *A. flavus* and *A. parasiticus*. The *nor*-1 gene, involved in the conversion of NA to AVN, and a *ver*-1 gene, involved in the conversion of VA to demethylsterigmatocystin, were cloned separately via functional complementation of aflatoxin blocked mutants of *A. parasiticus* (Chang et al., 1992; Skory et al., 1992). Genetic complementation in this instance is briefly defined as a

Figure 6. Schematic representation of the proposed aflatoxin biosynthetic pathway in *A. flavus* and *A. parasiticus* (adapted from Trail et al, 1995).

Figure 6.


norsolorinic acid
(NA)
averantin
(AVN)
averufanin
(AVNN)
averufin
(AVF)
versiconal hemiacetate
acetate (VHA)
versiconal
(VAL)
versicolorin B
(VB)
versicorolin A
(VA)
dihydrosterigmatocystin
(DHST)
sterigmatocystin
(ST)
dihydro-O-
methylsterigmatocystin
(DHOMST)
O-methylsterigmatocystin
(OMST)
aflatoxin B2
aflatoxin B1

process consisting of transformation of a genomic DNA library (genomic information cloned into shuttle vectors as thousands of short pieces of DNA) into aflatoxin blocked mutant strains to restore the functionality of the nonfunctional genes and resume the production of aflatoxins. A regulatory gene, *afl*-2, in *A. flavus* was also cloned via a similar strategy (Payne et al., 1993). The *apa*-2 gene in *A. parasiticus*, which was determined to be an analog to *afl*-2, was cloned on the basis of overproduction of aflatoxin pathway intermediates following transformation of a cosmid DNA to an OMST accumulating strain of *A. parasiticus* (Chang et al., 1993). Based on the sequence identity and functional interchangeability between *afl*-2 and *apa*-2, these two homologous genes were referred to as *afl*R (Woloshuk et al., 1994). The *omt*-1 gene, involved in the conversion of ST to OMST, was cloned by screening a cDNA library using polyclonal antibodies raised against the purified O-methyltransferase protein (Yu et al., 1993a; Keller et al., 1993). This cDNA library was constructed from RNA isolated from a wild type toxigenic strain of *A. parasiticus*.

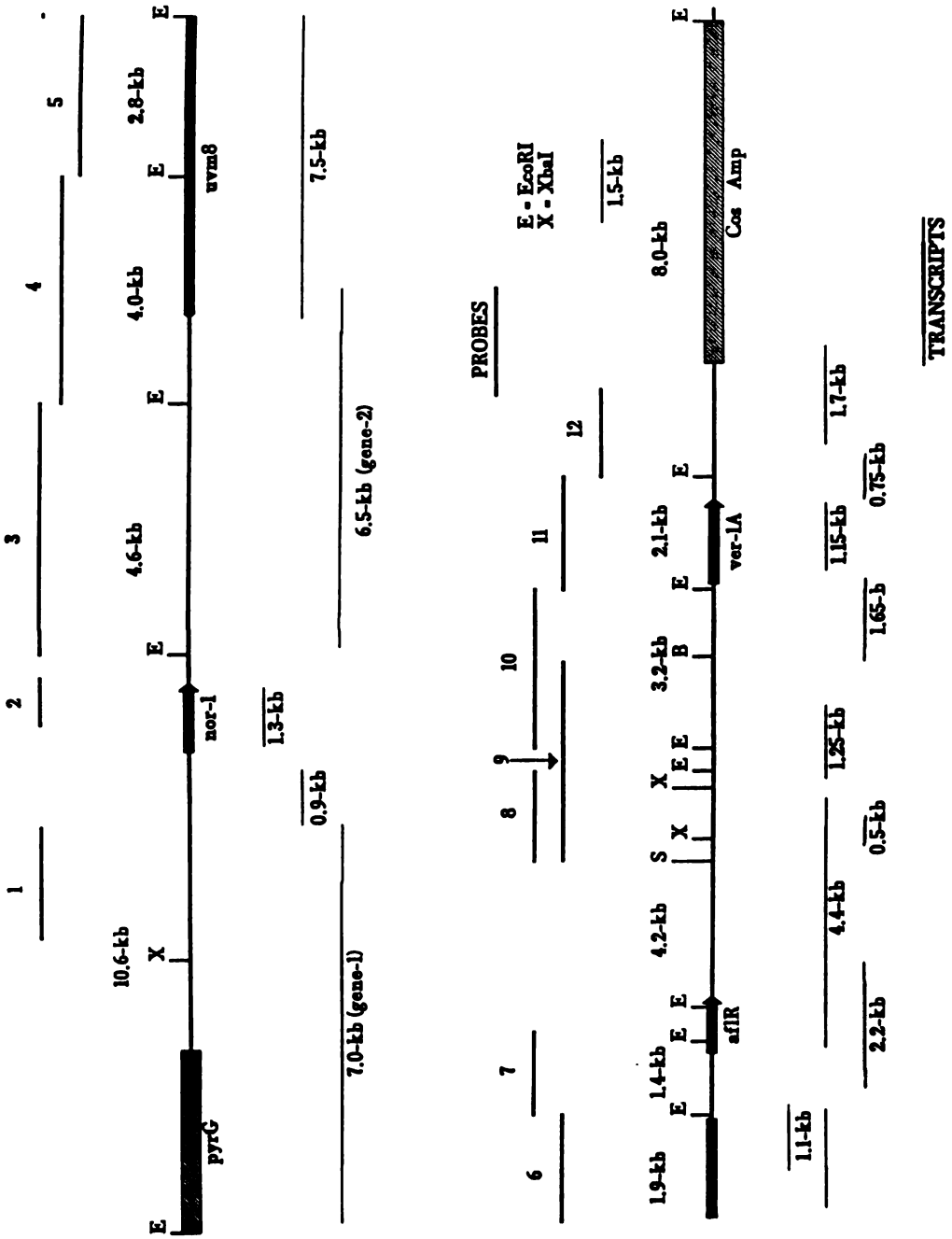
The *nor*-1, *ver*-1, *apa*-2/*afl*-2, and *omt*-1 genes were shown to be part of a gene cluster in the *A. parasiticus*/*A. flavus* genome (Figure 7) (Trail et al., 1995a; Trail et al, 1995b). Genes with cooperative functions are occasionally located adjacent to each others in a linkage group in the genome (gene cluster). Recently, the *uvm*8 gene and *pks*A (gene-1) were also identified in the same gene cluster as *nor*-1, *ver*-1, and *afl*R. By amino acid sequence comparison with other known polypeptides and recombinational inactivation studies (gene disruption), *uvm*8 and *pks*A were proposed

to encode enzymes with potential FAS and PKS activities, respectively (Mahanti et al., 1994; Trail et al., 1995a). The same studies also showed that these two genes are essential for aflatoxin biosynthesis.

In order to firmly establish the functionality of the cloned aflatoxin pathway genes, recombinational inactivation of *nor*-1 (Trail et al., 1994), *ver*-1 (Liang and Linz, 1994), and *uvm*8 (Mahanti et al., 1995) was performed using a similar strategy to that established in a model system described in this dissertation (Wu and Linz, 1993). In this model system, mitotically stable disruption of the *nia*D gene was successfully achieved through homologous recombination by either one-step gene replacement or integration via a truncated cloned gene in *A. parasiticus*. In addition, the functional roles of *nor*-1 and *omt*-1 were further established by comparing their predicted protein sequences with other known protein sequences and through enzymatic activity studies using fusion proteins expressed in *E. coli* (Yu et al., 1994; Zhou and Linz, 1994). The cloning, identification, and characterization of these aflatoxin associated genes is fundamental to the understanding of the regulation of aflatoxin production which may eventually lead us to establish strategies for control of toxin production. Experimental studies on production of aflatoxin and several other PKS secondary metabolites suggest the following: (1) aflatoxin production is in part regulated at the transcriptional level; (2) aflatoxin biosynthesis may be subject to coordinate regulation; (3) the phenomenon of clustering of aflatoxin pathway genes may be related to the regulation of toxin production.

Figure 7. Restriction endonuclease and transcript map of cosmid NorA. The size and location of transcripts are shown. The location of genes and the direction of transcription are indicated when information is available. The location of several restriction endonuclease sites is also indicated. E: *Eco*RI; X: *Xba*I; S: *Sac*I; and B: *Bam*HI (adapted from Trail et al., 1995a).

Figure 7.


PROBES
E - EcoRI
X - XbaI
TRANSCRIPTS
pyrG
nor-1
uvm8
ver-1A
aflR
Cos
Amp
10.6-kb
4.6-kb
4.0-kb
2.8-kb
1.3-kb
0.9-kb
7.0-kb (gene-1)
6.5-kb (gene-2)
7.5-kb
1.5-kb
8.0-kb
2.1-kb
3.2-kb
4.2-kb
1.4-kb
1.9-kb
1.1-kb
2.2-kb
4.4-kb
0.5-kb
1.25-kb
1.65-b
1.15-kb
0.75-kb
1.7-kb

Aflatoxin biosynthesis is proposed to be regulated in part at the transcriptional level

Gene expression in cells consists of the transfer of genetic information from genes to proteins through a sequential process comprising synthesis of RNAs from DNA templates (transcription) followed by synthesis of enzymes (proteins) from RNA templates (translation). Based on this scheme, the mechanisms governing gene expression are artificially divided into two broad classes: transcriptional regulation and translational regulation (Smale and Baltimore, 1989; Bettany et al., 1992; Rubin and Halim, 1993; Lovett, 1994; Standart and Jackson, 1994). In most cases, the availability of a specific DNA sequence (promoter), an RNA polymerase complex (transcription complex), and associated general transcription factors constitute the basic components of the transcription machinery. Transcription factors are any proteins that are necessary for initiation of transcription, but are not part of the RNA polymerase complex. Transcription factors bind to DNA to initiate their function. The binding sites on DNA for transcription factors are usually located 5' to the transcription initiation site. This region contains crucial information requisite for transcription initiation and is generally referred to as a promoter.

Aflatoxins are a group of secondary metabolites produced primarily in idiophase after production of primary metabolites and exponential growth (trophophase). Cleveland (1987) first demonstrated that the appearance of two aflatoxin metabolic enzymes, methyltransferase and oxidoreductase, coincides with the cessation of exponential growth of fungi and onset of aflatoxin production. A cyclase

activity involved in the conversion of VAL to VB also shared a similar temporal expression pattern with the methyltransferase and oxidoreductase (Anderson and Green, 1994). A similar temporal expression pattern of aflatoxin pathway enzymes was also observed for the *ver*-1 protein by Western analyses (Liang and Linz, 1994). Time course studies also indicated that the accumulation of *nor*-1, *ver*-1, and *omt*-1 transcripts was not detected until near the end of the trophophase (Chang et al., 1992; Skory et al., 1992; Skory et al., 1993; Yu et al., 1993b). These results are consistent with an early experiment in which addition of RNA or protein synthesis inhibitors during the late growth stage of aflatoxigenic *Aspergillus* resulted in no accumulation of transcripts or proteins of the genes associated with aflatoxin production, respectively. Together, these observations suggest that the regulation of aflatoxin production is in part at the transcriptional level.

Aflatoxin biosynthesis may be subject to coordinate regulatory control

The similar temporal expression pattern of the pathway enzymes described above and the pattern of expression of the *nor*-1 and *ver*-1 genes of *A. parasiticus* indicates that these genes may be under a coordinate regulatory control mechanism. The expression of several genes in a single metabolic pathway regulated by a common set of regulatory factors is referred to as coordinate regulation. A similar but distinct phenomenon is global regulation in which several metabolic or developmental pathways are under a common regulatory mechanism. Recently, regulatory genes associated with aflatoxin production have been identified in *A. flavus* (*afl*-2) and *A.*

parasiticus (*apa*-2) and designated together as *afl*R (Chang et al., 1993; Payne et al., 1993). Several lines of biochemical and genetic evidence suggest a coordinate regulatory role played by *afl*R: (1) metabolite feeding studies showed that *afl*-2 is required to complement the deficiency of a *A. flavus* mutant strain which cannot convert four aflatoxin pathway intermediates to aflatoxin. In addition, enzymatic activities required for conversion of three known aflatoxin pathway intermediates are restored in the same mutant strain after being transformed with *afl*-2 (Payne et al., 1993); (2) transformation of a wild type strain of *A. parasiticus* with *apa*-2 results in over-production of all aflatoxin pathway intermediates analyzed and a twofold increase in the enzymatic activities converting NA and ST (Chang et al., 1993); (3) the *afl*R protein contains a zinc cluster DNA-binding motif (Vallee et al., 1991) which was also identified in several transcription factors in other fungi (Giles et al., 1991; Nehlin et al., 1991). One example is GAL4, a transcription factor in galactose metabolism in *S. cerevisiae*. GAL4 can coordinately regulate several galactose metabolic genes by activating these genes (positive regulation). A consensus nucleotide sequence, CGG...GCC, involved in the regulatory control of GAL4 was identified in the promoter regions of structural genes involved in galactose metabolism. The identification of a similar sequence in the promoter region of *nor*-1 and *ver*-1 may indicate that *afl*R participates in coordinate regulation of aflatoxin pathway genes. This GAL4 cis-acting element was also identified in the promoter of *afl*R gene, indicating a potential role of the *afl*R in regulating its own expression.

Examples of coordinate regulation and global regulation are wide spread in

development and production of secondary metabolites of procaryotes and lower eucaryotes (Hopwood, 1988; Martín and Liras, 1989; Fernández-Moreno et al., 1992; Mayorga and Timberlake, 1993; Payne et al., 1993). Expression of numerous structural and regulatory genes (encoding transcription factors) involved in fungal sporulation in *A. nidulans* was found to be under coordinate control governed by the products of two regulatory genes *brl*A and *aba*A (Andrianopoulos and Timberlake, 1994). In *S. griseus*, an A-factor-binding protein synchronously represses genes encoding enzymes involved in streptomycin biosynthesis and genes involved in sporulation (Miyake et al., 1990). This A-factor-binding protein is considered to be a coordinate and global regulator affecting simultaneously the production of antibiotic and bacterial sporulation.

Global regulatory mechanisms are also implicated in the production of several antibiotics in certain bacteria. In *S. coelicolor* A3(2), regulatory loci associated with the production of the antibiotics actinorhodin, undecylprodigiosin, methylenomycin, and calcium-dependent antibiotic were identified.. Transformation of plasmids containing *afs*Q1-*afs*Q2 into *S. coelicolor* A3(2) or *S. lividans* resulted in simultaneous enhancement of actinorhodin, undecylprodigiosin, and A-factor production (Ishizuka et al., 1992). Subsequent protein sequence studies identified *afs*Q1 as a transcriptional regulator with significant similarity to other prokaryotic response regulators involved in osmoregulation, nitrogen regulation, and phosphate regulation indicating its potential role as a global regulator.

More evidence supporting global regulation in secondary metabolite production

is indicated in studies in which a mutation in each of the distinct loci *abs*A, *abs*B, *afs*B, or *aba*A abolishes the production of more than one antibiotic in *S. coelicolor* A3(2) (Adamidis and Champness, 1992; Fernández-Moreno et al., 1992). An *afs*R gene of *S. coelicolor* A3(2) complements the *afs*B mutation and simultaneously restores the production of four antibiotics, indicating that *afs*R is a global regulatory gene (Horinouchi et al., 1983; Horinouchi et al., 1990). The regulatory locus *act*II-ORF4 and *afs*Q1 can suppress the *abs*B and *abs*A mutations respectively and restore actinorhodin production, indicating a regulatory role of *abs*A and *abs*B in antibiotic production (Adamidis and Champness, 1992). The experimental evidence indicates that bacterial/fungal development and production of certain secondary metabolites may be related and under the control of coordinate or global regulatory mechanisms. Another interesting feature which may be related to the regulation of aflatoxin production is the occurrence of the cluster of genes involved in the synthesis of this secondary metabolite.

Clustering of aflatoxin pathway genes may contribute to the regulation of toxin synthesis

The phenomena of gene clustering is observed in genes involved both in primary and secondary metabolism (Orr and Timberlake, 1982; Ohnuki et al., 1985; Schwatz, 1987; Giniger and Ptashne, 1988; Butler et al., 1989; Giles et al., 1991; Nehlin, et al., 1991; Aharonowitz et al., 1992; Guilfoile and Hutchinson, 1992; MacNeil et al., 1992; Sophianopoulou et al., 1993; Scouras et al., 1994; Yu et al., 1994). The clustering of

pathway genes which encode enzymes leading to the production of numerous procaryotic antibiotics, i.e. actinorhodin, granaticin, tetracenomycin, oxytetracycline, frenolicin, erythromycin, and streptomycin, has been reported. Moreover, genes involved in numerous fungal secondary metabolic pathways or fungal development were also reported to be in corresponding clusters. These include the six spore-specific genes in *A. nidulans* (Orr and Timberlake, 1982), the penicillin biosynthetic pathway genes in *A. nidulans* or *P. chrysogenum* (Montenegro et al., 1992), three melanin producing genes in *Alternaria alternata* (Kimura and Tsuge, 1993), and trichothecene-pathway specific genes in *F. sporotrichioides* (Hohn et al., 1993). Of particular interest in these findings is that in several incidences the genes encoding positive-acting or negative-acting transcription factors are encompassed in the corresponding gene clusters. Examples are the *prn*A gene encoding a positive-acting regulator in the proline utilization gene cluster in *A. nidulans* (Sophianopoulou et al., 1993), the *tcm*R gene expressing a repressor in the tetracenomycin biosynthetic pathway gene cluster in *S. glaucescens* (Guilfoile and Hutchinson, 1992), *act*II-orf4 for the production of actinorhodin in *S. coelicolor* A3(2) (Fernández-Moreno et al., 1991), and *str*R encoding a positive regulator in the streptomycin gene cluster in *S. griseus* (Ohnuki et al., 1985). Despite the presence of the regulatory loci in the corresponding gene cluster, no evidence directly shows that the genes in a cluster are under a coordinate regulatory mechanism.

Geever et al (1989) proposed that clustering of genes may facilitate regulation by promoting the access of regulatory proteins to the corresponding protein-binding

nucleotide sequences (promoter or enhancer) on adjacent clustered genes. Several studies also indicate that genes in a cluster may be subject to coordinate regulatory control (Cove, 1979; Giniger and Ptashne, 1988; Kulmburg et al., 1993; Schwatz, 1987; Sophianopoulou et al., 1993). The positive-acting transcription factor GAL4 in galactose catabolism of *S. cerevisiae* (Nehlin et al., 1991) and the gene pair *qa*-1F/*qa*-1S (*qut*A/*qut*R in *A. nidulans*) encoding an activator/repressor in the quinic acid utilization pathway in *N. crassa* (Giles et al., 1991), respectively, are two examples in which positive acting regulating genes constitute part of the gene cluster and govern the expression of most of the pathway genes.

Most of the genes encoding enzymes involved in synthesis of PKS-derived secondary metabolites are clustered (Motamedi and Hutchinson, 1987; Sherman et al., 1989; Cortes et al., 1990; Donadio et al., 1991; Trail et al., 1995a). Transcript mapping together with gene complementation and inactivation experiments in *A. parasiticus* showed that the aflatoxin pathway genes *ver-1*, *nor-1*, *uvm*8, *omt*-1, *afl*R, and gene-1 (*pks*A) are clustered in one linkage group (Trail et al, 1995a; Yu et al, 1993b). Recent data generated in our laboratory as well as others demonstrate a common temporal expression pattern and level of expression among *nor*-1, *ver*-1, *omt*-1 and eight other transcripts in a 35-kb genomic DNA fragment cloned from *A. parasiticus*, indicating that these clustered genes are coordinately regulated (Chang et al., 1992; Skory et al., 1992; Skory et al., 1993; Yu et al., 1994).

II. Harmful effects of aflatoxins

The harmful effects of aflatoxins on the health of living organisms are generally divided into two broad categories, biochemical and biological effects (Ellis et al., 1991). These effects are mainly derived from adduct formation between activated aflatoxins and cellular macromolecules such as DNAs, RNAs, and proteins (Essigmann et al., 1982; Yu et al., 1988; Yu et al., 1990; Wild et al., 1990). However, nonactivated aflatoxins (AFB1 and AFG1) were also shown to interact with nucleic acids to form non-covalent, weak, and reversible bonds (Stark, 1980). This noncovalent binding contributes to the mutagenicity of nonactivated AFB1 in certain stains of *Bacillus subtilis* and *Salmonella typhimurium*.

Modes of action

Activation of AFB1 to its highly reactive and unstable intermediate AFB1-8,9-epoxide is mainly carried out by the cytochrome P450 monooxygenase enzyme systems (P450, CYP) (Koser et al., 1988; Kamataki et al., 1992; Massey et al., 1995). The activated AFB1-8,9-epoxide can form adducts with DNA, RNA, and proteins. The primary site on DNA for adduct formation is the N^7 position of the guanine nucleotide in a region with alternating G-C sequences (Essigmann et al., 1982; Yu et al., 1990) and the major aflatoxin-DNA adduct that forms is 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB1-N^7-Gua). DNA adduct formation between activated AFB1 and chromosomal DNA causes frameshift mutations (Yu et al., 1990) and/or G:C to T:A transversions. The AFB1-N^7-Gua adduct has been detected in biological systems

or *in vitro*, whereas the AFB1-8,9-epoxide formed via P450 mediated enzymatic activation has not been isolated (Gopalakrishnan et al., 1992).

Biochemical effects

AFB1 exerts numerous negative effects on cellular biochemical functions. The biochemical effects of aflatoxins are believed to reside in inhibition of the biosynthetic pathways affecting cellular energy metabolism and metabolism of carbohydrates, lipids, nucleic acids and proteins (Moss and Smith, 1985; Yu et al., 1988; Yu et al., 1990; Ellis et al., 1991).

AFB1, AFG1, and AFM1 have been shown to inhibit the adenosine triphosphatase (ATPase) activity in the electron transport system resulting in decrease of oxygen uptake and energy generation. The binding of AFB1-8,9-epoxide to DNA templates and RNA templates inhibits RNA and protein synthesis, respectively. Aflatoxins can also reduce hepatic glycogen levels due to the inhibition of glycogenesis and glucose transport into liver cells.

Biological effects

The biological effects of aflatoxins can be subdivided into carcinogenicity, mutagenicity, teratogenicity, hepatotoxicity, and aflatoxicosis. The severity of these biological effects in the target organism is influenced by tissue variation, species variation, gender, age, nutritional status, and the effect of other chemicals. In most cases, the activity of a specific P450 (CYP) isoform appears to play a prominent role

in determining the extent of the influence. One example is CYP3A4, a predominant P450 isoform expressed in adult human liver. β-naphthoflavone, a polycyclic aromatic hydrocarbon (PAH), which can induce CYP1A2 activity but not CYP3A4, does not significantly affect the ability of human liver microsomes (tissue with highest P450 activity) to generate DNA-binding AFB1, whereas glucocorticoid predominantly activates CYP3A4 and DNA adduct formation (Massey et al., 1995). As for the influence of gender on the response to aflatoxin exposure, the P450 activity in male and female rats has been shown to be under the differential control of sex hormones. Nutritional factors, including protein, fat, vitamins, trace elements and caloric intake may also influence aflatoxin-associated carcinogenicity via their effects on P450 activities (Bhattacharya et al., 1987; Francis et al., 1988). For example, the inhibition of a male rat specific isozyme, CYP2C2, by caloric restriction, resulted in decreased activation of AFB1 (Chou et al.,1993).

Carcinogenicity

Aflatoxins are the most potent naturally occurring carcinogens which have ever been studied. Aflatoxins have been shown to cause cancer in liver, colon, and kidney in laboratory animals such as rats, ducks, and monkeys (Andamson et al., 1976; Hulla et al., 1993). Epidemiological studies have also shown a consistent association between chronic intake of aflatoxins and development of primary liver cancer in humans. However, no evidence shows a direct correlation. Recently, epidemiological studies provided strong evidence of an association between chronic hepatitis B virus

(HBV) infection and primary hepatocellular carcinoma (HCC) in humans (Groopman et al., 1988, Yeh et al., 1989; Wild et al., 1990; Tanaka et al., 1991). Although hepatitis B virus was generally considered to be associated with HCC, one report showed that HBV and AFB1 do not appear to act synergistically in the genesis of HCC (Santella et al., 1993).

The carcinogenicity of AFB1 is believed to be derived from its capability to form DNA adducts and cause DNA mutations. Experimental evidence shows that AFB1 can cause mutations in the p53 tumor suppressor gene (Gopalakrishnan et al., 1992; Hollstein et al., 1993). Mutations in p53 may contribute to the development of 50% of human cancers (Kamb et al., 1994). The best known genetic changes in human HCC are mutations in p53 as well as integration of HBV DNA into the host genome. In HBV-related HCC, the wild type function of p53 may be inactivated by a viral protein (i.e. HBxAg, HBV X antigen protein) (Unsal et al., 1994). In addition to the mutations in the p53 tumor suppressor gene, AFB1 also exerts it carcinogenicity through induction of virus expression (Groudine et al., 1981; Rascati and McNeely, 1983) and activation of certain other oncogenes (McMahon et al., 1986a; McMahon et al., 1986b; Larson et al., 1993; Soman and Wogan, 1993).

Mutagenicity

AFB1 has also been found to be a potent mutagen. The formation of an epoxide derivative by metabolic activation has been shown to be mainly responsible for the mutagenicity of AFB1 (Stark, 1980). Stark suggested that the formation of an AFB1-

N^7-Gua adduct inhibits DNA polymerase activity resulting in a single-strand gap at the site of the adduct. This DNA lesion acts as a inducer to an error-prone repair system in mammalian cells analogous to the SOS repair in bacteria leading to mutations.

Teratogenicity and hepatotoxicity

AFB1 exerts its teratogenicity mainly due to its ability to inhibit protein synthesis. AFB1 impairs the normal differentiation of the primordial cells during the course of gestation resulting in teratogenicity. The liver becomes the target organ for AFB1 toxicity mainly because it is the primary organ for activation of AFB1. The biotransformation of AFB1 to its bioreactive intermediate and subsequent binding with macromolecules in liver cells result in the formation of liver necrosis and hemorrhage.

Aflatoxicosis

The acute effects of aflatoxins may lead to aflatoxicosis that is characterized by vomiting, abdominal pain, pulmonary edema, gastrointestinal hemorrhage, bile duct proliferation, jaundice, and fatty and pale liver. The cause of aflatoxicosis is obscure, though acute AFB1 toxicity may be related to the binding of various functional proteins with AFB1 biotransformation products (Massey et al., 1995). Biotransformation of AFB1 via a specific P450 isoform and epoxide hydrolase may lead to aflatoxin B_{2a} and AFB1-8,9-dihydrodiol production, respectively. AFB1-8,9-dihydrodiol undergoes opening of the furan rings to yield a dialdehydic phenolate ion.

Aflatoxin B_{2a} and the dialdehydic phenolate ion react with primary amino groups in protein to form a Schiff base and results in reduced protein activity and acute AFB1 toxicity.

Immunotoxicity

B lymphocytes have been shown to be more sensitive to the sister chromatid exchange-inducing property of AFB1 than T cells. B cell proliferation was also dramatically reduced by AFB1 in contrast to the negligible effects of AFB1 on T lymphocyte proliferation (Potchinsky and Bloom, 1993). However, experimental evidence indicates that animals receiving different amounts of AFB1, e.g. swine (500 ppm in feed), rabbits (24 ppm in feed), and guinea pig (0.045 mg/kg body weight) showed no significant effects in antibody production (review, Pestka and Bondy, 1994). In spite of the greater sensitivity of B lymphocytes to AFB1 geno- and cytotoxicity, AFB1 suppresses cell-mediated immunity to a greater extent than humoral immunity. The effects of AFB1 on cell-mediated immunity have been experimentally indicated in the reduction in thymus weight and T lymphocyte numbers as well as suppression of delayed type hypersensitivity in laboratory mice (for review, see Pestka and Bondy, 1994). In summary, the immunotoxicity of AFB1 is mainly a characteristic of the suppression of cell-mediated immunity. Aflatoxins usually exert their harmful effects not independently but additively. Therefore, the highly toxic effects of aflatoxins to test animals and the potential health threat to humans that made the Food and Drug Administration (FDA) impose an action level 20 ppb for

total aflatoxins in food and feed in the United States. The current action level for AFM1 is 0.5 ppb because of the potential risks for children who consume a large quantity of milk. In order to achieve the goal of delivery of aflatoxin-free food and feed or products with aflatoxin concentrations below the regulatory level, there is an urgent need for appropriate strategies to control aflatoxin contamination.

III.Control of aflatoxin contamination

Strategies to control aflatoxin contamination are generally classified into two broad categories; (1) prevention of fungal growth and crop contamination by the toxigenic fungi, which is generally referred to as preharvest prevention and (2) postharvest remediation (Ellis et al., 1991). Preharvest prevention includes control of the environmental conditions, improved farm management, breeding of host plants to resistance, and the use of nontoxigenic biocompetitive microorganisms (biocontrol agents). Postharvest remediation can be achieved by the use of rapid aflatoxin screening techniques and decontamination of aflatoxins (detoxification) or destruction of contaminated materials.

Postharvest remediation

In order to prevent aflatoxins from getting into feed or food, techniques of rapid detection and decontamination of aflatoxins in peanuts, corn, and cottonseed were developed. Rapid screening of aflatoxin-containing crops has been conducted by color sorting, fluorescence sorting (Pelletier and Reizner, 1992), and flotation separation

(Gnanasekharan et al., 1992). Rapid detection methods have the problem of low precision, although employment of those methods usually results in removal of contaminated crops. Recently, complete removal of aflatoxin contaminated peanuts by a rapid screening method was reported (Chiou et al., 1994). In this study, peanuts were deskinned and lightly roasted followed by hand or electronic color sorting. The resulting commercial peanut lots did not contain any detectable aflatoxins at the ppb level based on thin layer chromatography (TLC).

Detoxification methods include physical, chemical, and biological methods (for review, see Ellis et al., 1991; Piva et al., 1995). Physical methods include solvent extraction, heat inactivation (250°C), and adsorption removal of aflatoxins (Piva et al., 1995). Chemical methods employ acids, bases, oxidizing agents, or sodium bisulfite to detoxify aflatoxins. Of the detoxification methods used, anhydrous ammonia gas appears to be the most effective (Cole, 1988). One example of detoxification of aflatoxins by ammonia gas was reported in a feeding study using rainbow trout as the test animal. The result of this study showed that the incidence of hepatoma dropped from 98% in trout fed with untreated aflatoxin contaminated corn to 3% in trout fed with ammonia treated contaminated corn (Brekke et al., 1977).

Detoxification of aflatoxins in contaminated foods or animal feeds is not economical and usually results in a decrease in nutritional quality, left-over toxic residue or bad odor. Although detoxification of aflatoxin contaminated feed with ammonia is legal in certain states in the United States such as Arizona, California, Georgia, and Alabama, no detoxification methods for food are FDA-approved.

Preharvest prevention provides an alternative approach to achieve control of aflatoxin contamination without having the unwanted side-effects of detoxification.

Preharvest prevention

Preharvest prevention of aflatoxin contamination is focused on the control of aflatoxin in the field before harvest. This can be achieved by adequate agronomic practices via control of environmental conditions and improved farm management, increasing host resistance to aflatoxin producing fungi via breeding or genetic engineering of plants, and use of nontoxigenic biocompetitive microorganisms.

Agronomic practices

Adverse environmental conditions such as drought is the most significant factor in preharvest contamination. The most significant aflatoxin contamination of peanuts occurred during periods of late season drought stress as peanuts are maturing (Cole, et al., 1982; Dorner et al., 1991), though contamination also occurred during postharvest curing (storage). Summer drought stress was also observed in aflatoxin contaminated field corn (Dunlap, 1991; Wicklow et al., 1991). Drought stress has direct effects on almost every other factor in the aflatoxin contamination profile. These include soil temperature and moisture. Elevated soil temperature and a decline in moisture favors the population build up of toxigenic fungi and crop damaging insects. Studies have shown that insect or mechanical damage can enhance fungal infection and aflatoxin contamination on corn and cottonseed (Wicklow et al., 1988;

Bowen and Mack, 1991; Cotty, 1991; Dunlap, 1991; Wicklow, 1991). Therefore, agronomic practices such as control of environmental conditions and improved farm management, including adequate irrigation in the field, application of fungicides or insecticides, and proper handling of the harvested crops play a determinant role in prevention of aflatoxin contamination.

Although preharvest prevention strategies have been shown to be effective in preventing aflatoxin contamination, most of them are too expensive or unavailable to farmers and are therefore considered uneconomical and impractical. Preharvest prevention of aflatoxin contamination, an alternative to postharvest control, has recently focused on two areas; the development of resistant host plants and generation of nontoxigenic competitive microorganisms.

Increasing host resistance against aflatoxin producing fungi

Development of resistant plants is based on two genetic technologies, classic plant breeding and modern genetic engineering. An understanding at the molecular level of the interactions between pathogens and host plants can facilitate the development of genetic technologies to increase plant resistance. The interrelationship between plants and pathogens is artificially classified into incompatible and compatible interactions. In the most frequent case, the genotypes of the interacting organisms are all incompatible with each other, resulting in either no interaction taking place or nonhost/nonpathogen (induced resistance) interaction. In the rarest cases, some of the genotypes of these interacting organisms are compatible in which the virulent

pathogen can infect the susceptible host.

The nonhost/nonpathogen incompatible interaction between plants and pathogens has been demonstrated in many host-pathogen interactions (Cervone et al., 1989; Bent et al., 1990; De Wit et al., 1990; Hahlbrock et al., 1991; Scheel et al., 1991; Jones et al., 1994). Attack of insects or microorganisms on plants results in the release of hydrolyzing enzymes endopolygalaturonases (EPGases) by the microorganisms. EPGases digest plant cell walls to oligogalacturonides which can serve as elicitors to activate the plant genes involved in production of defensive chemicals, phytoalexins and antinutritive proteinase inhibitors (Ryan et al., 1986). Upon pathogen infection, a variety of pathogenesis-related (PR) proteins, including a variety of plant-derived chitinases or β-glucanases, are also induced by fungal elicitors and accumulate in plant tissues. These enzymes hydrolyze fungal cell walls to polyglucosamine (chitosan), which also serves as an elicitor in activation of plant defensive mechanisms (Joosten and De Wit, 1989). A plant-derived EPGase-inhibiting protein (PGIP) has been isolated from a variety of dicotyledonous plants such as beans, peas, tomatoes, cucumbers, apples, oranges, and pears. The PGIP maintains a stable and prolonged occurrence of oligogalaturonides with a degree of polymerization between 10 to 13, a range of oligogalaturonides polymers that are able to elicit phytoalexin synthesis in plants (Cervone et al., 1989). Therefore, the EPGases are both fungal pathogenesis factors and a potential avirulence factor.

The nonhost/nonpathogen incompatible interaction between plants and pathogens can also be explained using an avirulent gene for resistant gene (gene-for-

gene) interaction model. In this model, the resistant host has one or more resistance genes incompatible with the avirulence gene(s) in the pathogen, resulting in the resistant interaction. One example of gene-for-gene interaction was observed in the interaction between the host *Lycopersicon esculentum* (tomato) and the fungal pathogen *Cladosporium fulvum* (De Wit et al., 1994). A functional avirulent gene, *avr*9, which encodes a protein elicitor in *C. fulvum* was identified in an avirulent race but not in a virulent race (De Wit et al., 1990). This elicitor interacts with a product of the resistance gene *cf*-9 (Jones et al., 1994) in the tomato. Recently, the *cf*-9 gene has been cloned from a resistant tomato strain. The deduced amino acid sequence reveals that this is a transmembrane protein and contains an extracytoplasmic leucine rich region which is considered to interact with the *avr*9 ligand (Jones et al., 1994). The leucine rich region of *cf*-9 protein has a conserved region with a high degree of similarity to PIGP, indicating the possible role of PGIP as a resistance gene in the gene-for-gene interaction.

Understanding the molecular basis for nonhost/nonpathogen interactions between pathogens and host plants can enhance the development of strategies to increase plant resistance. This depends on the accomplishment of the following goals; identification of genes encoding fungal-growth inhibiting factors (e.g. fungal cell wall digesting enzymes) or proteinase inhibitors (PGIP) which interfere with fungal-encoded plant cell wall digesting enzymes (e.g. pectinase and polygalacturonases), cloning of these inhibitory genes, and development of techniques to stably transform plants with those genes (Cleveland et al., 1991).

Genetic improvement of plants depends on the selection of beneficial genotypes of plants (or fungi) leading to an increase in plant resistance. This genetic improvement can be achieved by classic plant breeding and genetic engineering.

Classic breeding of crops

One-half of the increase in crop production achieved during the last decade is attributed to genetic improvement (Phillips, 1993). This is achieved by both classic plant breeding and genetic engineering techniques. Classic plant breeding to develop populations with agronomic traits of interest usually covers a time range of ten years. Additionally, the cost, space consideration, extensive evaluation (multi-environmental testing) of the new generations, and the unstable genotype-by-environment interaction make classic plant breeding extremely tedious and time-consuming (Phillips, 1993).

In spite of the problematic features of classic breeding, studies to identify corn and peanut hybrids resistant to preharvest aflatoxin contamination are constantly undertaken (Holbrook et al., 1991; Wicklow, 1991). Zummo and Scott (1992) reported two corn (*Zea mays* L.) genotypes, Mp313E and Mp420, to be the first identified maize inbreds resistant to aflatoxin producing *A. flavus*. A number of corn hybrids which show resistance to *A. flavus* infection have also been identified and tested in a multi-environmental testing experiment. Research conducted during the years 1990-91 in six states of the U.S. showed that the resistant hybrids contained about 45% fewer kernel infections and 58% less aflatoxin than susceptible hybrids (Scott and Zummo, 1990; Scott et al., 1991).

The approach to achieve genetic improvement of plants is an integration of classic breeding and genetic engineering. Genetic engineering can be considered to be an enhancement to classic breeding which improves the crop genotype at specific genes in a complex genetic trait (described below).

Genetic engineering of crops

Genetic manipulation of plants requires efficient DNA transformation systems. Introduction of DNA into dicotyledonous plants was first achieved through Ti plasmid mediated transformation systems (Hohn et al., 1990; Nester and Gordon, 1990). Oncogenic strains of *Agrobacterium tumefaciens* carry a large tumor-inducing (Ti) plasmid (180 kb). This plasmid integrates into the nuclear genome of a susceptible plant after infection. The transfer and integration process is facilitated in *trans* by a variety of virulence proteins encoded by virulence genes (*vir*) on the Ti plasmid. Modified vectors, which possess exogenous DNA along with Ti plasmid sequences have been constructed for the efficient transformation of higher plants (Bevan, 1984). Transformation of these vectors into higher plants is usually carried out via *A. tumefaciens* infection, fusion of Ti-derived plasmid containing bacteria spheroplast with plant protoplasts, or fusion of Ti-plasmid-containing liposome with plant protoplasts. Transformation of dicotyledonous protoplasts by *E. coli* plasmid vectors has also been described (Davey et al., 1989). A high-velocity microprojectile transformation system for monocotyledonous plants was recently developed. DNA-coated gold or tungsten particles (4 μm in diameter) have been used to deliver DNA

into intact tissues of peanuts (Weissinger et al., 1991).

An example of using genetic engineering techniques to enhance plant resistance against aflatoxin contamination has recently been reported (Cleveland et al., 1991). A pectinase inhibitor and a wound-inducible proteinase inhibitor II were isolated from orange and potato, respectively. These inhibitors were shown to inhibit a pectinase and an *A. flavus* proteinase activity which digests plant cell walls. Transformation of cotton and peanuts with chimeric genes containing proteinase inhibitor II wound-inducible promoter fused to genes encoding chitinase, orange-derived pectinase inhibitor and potato-derived proteinase inhibitor II to enhance resistance to invasion by *A. flavus* and *A. parasiticus* is in progress (Cleveland et al., 1991).

Application of crop genetic engineering to control aflatoxin contamination in the field is restricted for several reasons. First, the majority of agronomic traits of interest in plants are quantitative traits. These traits include genetic information which determines pathogen resistance, high yield, and stress resistance. Quantitative traits are the result of the function of many genes, each of which has a relatively small effect. This genetic complexity raises the difficulty to genetically improve the crops through manipulation of individual genes. Secondly, transgenic plants developed through classic breeding or genetic engineering are subject to "genotype-by-environment instability" because characteristics advantageous to the plants vary to a great extent in distinct environments (Allard and Bradshaw, 1964). Third, unlike most of the gene-for-gene interactions in which a pathogen has a restricted number of host plants (usually only one host plant), the toxigenic *A. flavus* and *A. parasiticus* can

infect and contaminate a wide variety of host plants. Furthermore, *A. flavus* and *A. parasiticus* are not typical pathogens but are considered opportunistic pathogens or saprophytes which often infect the plant when they are subject to damage or environmental stresses. These two unique features in the interaction of aflatoxigenic *Aspergillus* and host plants make the development of strategies to increase plant resistance very difficult. However, genetic manipulation of the host plants to produce specific products to repress aflatoxin biosynthesis during the interaction of the organisms appears to be a logical alternative approach to generation of plants resistant to fungal infection. Since genetic engineering of plants to aflatoxin resistance will likely be successful as a long term approach, researchers sought an alternative short term strategy to help prevent aflatoxin contamination. This strategy involves the use of biocontrol microorganisms to reduce or completely inhibit aflatoxin production in the field.

Use of nontoxigenic biocontrol agents to control aflatoxins

It is well known that aflatoxin production in the field is influenced by a variety of environmental factors. These factors include temperature, relative humidity, availability and types of nutrients, light, pH, and atmospheric gases (which have been previously described), and competing microflora. The competing microflora (competitive microorganisms) are generally referred to as the "normal flora" that share a common ecological niche as the target microorganisms and are capable of interfering with growth or toxin production by the target microorganisms. In this regard,

competing microflora can be used as biocontrol agents to reduce toxin accumulation in the field or after harvest. Biocontrol agents are either naturally occurring competing microflora or laboratory generated competitive microorganisms. In this report, the hypothesis and criteria for the use of biocontrol agents at the preharvest stage as well as the mechanisms and factors affecting the efficacy of biocontrol agents are discussed.

The hypothesis associated with the use of nontoxigenic biocontrol agents to achieve preharvest control of aflatoxin contamination is that application of highly competitive and nontoxigenic biocontrol agents in the field can result in a significant decrease in aflatoxin production on crops (Ehrlich et al., 1984; Dorner et al., 1992). The rationale for the use of biocontrol agents is that the biocontrol agents would dominate the microflora and therefore prevent the buildup of the toxigenic strains. Several criteria requisite for the use of biocontrol agents have been proposed; (1) the biocontrol agents are nontoxigenic and should not induce adverse effects on host plants, (2) they should occupy the same ecological niche as the toxic strains of *A. flavus*/*A. parasiticus*, (3) they should be genetically stable, (4) they should be persistent in the environment as well as capable of outcompeting the toxigenic strains under the environmental conditions conducive to aflatoxin production.

A variety of mechanisms associated with the ability of biocontrol agents to reduce toxin accumulation have been proposed. The biocontrol agents may dominate the microflora by competing for the substrates necessary for the growth or toxin production by the toxigenic organisms (BjÖrnberg and SchnÜrer, 1993) or by

rendering conditions unfavorable for growth and toxin biosynthesis (biocompetitive exclusion). Biocontrol agents may also metabolize the toxins produced (Cole et al., 1972; Weckbach and Marth, 1977; Dole and Marth, 1978; Samson, 1992). One example to demonstrate the use of biocontrol agents by competitive exclusion is the suppression of the growth of grain-spoilage molds *P. roqueforti* and *A. candidus* in the presence of the yeast *Pichia anomala*. It is likely that competition for nutrients plays a major role in this yeast-mold interaction. *P. anomala* also produces large amounts of ethyl acetate, which reduces mold growth and spore germination by *P. roqueforti* and *A. candidus* (BjÖrnberg and SchnÜrer, 1993). Competitive exclusion is also exemplified by the effects of chitinolytic bacteria and mycoparasitic fungi on the growth of toxigenic fungi. The chitinolytic bacteria *Serratia marcescens* and *Aeromonas hydrophila* (Gay et al., 1991) and the mycoparasitic fungus *Paecilomyces lilacinus* (Gupta et al., 1993) secrete degradative enzymes inhibiting growth of *Aspergillus* sp. In contrast, several fungal strains which are effective in reducing aflatoxin production appear to have no effect on the growth of toxigenic fungi. This was observed in the interaction between the aflatoxin producing *Aspergillus* sp. and *A. niger* (Wicklow et al., 1980; Shantha et al., 1990) and aflatoxin production blocked mutants *A. parasiticus* ATCC24690 and ATCC36937 (Ehrlich, 1987). A variety of *A. niger* strains were shown to possess the ability to reduce the substrate pH or produce a water-soluble component that is inhibitory to aflatoxin formation without affecting fungal growth (Horn and Wicklow, 1983; Shantha et al, 1990). This type of interaction may also be attributed to the degradation of the toxins. *Rhizopus spp.*

(Cole et al, 1972) and *A. niger* (Shantha et al, 1990) can metabolize aflatoxin to less toxic compounds thereby lowering the concentration of aflatoxin. A study by Doyle and Marth (1978) also showed that aflatoxins are degraded by a mycelial macerate derived from toxigenic or nontoxigenic strains of *A. flavus* and *A. parasiticus*, rendering a lower concentration of aflatoxin in corn.

In addition to the previously described direct or indirect interaction between the biocontrol strains and the toxigenic strains, the addition of biocontrol agents also has the potential to induce the defense mechanisms of host plants to prevent the growth or toxin production by the toxigenic strains. Examples of plant defense mechanisms which might be affected are thickening of plant cell walls, phytoalexin production, or gene-for-gene defense mechanisms. However, these types of biocompetitive mechanisms are unavailable or are currently under study but have not been concluded.

The use of biocontrol agents to control aflatoxin production has been under intense investigation for decades. Although some success has been reported in lowing aflatoxin production, potential problems are associated with the currently used biocontrol strategies.

Biocontrol agents have been used in controlling aflatoxins

Recently, numerous biocontrol agents have been tested for the ability to reduce aflatoxin production by toxigenic strains on corn, cottonseed, peanuts, and tree nuts in the greenhouse or in field studies (Wicklow et al, 1988; Cole et al, 1991; Cotty,

1990; Cotty and Daigle, 1991). A wide variety of bacterial species (Weckbach and Marth, 1977; Misaghi and Cotty, 1991) and yeasts (Weckbach and Marth, 1977; Sommer et al, 1991) have been tested. Fungi, including mycoparasitic fungi (Gupta et al, 1993), fungi isolated from the same ecological niche as toxigenic *Aspergillus* (Wicklow et al, 1980), aflatoxin-blocked mutants of toxigenic *A. flavus* and *A. parasiticus* (Ehrlich, 1987; Horn et al, 1994), and naturally occurring nontoxigenic variants of *A. flavus* and *A. parasiticus*, were also tested for their ability to reduce aflatoxin contamination in the field (Cotty and Bhanagar, 1994; Dorner et al, 1994). Biocontrol agents were also tested using in vitro competition studies in which toxigenic and biocontrol agents were co-cultivated in the laboratory in the absence of host plants (Weckbach and Marth, 1977; Ehrilch, 1987; Shantha et al., 1990).

The experimental evidence derived from the field/greenhouse studies and in vitro competition tests suggests that the effectiveness for the use of biocontrol agents appears to depend to a large extent on the type and amount of inocula. The sequence of inoculation as well as the specific biocontrol strains used are also considered to be important for effective biocompetition.

Amount of inoculum

The efficacy of biocontrol agents may have a quantitative relationship with the magnitude of the reduction in growth or toxin production by *Aspergillus*. A one year study conducted by Horn et al (1994) in the state of Georgia using peanuts as the host plant, showed that the most significant reduction in percentage infection of peanuts

by toxigenic strains of *A. flavus* and *A. parasiticus* was observed when a larger quantity of a biocontrol inoculum, a NA accumulating strain of *A. parasiticus* NRRL6111 (ATCC24690), was used. Higher levels of biocontrol inocula also resulted in most effective aflatoxin reduction in a three year study (1987-1989) conducted by Dorner and his coworkers in 1992 in which a naturally occurring OMST accumulating strain of *A. parasiticus* (NRRL 13539) was used as the biocontrol agent. The experimental evidence indicate that effective control of aflatoxin contamination can be achieved by applying an appropriate population of biocontrol propagules, which should be determined before their release in the field. This is especially important for proposed commercial use.

Type of inoculum

In the study conducted by Horn and coworkers (1994), a significant reduction in the percentage infection by toxigenic fungi on peanut seeds was observed when biocontrol inocula in the form of mycelia and sclerotia were used, whereas conidia had less effect. However, this study was unable to show a consistent relationship between the percentage infection by toxigenic *Aspergillus* and aflatoxin accumulation.

Sequence of inoculation

Numerous studies indicate that the successful establishment of aflatoxigenic fungi and subsequent aflatoxin contamination may depend to a great extent on the sequence of colonization by toxic *A. flavus* and *A. parasiticus* in relation to that by biocontrol

fungi. *In vitro* competition studies using biocontrol agents capable of interfering with aflatoxin production indicate that early establishment of the biocontrol agent has greater effect in reducing toxin production than simultaneous inoculation (Weckbach and Marth, 1977; Wicklow et al., 1980; Ehrlich, 1987; Cotty, 1990; Cotty and Bhatnagar, 1994). A similar observation was obtained by Wicklow and coworkers (1988) in a greenhouse test. In this test, a mixture of five naturally corn-infesting competitor fungi was applied together with *A. flavus* on corn at different maturation stages (Wicklow et al., 1988). Inoculation with *F. moniliforme* before or together with *A. flavus* during the first 21 days after silking could efficiently repress growth and toxin production by *A. flavus* on corn, whereas with *Acremonium strictum*, significant interference with growth and aflatoxin production was observed only when the biocontrol agent was inoculated before the toxic strain. In studies using cotton cultivated in controlled environmental plots, Cotty also showed that inoculation of one of five atoxigenic *A. flavus* strains 24 hours prior to a toxigenic strain resulted in a greater reduction in toxin accumulation on cotton bolls compared with simultaneous treatment or delayed challenge with the atoxigenic strains (Cotty, 1990; Cotty and Bhatnagar, 1994).

Efficacy of specific biocontrol strains

Different biocontrol agents appear to have different abilities to reduce aflatoxin accumulation (Cotty, 1990; Cotty and Bhatnagar, 1994). When five atoxigenic strains of *A. flavus* were tested for use as biocontrol agents, Cotty and coworkers (1990)

observed a significant variation in efficacy at reducing aflatoxin accumulation on cotton bolls. A similar variation in strain efficacy was also observed in *A. parasiticus.* Blocked mutants accumulating NA (ATCC24690) (Horn et al, 1994), VA (ATCC36597) (Ehrlich, 1987) or OMST (NRRL13539) appeared to interfere with aflatoxin production by the wild-type strain, whereas the AVF (ATCC24551) or AVN (SRRC163) accumulating strains did not. Thirteen naturally occurring fungal populations isolated from a common ecological niche as *A. flavus* on corn, two strains of *Trichoderma viridie* and *A. niger* (NRRL 6411) had the most significant adverse effect on aflatoxin production (Wicklow et al., 1980). Other common fungal contaminants of field corn such as *F. moniliforme* (Wicklow, et al., 1980; Wicklow et al., 1988), *P. oxalicum* (Wicklow et al., 1980; Ehrlich et al., 1984), *P. funiculosum* had lower but still significant effects. Inhibitory effects were not observed for some pathogenic fungi such as *Candida guillermondii* and *Alternaria alternata* (Wicklow et al., 1988).

There are several other determinants which may have an impact on the use of biocontrol agents. First, there appears to be no positive correlation between the soil population of toxigenic strains/biocontrol agents and the percentage infection of peanuts by the toxigenic fungi. Horn et al. (1994) demonstrated that there is no significant change in the soil population of toxigenic *A. flavus* and *A. parasiticus* after application of atoxigenic *A. parasiticus* biocontrol strains, despite the significant decrease in percentage infection of toxigenic fungi on peanuts. The ineffectiveness of biocontrol agents on reducing the soil population of toxigenic *A. parasiticus* was

also observed in a three year study conducted by Dorner et al. (1992). In contrast, in a two year cotton field study in Arizona, Cotty and Daigle (1991) observed population shifts in the *A. flavus* soil population in a intraspecific competition study, indicating displacement of endemic toxigenic strains of *A. flavus*. Second, the biocontrol agents may remain effective for several consecutive years after initial application in the field (Dorner et al., 1992). In a three year consecutive study, the most significant decrease in the soil population of the native strains and aflatoxin production was only observed in the year following inoculation of the biocontrol agents. The soil population as well as the production of aflatoxin for the remaining one year remained the same (Dorner et al., 1992). Third, many studies have shown that *A. flavus* is more invasive in infecting plant hosts than *A. parasiticus*. However, one study offers evidence that *A. parasiticus* is most effective in a intraspecific competition to fight off *A. parasiticus* (Horn et al., 1994).

In spite of numerous reports indicating that currently used biocontrol agents are effective in partially controlling aflatoxin accumulation in the fields, there are several potential problems associated with the use of microorganisms.

Currently available biocontrol agents have potential problems

The problems associated with the use of currently available fungal biocontrol strains are: (1) Naturally occurring or lab-isolated atoxigenic isolates of *A. flavus* or *A. parasiticus* and related *Aspergilli* may produce toxins under certain conditions (Schindler et al., 1980; Lee, 1989; Cotty, 1990; Cotty and Bhatnagar, 1994). Cotty

(1990) showed a potential for a very effective atoxigenic *A. flavus* (AF36) to become aflatoxin producing. He also showed that this naturally occurring atoxigenic strain could convert NA to aflatoxin, indicating the presence of pathway enzymatic activities and the potential disadvantage in using this strain. (2) Aflatoxin-blocked mutants accumulate toxic intermediates. (3) Nontoxigenic isolates may mutate to toxigenicity during production or inoculum for biocontrol studies. Several studies have shown a progressive attenuation of toxin producing capacity after repeated transfer under laboratory conditions of certain naturally occurring toxin producing strains of *A. flavus* and *A. parasiticus* (Mayne et al., 1971; Torres et al., 1980; Lee, 1989). Mayne et al (1971) also indicated a potential increase in aflatoxin production after successive subculturing of strains of *A. parasiticus*. Recently, genetic data indicated the potential for naturally occurring atoxigenic strains of *A. flavus* to produce aflatoxins (Rarick et al., 1994). Therefore, it is logical to reason that nontoxigenic isolates may mutate to toxigenicity during production of inoculum for biocontrol studies. (4) Application of other bacteria, yeast, or fungal strains other than toxigenic *Aspergillus* to fight off *A. flavus* or *A. parasiticus* creates the risk of ruining the ecological system by artificially bringing in a more dominant microflora which initially occupy a small part of the biohabitat. These potential problems can be solved by the generation of nontoxigenic but genetically stable biocontrol strains via recombinational inactivation (gene disruption).

Genetically stable, nontoxigenic biocontrol strains can be generated by gene disruption

Gene disruption (described in Chapter III) as a mechanism to generate null mutations in target genes has been reported in several filamentous fungi (Scherer and Davis, 1979; Miller et al., 1985; Paietta and Marzluf, 1985; Orr-Weaver and Szoatak, 1985; Wernas et al., 1987; Dunne and Oakely, 1988; Finchman, 1989; Goosen et al., 1989; O'Hara and Timberlake, 1989; Chevalet et al., 1992). In *A. nidulans* and *A. parasiticus*, the frequency of genetic transformation is higher when homologous sequences between the transforming vectors and their genomic counterparts are present (Dunne and Oakely, 1988; Horng et al, 1990; Skory et al., 1990). Recently, homologous recombination in *A. parasiticus* was successfully used in a gene disruption experiment (Wu and Linz, 1993). The strategies established in this study have been applied to disrupt numerous aflatoxin pathway genes to produce genetically stable, nonaflatoxin producing mutants of *A. parasiticus* (Liang et al., 1994; Trail et al., 1995a; Mahanti et al., submitted). These nontoxigenic and genetically stable mutants can now be used in *in vitro* competition studies and greenhouse/field tests to test their efficacy as biocontrol agents. The release of genetically engineered *A. flavus* and *A. parasiticus* in commercial fields may help to avoid the problems associated with the use of currently available biocontrol agents.

CHAPTER II

CLONING OF A ß-TUBULIN GENE FROM A BENOMYL RESISTANT MUTANT OF *ASPERGILLUS PARASITICUS* AND ITS USE AS A DOMINANT SELECTABLE MARKER

INTRODUCTION

Genetic transformation provides one method to isolate specific genes and allows the study of gene expression and function by in vitro manipulation of the cloned genes and reintroduction into the organism. In previous work, the nitrate reductase structural gene (*niaD*) and the gene encoding orotidine monophosphate decarboxylase (*pyrG*) in *A. parasiticus* have been cloned and used to transform the corresponding auxotrophic fungal strains (Horng et al., 1990; Skory et al., 1990). These auxotrophic markers have been used recently to clone genes associated with aflatoxin biosynthesis by constructing cosmid genomic DNA libraries and screening them by complementation of mutants blocked in aflatoxin biosynthesis (Chang et al., 1992; Skory et al., 1992). These two genes were also used as selectable markers in experiments involved in the study of gene function (integrative inactivation) or gene expression in *A. parasiticus* (Wu and Linz, 1993; Liang and Linz, 1994; Trail et al., 1994; Trail et al., 1995a; Mahanti et al., 1995 in press). Even though these transformation systems were successfully applied to genetic studies in *A. parasiticus*, it would be advantageous to obtain a dominant selectable marker for use in transformation without requiring the generation of a particular mutation in the recipient strain. A dominant selectable marker would be especially important in developing gene disruption constructs which will allow the study of the function of the cloned aflatoxin associated genes by introducing directed mutations in a wild type genetic background.

Benomyl is a fungicide which inhibits the polymerization of tubulin monomers

into functional microtubules. Most mutations conferring benomyl resistance in fungi such as *Neurospora crassa* and yeast result from a single amino acid change which maps in the ß-tubulin structural gene (Thomas et al., 1985; Orbach et al., 1986). Preliminary investigations demonstrated that growth of *A. parasiticus* is completely inhibited by benomyl at a concentration of 5 µg/ml (Horng, 1989, personal communication). Because it is sensitive to benomyl, *A. parasiticus* potentially could be transformed with a gene conferring benomyl resistance. The amino acid sequences of proteins in the tubulin superfamily are highly conserved among evolutionarily diverse organisms (Cleveland et al., 1980; Orbach et al., 1986; Oakley and Oakley, 1989). This conservation allowed tubulin genes to be cloned and characterized from many different organisms using heterologous genes as probes to screen genomic DNA libraries (Cleveland and Sullivan, 1985; Oakley and Oakley, 1989; Orbach et al., 1986; Ossanna and Mischke, 1989; Cameron et al., 1990; Seip et al., 1990; Cooley and Caten, 1993; Goldman et al., 1993).

The hypothesis associated with this study is that an efficient transformation system in *A. parasiticus* can be developed using a ß-tubulin gene conferring benomyl resistance. In this report, we describe the isolation of a DNA fragment from a benomyl resistant mutant of *A. parasiticus* which has a high degree of identity to the *N. crassa tub*-2^r gene. The portion of this DNA fragment carrying the *ben*^{r} gene was completely sequenced and successfully used as a dominant selectable marker in transformation of *A. parasiticus*.

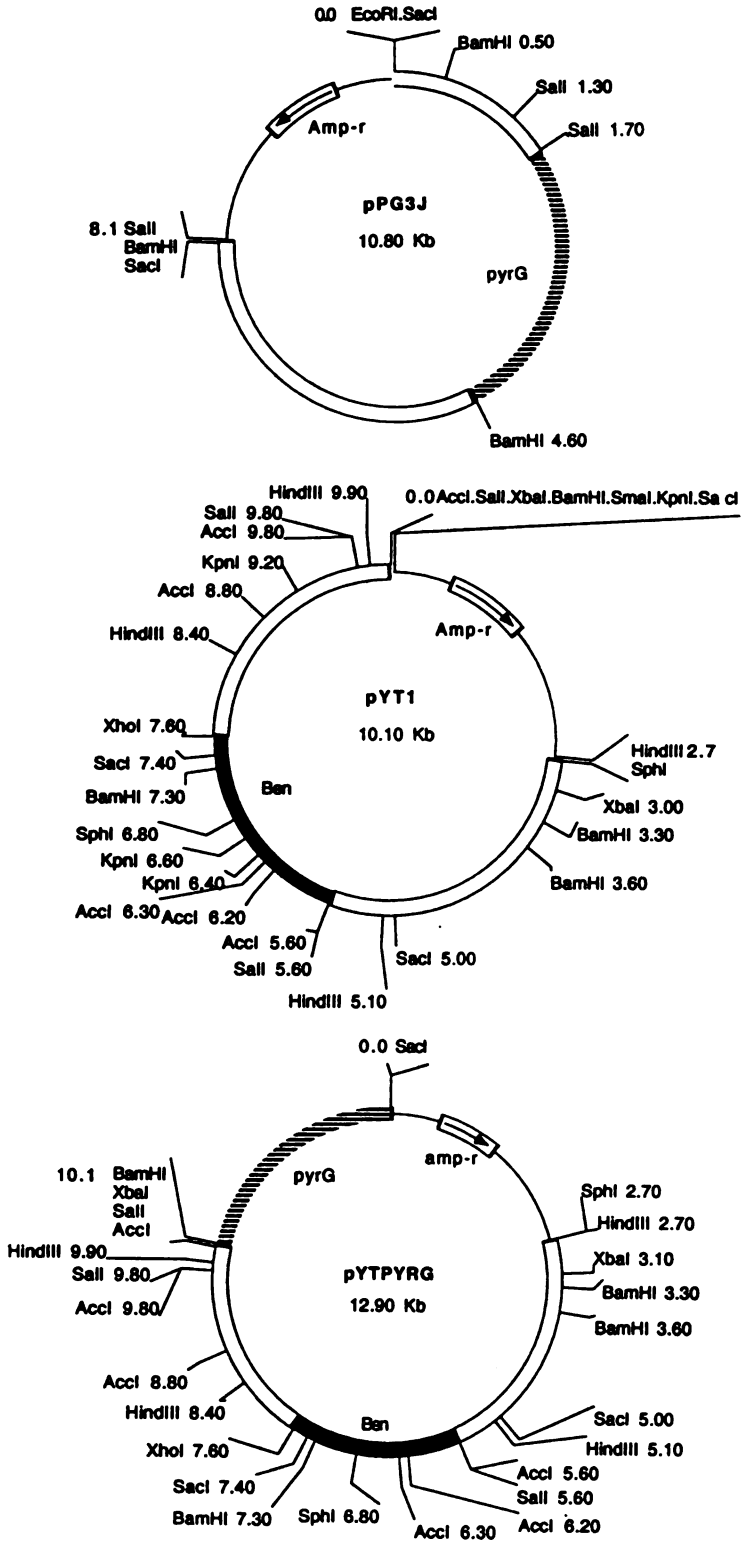
MATERIALS AND METHODS

Strains and plasmids

Escherichia coli DH5α F'^{qe} [F'/*endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1*Δ (*lacZYA-argF*)$_{u169}$:(m80 *lacZ*Δ *M15*)] (Gibco BRL, Life Technologies, Inc. Gaithersburg, MD) was used to propagate plasmid DNA. *A. parasiticus* NRRL5862 (SU-1, Bennett, 1979), a wild type aflatoxin-producing strain and *A. parasiticus* CS10 (Skory et al., 1990), an orotidine monophosphate decarboxylase deficient (*pyr*G) mutant strain derived from *A. parasiticus* ATCC36537 (Bennett and Goldblatt, 1973) (*wh*-1, *ver*-1; accumulates versicolorin A) were the recipient strains in transformation experiments. Plasmid pYT1 (Figure 8), containing a putative mutant ß-tubulin allele (*ben*^{r}) from a benomyl resistant (Ben^R) mutant strain of *A. parasiticus*, or plasmid pYTPYRG (Figure 8), containing the *A. parasiticus ben*^{r} plus the *A. parasiticus pyr*G gene, were used to transform *A. parasiticus* CS10. pYT1 was generated by subcloning a 7.4-kb *Nsi*I genomic DNA fragment isolated from a benomyl resistant mutant of *A. parasiticus* into the *Pst*I site of pUC19. This subcloned fragment carries a putative *ben*^{r} gene. pYTPYRG was constructed by subcloning the 2.9-kb *Sal*I/*Bam*HI fragment from pPG3J, which contained a functional copy of the *pyr*G gene, into the *Sma*I site of pYT1. For cotransformation experiments pYT1 was used in combination with pPG3J (Figure 8) (Skory et al., 1990) which contains a functional *pyr*G gene. In addition a heterologous *ben*^{r} gene from *N. crassa*, carried on plasmid pBT3, was tested for expression in *A. parasiticus*. Plasmid pBT3 (kindly provided by Marc J. Orbach) contains a mutant ß-tubulin allele (a 3.1-kb *Hin*dIII fragment) from

Figure 8. Restriction endonuclease maps of plasmids pPG3J, pYT1, and pYTPYRG.

Figure 8.


0.0 EcoRI.SacI
BamHI 0.50
SalI 1.30
SalI 1.70
Amp-r
pPG3J
10.80 Kb
pyrG
8.1 SalI
BamHI
SacI
BamHI 4.60
HindIII 9.90
0.0 AccI.SalI.XbaI.BamHI.SmaI.KpnI.Sa cI
SalI 9.80
AccI 9.80
KpnI 9.20
AccI 8.80
HindIII 8.40
Amp-r
pYT1
10.10 Kb
XhoI 7.60
SacI 7.40
BamHI 7.30
Ben
SphI 6.80
KpnI 6.60
KpnI 6.40
AccI 6.30
AccI 6.20
AccI 5.60
SalI 5.60
HindIII 5.10
SacI 5.00
HindIII 2.7
SphI
XbaI 3.00
BamHI 3.30
BamHI 3.60
0.0 SacI
amp-r
pyrG
10.1 BamHI
XbaI
SalI
AccI
HindIII 9.90
SalI 9.80
AccI 9.80
pYTPYRG
12.90 Kb
AccI 8.80
HindIII 8.40
XhoI 7.60
SacI 7.40
BamHI 7.30
SphI 6.80
Ben
AccI 6.30
AccI 6.20
SalI 5.60
AccI 5.60
HindIII 5.10
SacI 5.00
SphI 2.70
HindIII 2.70
XbaI 3.10
BamHI 3.30
BamHI 3.60

a benomyl-resistant strain of *N. crassa* subcloned into the *Hin*dIII site of pUC19 (Orbach et al., 1986).

Media and culture conditions

YES (2% yeast extract, 6% sucrose, pH 5.8) supplemented with 100 µg/ml uridine was used as a liquid culture medium for *A. parasiticus* CS10. Protoplasts of *A. parasiticus* CS10 transformed with *pyr*G containing plasmids were selected for uridine prototrophy on Czapek-Dox agar (Difco, Detroit, MI) containing 0.6 M KCL. The level of benomyl resistance of uridine prototrophs was determined on Czpaek-Dox agar (CZ agar) containing 0, 1.0, 2.0, 3.0, or 4.0 µg/ml of benomyl (Dupont CO, technical grade, 98% pure). Benomyl was prepared and added to the media as described by Orbach (Orbach et al., 1986). Benomyl resistant transformants were cultured and harvested on potato-dextrose agar (Difco, Detroit, MI) supplemented with 0.5 µg/ml of benomyl.

Isolation of benomyl resistant mutant strains (by Dr. Skory)

Conidia of *A. parasiticus* ATCC36537 were grown in CZ media for 6-8 hours, harvested by resuspension in 0.1 M Tris-maleate [pH 8.0] supplemented with 1.5% (w/v) sucrose and 0.1% (w/v) $(NH_4)_2SO_4$ and mutagenized using N-methyl-N'-Nitro-N-Nitrosoguanidine (Sigma Chemical Co. St. Louis, MO) at a concentration of 100 µg/ml for one hour. Spores were mixed with 10 ml of PDA and screened for resistance to benomyl on PDA containing 7.5 µg/ml benomyl. Conidia from benomyl resistant colonies which appeared after incubation at 30°C for 4 days were subjected to a second round of mutagenesis under the same conditions, incubated in PDB for

one and a half hours, and screened for benomyl resistance (20 µg/ml) on PDA agar. One benomyl resistant isolate was selected for genomic DNA purification to generate a DNA library.

Isolation of fungal genomic DNA

High molecular weight genomic DNA was isolated from mycelia using the procedure of Cihlar and Sypherd (1980) as modified by Horng (1989). Mycelia were ground in a mortar and pestle in the presence of liquid nitrogen and resuspended in TSE buffer (0.1 M Tris, 0.15 M NaCl, and 0.1 M EDTA, pH 8.0). Then sodium dodecylsulfate (SDS) and proteinase K were added to 1% (w/v) and 100 µg/ml, respectively.

Construction and screening of the genomic DNA library (by Dr. Horng)

High molecular weight genomic DNA purified from the benomyl resistant strain of *A. parasiticus* ATCC36537 (*wh*-1, *ver*-1, *ben*^{r}) was partially digested with *Sau*3AI. DNA fragments, 15 to 20-kb in size, were isolated by sucrose gradient centrifugation and cloned into the *Bam*HI arms of lambda EMBL3 (Promega Biotec). Recombinant phage DNAs were packaged according to the protocol provided by the supplier. The lambda DNA library was screened by *in situ* plaque hybridization (Maniatis et al., 1989) with a radiolabeled 2.6-kb *Sal*I fragment (*tub*-2^r) excised from pBT3. This DNA fragment contained a mutant allele of a *N. crassa* ß-tubulin gene (*tub*-2) conferring resistance to benomyl.

Transformation of fungal protoplasts

Transformation of *A. parasiticus* protoplasts in the presence of polyethylene glycol (av.mol.wt.3350; Sigma Chemical Co. St.Louis, MO) was performed essentially as

described by Oakley (Oakley et al., 1987), with modifications (Skory et al., 1990). Plasmid DNA used for transformation was prepared by alkaline lysis and CsCl density gradient centrifugation (Maniatis et al., 1989). In some transformation experiments pYT1 was linearized by the restriction endonuclease *Sma*I prior to transformation. Other plasmids were used in circular form. Protoplasts from *A. parasiticus* CS10, transformed by pYT1 or pBT3, were regenerated in 5 ml YES containing uridine (100 μg/ml) at 30°C for 24 h, washed twice with distilled water, and spread on CZ agar containing benomyl (0.0 to 4.0 μg/ml) and uridine (100 μg/ml) for direct selection for benomyl resistance.

Southern hybridization analysis

Southern hybridization analyses were performed as described by Maniatis (Maniatis et al., 1989) using radiolabeled probes.

Study of the expression of *ben*^{r} and mitotic stability

One *ben*^{r} transformant, transformant 80 was subjected to single spore isolation and allowed to complete the full vegetative growth cycle on either PDA containing 1.0 μg/ml benomyl or PDA containing 100 μg/ml uridine. The percentage of Ben^r viable spores was estimated by parallel plating of appropriate dilutions onto selective and nonselective media.

Restriction enzymes and chemicals

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN (BMB). Enzyme digestion and agarose gel electrophoresis were performed according to standard procedures (Maniatis et al., 1989). Radiolabeled

DNA probes were generated with a Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) with [α-^{32}P] dCTP (Dupont) according to the supplier's instructions.

Nucleotide sequence analysis

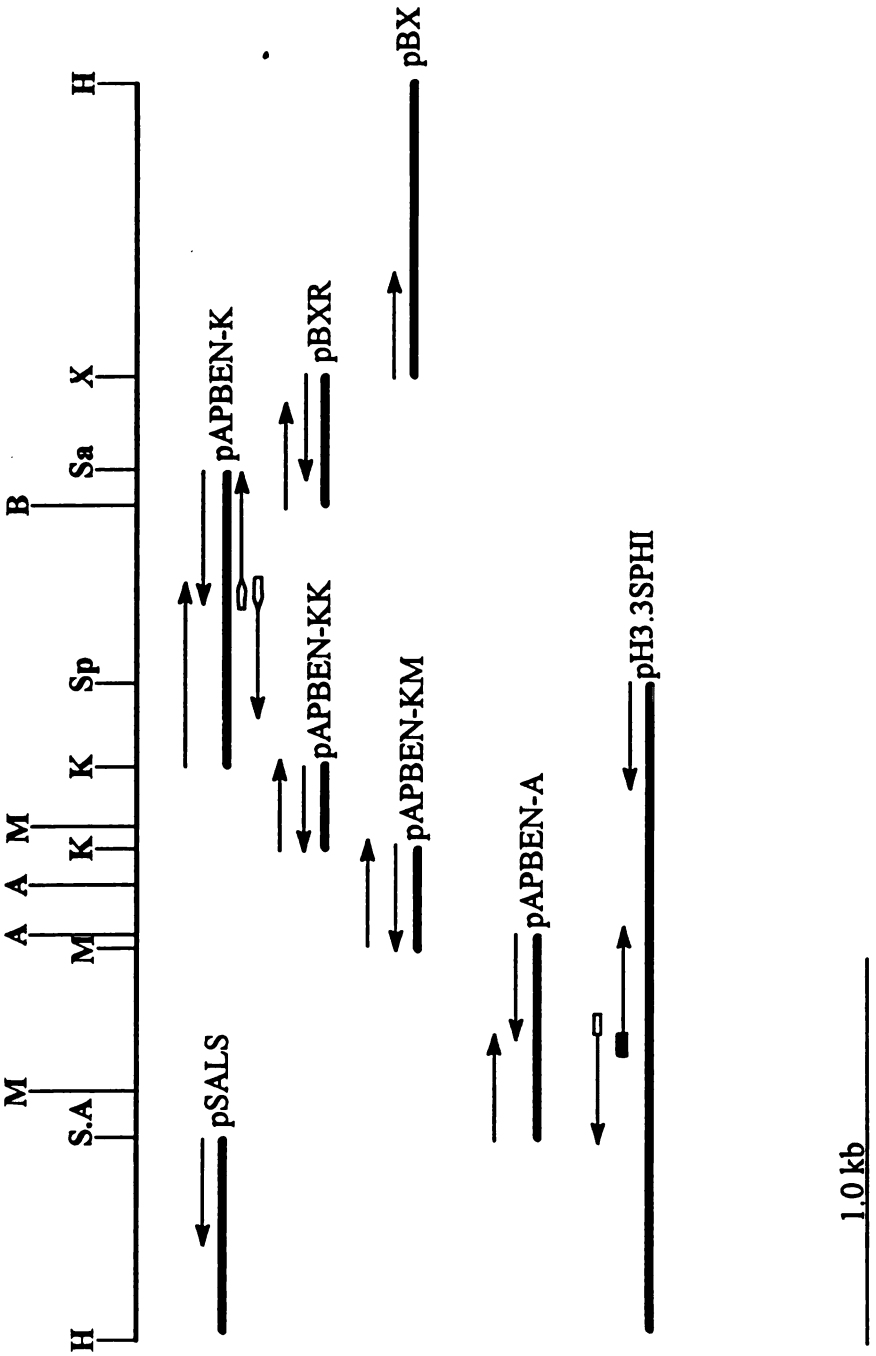
Nucleotide sequence analysis was performed by the dideoxy chain termination method (Sanger et al., 1977). DNA fragments derived from the *ben*^{r} gene in pYT1 were generated by appropriate restriction endonucleases and subcloned into pBLUESCRIPT SKII (Strategene Cloning systems, La Jolla, Calif.) or pUC19 to generate the plasmids pSALS (a 543 bp *Hin*dIII/*Sal*I subclone), pH3.3SPHI (a 1.73-kb *Hin*dIII/*Sph*I subclone), pAPBEN-A (a 530 bp *Acc*I/*Acc*I subclone), pAPBEN-KM (a 258 bp *Msp*I/*Kpn*I subclone), pAPBEN-KK (a 213 bp *Kpn*I/*Kpn*I subclone), pAPBEN-K (a 768 bp *Kpn*I/*Sac*I subclone), pBXR (a 330 bp *Bam*HI/*Xho*I subclone), and pBX (a 760 bp *Xho*I/*Hin*dIII subclone) (Figure 9). Cloned inserts were sequenced on both strands except pSALS and pBX. Primers used for sequencing are T_7, T_3, M13/pUC 17 mer sequencing primer (-20) 5'd(GTAAAACGACGGCCAGT)3' (New England Biolabs, Beverly, MA), and four synthetic oligonucleotides 5'd(CG(AGCT)CTATGATTTGATGTT)3', 5'd(TTTGTTTCATCATATAGGTT), 5'd(GCTTCGCTCCTCTGACCAGC)3',and5'd(GCTGGTCAGAGGAGCGAAGC)3'.

Computer analysis of sequence data

Computer analysis of sequence data was performed using the Wisconsin Genetic Computer Group package. The locations of the translation start codon, introns, the translation stop codon, and deduced amino acids were predicted by the

Figure 9. Strategy used for sequencing a 2.16-kb DNA fragment containing the *ben*^{r} gene isolated from a benomyl resistant mutant strain of *A. parasiticus*. The direction of each arrow indicates the strand which was sequenced. The open-box, filled-box, and open arrows indicate regions sequenced with the synthetic oligonucleotide primers 5'd(CG(AGCT)CTATGATTTGATGTT)3', 5'd(TTTGTTTCATCATATAGGTT), 5'd(GCTTCGCTCCTCTGACCAGC)3',and5'd(GCTGGTCAGAGGAGCGAAGC)3', respectively. Abbreviations: H, *Hin*dIII; S, *Sal*I; A, *Acc*I; M, *Msp*I; K, *Kpn*I; Sp, *Sph*I; B, *Bam*HI; Sa, *Sac*I; X, *Xho*I.

Figure 9.


H
M
S.A
A
M
A
A
M
K
K
Sp
B
Sa
X
H
pSALS
pAPBEN-K
pAPBEN-KK
pAPBEN-KM
pBXR
pBX
pAPBEN-A
pH3.3SPHI
1.0 kb

softwareprograms Frame, Test-code and Codon preference.

RESULTS

Isolation of a putative *A. parasiticus* ß-tubulin gene (by Dr. Horng)

A strain of *A. parasiticus* ATCC36537, resistant to 20 µg/ml of benomyl, was isolated. Southern hybridization analyses of genomic DNA purified from this Ben^R strain and the Ben^S parental strain were performed using the *N. crassa tub*-2^r gene as the probe. No differences in hybridization pattern were observed in these two strains, indicating that the Ben^R phenotype was not due to a major genetic rearrangement. A genomic DNA library was constructed in lambda EMBL3 using genomic DNA purified from this mutant strain. A total of 12,000 individual clones from the library were screened by *in situ* plaque hybridization under high stringency (Maniatis et al., 1989) using the *N. crassa tub*-2^r gene as the probe. Three recombinant phage clones hybridized to the *N.* crassa *tub*-2^r probe. DNAs were purified from each of these three clones and compared by restriction enzyme analysis. Two of the clones were shown to be identical. Southern hybridization analysis was used to compare DNA isolated from the 2 unique phage clones with genomic DNA purified from wild type *A. parasiticus* NRRL5862. Under high stringency hybridization and wash conditions, these DNAs showed identical hybridization patterns using the *N. crassa tub*-2^r probe suggesting that the two phage clones contained at least a portion of a ß-tubulin gene and that no major genetic rearrangement occurred during library construction. In individual digests of the DNA with several different restriction enzymes, only one

restriction fragment hybridized to the heterologous *N. crassa tub-2*^{r} probe, indicating that either there is only one ß-tubulin gene in *A. parasiticus* or two copies of the gene are tightly linked together. A 7.4-kb *Nsi*I *A. parasiticus* genomic DNA fragment in both unique phage clones was found to hybridize strongly to the *N. crassa tub-2*^{r} probe. This 7.4-kb fragment was excised and subcloned into pUC19 to generate pYT1.

Transformation and expression of the putative *A. parasiticus ben*^{r} gene

In preliminary experiments, the growth of *A. parasiticus* CS10 was shown to be completely inhibited by benomyl at a concentration of 5.0 µg/ml. A more precise analysis of the minimum inhibitory concentration (MIC) determined that conidia of *A. parasiticus* CS10 were sensitive to as little as 1.0 µg/ml benomyl on PDA agar. *A. parasiticus* CS10 (Ben^s) was transformed using plasmid pYTPYRG (5 µg) or pYT1 (8 µg) in combination with pPG3J (2 µg). The frequency of transformation to *pyr*G$^+$ with pYTPYRG was approximately 10 transformants per µg of DNA. Of the 225 *pyr*G$^+$ transformants analyzed 103 (46%) were resistant to benomyl at a concentration of 1.0 µg/ml (Table 1). When *A. parasiticus* CS10 was cotransformed by pYT1 (8 µg) in combination with pPG3J (2 µg), the frequency of transformation to *pyr*G$^+$ was similar to that of pYTPYRG alone (approximately 10 transformants per µg of pPG3J), but a very low cotransformation efficiency (less than one transformant per µg of pYT1) was observed when these clones were screened for resistance to benomyl. When equal quantities of pYT1 and pPG3J (2 µg each) were used in cotransformation, none of 40 *pyr*G$^+$ clones were found to be benomyl resistant.

Table 1. Transformation of *A. parasiticus* CS10 with pPG3J, pYTPYRG, or pYT1 in combination with pPG3J*.

	plasmid used and days of growth								
benomyl	pPG3J			pYTPYRG			pYT1/pPG3J		
(µg/ml)	3d	5d	7d	3d	5d	7d	3d	5d	7d
0.0	42	42	42	225	225	225	246	246	246
1.0	0	0	0	31	71	103	32	55	59
2.0	0	0	0	9	13	15	6	8	17
3.0	0	0	0	1	1	3	2	6	7
4.0	0	0	0	0	0	0	0	0	0

*A. *parasiticus* CS10 was transformed with pPG3J, pYTPYRG, or pYT1 in combination with pPG3J. Uridine independent colonies on CZ agar were replica plated on CZ agar containing various concentrations of benomyl. The numbers of benomyl resistant transformants after 3, 5, and 7 days of growth are indicated.

A. parasiticus CS10 was also transformed by circular pYT1, linear pYT1 or pBT3. Transformants were selected directly for resistance to benomyl on CZ agar containing uridine (100 μg/ml). The frequency of transformation by circular pYT1 to benomyl resistance (approximately 5 transformants per μg of DNA) was similar to that of pYTPYRG. With linear pYT1, the transformation frequency was approximately two fold higher than that of circular pYT1. The frequency of transformation of *A. parasiticus* with pBT3 was very low (less than one transformant per μg of DNA).

A. parasiticus CS10 was also transformed with pPG3J as a control. Of 42 *pyrG*$^+$ transformants, no benomyl resistant transformants were observed (at 1.0 μg/ml). Growth on benomyl was only observed when *A. parasiticus* protoplasts were transformed with a construct containing a *ben*^{r} gene.

Southern hybridization analysis

Southern hybridization analyses were performed on genomic DNAs isolated from the recipient strain (*A. parasiticus* CS10), Ben^S, and Ben^R transformants. Genomic DNAs were digested with *Sac*I and hybridized to the cloned *A. parasiticus ben*^{r} gene fragment (a 2.6-kb *Acc*I fragment excised from pYT1) or pUC19 (Figure 10A and 10B). The 2.6-kb *Acc*I fragment contains the region of pYT1 which hybridized to the *tub*-2^r gene from pBT3. Two DNA fragments which hybridized to the *N. crassa tub*-2^r probe (2.3-kb and 6.3-kb) were observed in genomic DNAs from the untransformed recipient strain or cells transformed with pPG3J. Since *Sac*I cuts once within the 2.6-kb *A. parasiticus ben*^{r} fragment, these fragments represent the native *ben*^{s} genomic

Figure 10. Southern blot analysis of benomyl-sensitive (Ben^S) and resistant (Ben^R) transformants of *A. parasiticus* CS10. Lane pPG3J, genomic DNA from *A. parasiticus* CS10 (isolate 2) transformed with pPG3J; lane CS10, genomic DNA from the untransformed *A. parasiticus* CS10; lanes 1 to 7, seven *pyr*G^+ transformants (isolates 155, 156, 49, 80, 94, 136, and 219, respectively) of *A. parasiticus* CS10 transformed with circular pYTPYRG, including two Ben^S isolates (isolates 155 and 156) and five Ben^R isolates (isolates 49, 80, 94, 136, and 219); lanes 8 to 13, six *pyr*G^+ transformants transformed with pYT1 in combination with pPG3J (isolates 1, 3, 95, 120, 173, and 178, respectively), including two Ben^S isolates (isolates 1 and 3) and four Ben^R isolates (isolate 95, 120, 173, and 178). Molecular weight markers are indicated at left (*Hin*dIII digested lamda DNA).

Figure 10A.

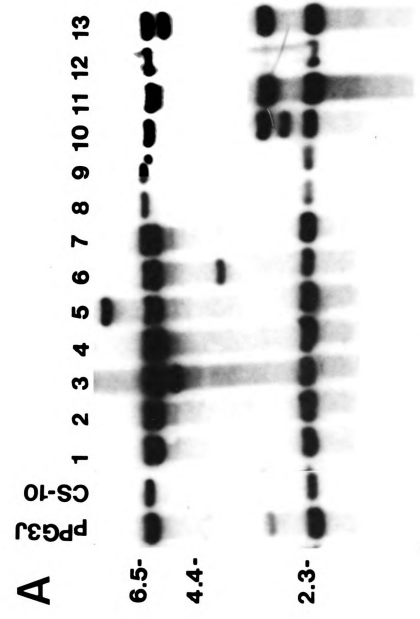
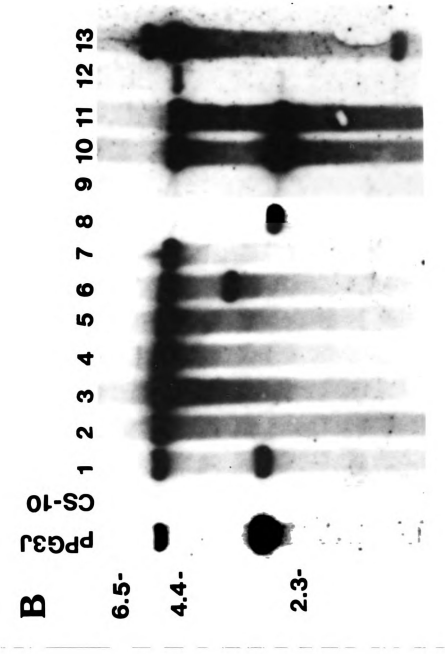


Figure 10B.



DNA fragments. The benr probe hybridized to three DNA fragments (6.3-kb, 6.0-kb, and 2.3-kb) in genomic DNA from transformants 155, 156, 80 and 219 consistent with fragment sizes predicted by a single crossover homologous recombination event at the ß-tubulin locus. Transformants 49, 94 and 136 produced more complicated restriction patterns with the benr probe. In each of these transformants an additional fragment was observed, suggesting that an additional copy of the benr gene may have integrated at a heterologous site. In support of this observation, transformants 49 and 136 were found to be resistant to slightly higher levels of benomyl (4.0 µg/ml), consistent with a gene dosage effect. Surprisingly, the integration of pYTPYRG at the bens locus could result in either Ben^S or Ben^R phenotype. This result suggested that in Ben^S transformants 155 and 156 gene conversion may have occurred before or after integration of pYTPYRG, causing the conversion of the benr allele on the plasmid to the wild-type allele, and resulting in *pyr*G^+, bens daughter nuclei. The Ben^S phenotype of transformants 155 and 156 could also result from the inactivation of the integrated gene, a phenomonom observed in *N. crassa* and higher plants (Selker et al., 1987; Selker and Garrett, 1988; Castro-Prado and Zucchi, 1993). When genomic DNAs from these transformants were hybridized to pUC19 (Figure 10B), a 4.7-kb *Sac*I fragment was observed, consistent with integration of plasmid pYTPYRG at the chromosomal bens locus. In addition to the 4.7-kb fragment, 2.7-kb and 4.0-kb fragments were observed for transformants 155 and 136, respectively (Figure 10B). These fragments were most likely from the heterologous integration at the 5.0-kb *Sac*I fragment of pYTPYRG.

Transformants 1 and 3 isolated from a cotransformation experiment were *pyr*G$^+$ and Ben^S. Southern analysis of genomic DNAs from transformants 1 and 3 showed two bands which hybridized to the *A. parasiticus ben*^{r} gene probe in a pattern identical to that of the recipient strain (Figure 10A). One 2.7-kb band was observed in transformant 1 using a pUC19 probe (Figure 10B). No bands hybridizing to pUC19 were observed for transformant 3. These data suggested that pPG3J integrated into the chromosomal DNA at a site other than the *ben*^{s} locus in transformant 1. In transformant 3, the data are consistent with a gene replacement or gene conversion event between the *pyr*G gene on pPG3J and the genomic *pyr*G locus. The data also suggested that pYT1 containing the *ben*^{r} gene did not integrate resulting in the Ben^S phenotype in transformant 1 and 3. The restriction patterns of *pyr*G$^+$, Ben^R transformants 95, 120, 173 and 178 with the *ben*^{r} probe suggested that pYT1 integrated at the chromosomal *ben*^{s} locus and pPG3J integrated at another site. The hybridization data with the pUC19 probe (Figure 10B) are consistent with this interpretation. The additional bands observed for transformants 95 and 178 with the *ben*^{r} probe suggested that pYT1 may have integrated at a heterologous site in addition to the homologous site. These multiple integrations of pYT1 appear to have resulted in multiple copies of the *ben*^{r} gene in the chromosome. Transformant 95 and 178 were found to be more resistant to benomyl (3.0 μg/ml) than transformants 120 and 173 (1.0 μg/ml), consistent with this observation.

The genomic DNAs from benomyl resistant isolates transformed with circular pYT1 or pBT3 showed no pUC19 sequences (Figure 11A). No difference in the

Figure 11. Southern hybridization analysis of genomic DNAs from *A. parasiticus* CS10 (recipient) and isolates transformed with pBT3, pYT1, and linear pYT1. (A) Genomic DNAs were digested to completion with *Acc*I and hybridized with a radiolabeled pUC19 probe. (B) Identical genomic DNA samples were hybridized with a radiolabeled *ben*^{r} probe (a 2.6-kb *Acc*I fragment from pYT1). Lane CS10, genomic DNA from the untransformed *A. parasiticus* CS10; lane 1, a Ben^{R} pBT3 transformant (isolate 3); lane 2 to 4, three pYT1 transformants (isolates 1, 6, and 7, respectively) including a Ben^{S} transformant (isolate 1) and two Ben^{R} transformants (isolates 6 and 7); lane 5 to 9, five *A. parasiticus* CS10 isolates transformed with linear pYT1 (isolates 8, 11, 12, 14, and 15, respectively) including a Ben^{S} transformant (isolate 8) and four Ben^{R} transformant (isolates 11, 12, 14, and 15). DNA size standards were *Hin*dIII-digested lambda DNA (in kilobases).

Figure 11.

hybridization pattern in these isolates was observed from that of the recipient strain (CS10) when hybridized to the *ben*^{r} probe (Figure 11B), suggesting that a gene replacement or gene conversion may have occurred at the *ben*^{s} locus, resulting in a single copy of the *ben*^{r} allele. pYT1 transformants were resistant to higher levels of benomyl (up to 3.0 µg/ml) than pBT3 transformants, which were only resistant to low levels of benomyl (1.0 µg/ml). Multiple integrations of the *ben*^{r} gene were observed in cells transformed with linear pYT1. These transformants were also resistant to relatively high levels of benomyl (3.0 µg/ml).

Study of the expression of *ben*^{r} and mitotic stability

Based on two independent experiments, 80 to 100% of the viable spores of pYTPYRG transformant isolate 80 were able to grow on CZ containing 1.0 µg/ml of benomyl, indicating that expression of *ben*^{r} in either the *ben*^{r}/*ben*^{r} or the *ben*^{s}/*ben*^{r} genetic background is not significantly repressed under the conditions applied. Spores parallel plated on CZ or CZ plus uridine were counted to estimate the excision rate. Based on two independent experiments, the transformed phenotype (Ben^R) of transformant 80 remained stable after one generation on the nonselective medium, indicating that the mitotic stability is relatively high.

Nucleotide sequence analysis

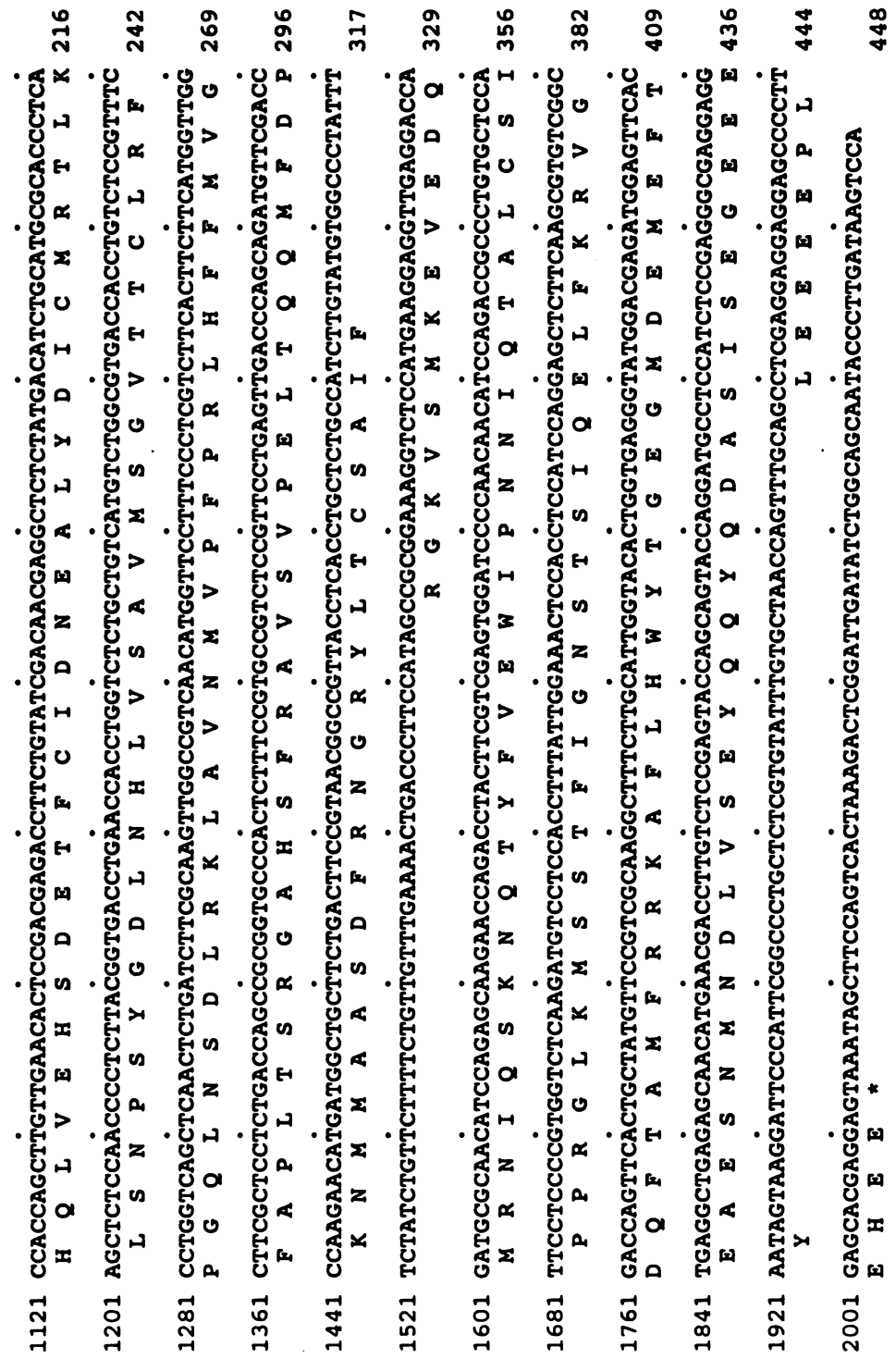
A 1,986 bp fragment of the *ben*^{r} gene was sequenced on both strands. The sequence of 91 bp at the 3'-end of *ben*^{r} (Figure 12) was obtained on the noncoding strand. The coding region of *ben*^{r} gene was located by making a comparison to the published sequence of a benomyl resistant β-tubulin gene from *A. flavus* (Seip et al., 1990).

Figure 12. Nucleotide sequence and deduced amino acid sequence of the noncoding strand of the putative *A. parasiticus* benomyl resistant β-tubulin gene (*ben*^{r}). Numbers indicated in the left-hand margin represent the position of the first nucleotide in each line; numbers indicated in the right-hand margin represent the position of the last amino acid in the line.

Figure 12.

```
   1 CGACTGTCTCCTCCAGTCTATCCAACAACCCCTCTCCAACGACCTCTTCGCCTTTTTCAAACCCACCTTTCCTACCAAC
                                                                                    .
  81 AACGCCAATATTCCCTCCACAATGCGTGAGATCGTATGTTGCTCCCTACCCCCCCGGTCCCCCCTCTTCAGACGAGTTTT
                M  R  E  E  I                                                       4
 161 TCGGGGATACCCCCCCCTGATAGGGACCCCACCCGTTCTCCAATACTACAAGGTTGCTGACGGAGTTTGTTTCATCATATA
                                                                                    .
 241 GGTTTACCTTCAGACCGGCCAGTGTGTAAGTTCGACTATGATTTGATGTTTAGAAGGACCATGGCGACGGATACTAAATG
       V  Y  L  Q  T  G  Q  C                                                      12
 321 TGATATTGGTGATAGGGTAACCAAATAGGTGCCGCTTTCTGGTATGTCTCGATACCTTCGAGTTAATATGCTTTGGACCA
                G  N  Q  I  G  A  A  F  W                                          21
 401 AGGAACTCCTCAAAAGCATGATCTCGGATGTGTCCTATTATATCTGCCACGTGTTTGCTAACAACTTTGCAGGCAAACCA
                                                                  Q  T  I           24
 481 TCTCTGGCGAGCACGGCCTTGACGGCTCCGGTGTGTAAGTACAGCCTGTATACACCTCGAACGAACGACGACCATATGGC
          S  G  E  H  G  L  D  D  G  S  G  V                                       35
 561 ATTAGAAGTTGGAATGGATCTGACGGCAAGGATAGTTACAATGGCTCCTCTGATCTCCAGCTGGAGCGTATGAACGTCTA
                                                Y  N  G  S  S  D  L  Q  L  E  R  M  N  V  Y  50
 641 CTTCAACGAGGTGCGTACCTCAAAATTTTCAGCATCTATGAAAACGCTTTGCAACTCCTGACCGCTTCTCCAGGCCAGCG
       F  N  E                                                            A  S  G     56
 721 GAAACAAGTATGTCCCTCGTGCCGTCCTCGTGGATCTTGAGCCTGGTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGT
          N  K  Y  V  P  R  A  V  L  V  D  L  E  P  G  T  M  D  A  V  R  A  G  P  F  G     82
 801 CAGCTCTTCCGTCCCGACAACTTCGTTTTCGGCCAGTCCGGTGCTGGTAACAACTGGGCCAAGGGTCACTATACTGAGGG
          Q  L  F  R  P  D  N  F  V  F  G  Q  S  G  A  G  N  N  W  A  K  G  H  Y  T  E  G  109
 881 TGCCGAACTTGTTGACCAGGTTGTCGATGTTGTTCGTCGCGAGGCTGAGGGCTGCGACTGCCTCCAGGGTTTCCAGATTA
          A  E  L  V  D  Q  V  V  D  V  V  R  R  E  A  E  G  C  D  C  L  Q  G  F  Q  I  T  136
 961 CCCACTCCCTCGGTGGTGGTACCGGTGCCGGTATGGGTACTCTCCTGATCTCCAAGATCCGTGAGGAGTTCCCCGACCGT
             H  S  L  G  G  G  T  G  A  G  M  G  T  L  L  I  S  K  I  R  E  E  F  P  D  R  162
1041 ATGATGGCCACCTTCTCCGTTATGCCCTCCCCCAAGGTCTCCGACACCGTTGTTGAGCCTTACAACGCCACTCTTTCCGT
       M  M  A  T  F  S  V  M  P  S  P  K  V  S  D  T  V  V  E  P  Y  N  A  T  L  S  V  189
```

Figure 12. (Cont'd)



Seven introns were predicted in the *ben*^{r} gene and the positions of these introns are identical to those in the *A. flavus* β-tubulin gene. The *ben*^{r} gene in *A. parasiticus* codes for a protein of 448 amino acids. An amino acid sequence comparison was made between the benomyl resistant β-tubulin genes from *A. parasiticus* (*ben*^{r}), *A. flavus*, *Septoria nodorum* (*tub*A^R), and *Trichoderma viridie* (*tub*2) (Figure 13).

Discussion

The imperfect fungi *A. flavus* and *A. parasiticus* are strictly anamorphic species and thus lack sexual reproduction. The lack of a sexual reproduction cycle makes traditional Mendelian genetic analysis of aflatoxin biosynthesis problematic. The parasexual cycle has been demonstrated in *A. parasiticus* (Papa, 1978; Bennett et al., 1980). Parasexual analysis allows crude genetic analysis of aflatoxigenic molds. However, it is unreliable and laborious to perform and several technical problems are inherent with this analysis (Bennett,1979; Bennett et al., 1980; Papa, 1986). The lack of convenient classical genetic analysis makes the development of modern genetic engineering techniques necessary in *A. flavus* and *A. parasiticus*. A transformation system is generally considered an essential step in modern genetic engineering techniques (Johnstone, 1985; Wernas et al., 1987; Frederick et al., 1989; Timberlake and Marshall, 1988). Transformation requires the presence on the transforming plasmids of a selectable marker allowing selective growth of transformed colonies. Selectable markers fall into two broad classes: auxotrophic markers and dominant markers. The use of auxotrophic markers in a transformation system is often tedious

Figure 13. Comparison of the predicted amino acid sequences of benomyl resistant β-tubulins from filamentous fungi. The amino acids conferring benomyl resistance in each β-tubulin gene are represented in boldface type and underlined. A. f: *A. flavus*; A. p: *A. parasiticus*; S. n: *Septoria nodorum*; T. v: *Trichoderma viride.*

Figure 13.

```
  1                                                                  50
A. f  MREIVHLQTG QCGNQIGAAF WQTISGEHGL DGSGVYNGSS DLQLERMNVY
A. p  MREIVYLQTG QCGNQIGAAF WQTISGEHGL DGSGVYNGSS DLQLERMNVY
S. n  MREIVYLQTG QCGNQIGAAF WQTISGEHGL DGSGVYNGTS DLQLERMNVY
T. v  MREIVYIQTG QCGNQIGAAF WQTISGEHGL DSNGIYNGSS ELQLERMNVY

      51                                                        100
A. f  FNEASGNKYV PRAVLVDLEP GTMDAVRAGP FGQLFRPDNF VFGQSGAGNN
A. p  FNEASGNKYV PRAVLVDLEP GTMDAVRAGP FGQLFRPDNF VFGQSGAGNN
S. n  FNEASGNKFV PRAVLVDLEP GTMDAVRAGP FGQLFRPDNF VFGQSGAGNN
T. v  FNEASNNKYV PRAVLVDLEP GTMDAVRAGP FGQLFRPDNF IFGQSSAGNN

      101                                                       150
A. f  WAKGHYTEGA ELVDQVVDVV RREAEGCDCL QGFQITHSLG GGTGAGMGTL
A. p  WAKGHYTEGA ELVDQVVDVV RREAEGCDCL QGFQITHSLG GGTGAGMGTL
S. n  WAKGHYTEGA ELVDQVLDVV RREREGCDCL QGFQITHSLG GGTGAGMGTL
T. v  WAKGHYTEGA ELVDQVLDVV RREAEGCDCL QGFQITHSLG GGTGSGMGTL

      151                                                       200
A. f  LISKIREEFP DRMMATYSVV PSPKVSDTVV EPYNATLSVH QLVEHSDETF
A. p  LISKIREEFP DRMMATFSVM PSPKVSDTVV EPYNATLSVH QLVEHSDETF
S. n  LISKIREEFP DRMMATFSVV PSPKVSDTVV EPYNATLSIH QLVENSDETF
T. v  LLSKIREEFP DRMMATFSVV PSPKVSDTVV EPYNATLSVH QLVENSDETF

      201                                                       250
A. f  CIDNEALYDI CMRTLKLSNP SYGDLNHLVS AVMSGVTTCL RFPGQLNSDL
A. p  CIDNEALYDI CMRTLKLSNP SYGDLNHLVS AVMSGVTTCL RFPGQLNSDL
S. n  CIDNEALYDI CMRTLKLNNP SYGDLNHLVS AVMSGVTTCL RFPGQLNSDL
T. v  CIDNEALYDI CMRTLKLNNP AYGDLNYLVS AVMSGITTCL RFPGQLNSDL

      251                                                       300
A. f  RKLAVNMVPF PRLHFFMVGF APLTSRGAHS FRAVSVPELT QQMFDPKNMM
A. p  RKLAVNMVPF PRLHFFMVGF APLTSRGAHS FRAVSVPELT QQMFDPKNMM
S. n  RKLAVNMVPF PRLHFFMVGF APLTSRGAHS FRAVTVPELT QQMFDPKNMM
T. v  RKLAVNMVPF PRLHFFMVGF APLTSPGAHS FRAVTVPELT QQMFDPKNMM

      301                                                       350
A. f  AASDFRNGRY LTCSAIFRGK VSMKEVEDQM RNIQSKNQTY FVEWIPNNIQ
A. p  AASDFRNGRY LTCSAIFRGK VSMKEVEDQM RNIQSKNQTY FVEWIPNNIQ
S. n  AASDFRNGRY LTCSAYFRGK VSMKEVEDQM RNVQNKNSSY FVEWIPNNVQ
T. v  AASDFRNGRY LTCCSIFRGK VAMKEVEDQM RNVQNKNSTY FVEWIPNNIQ

      351                                                       400
A. f  TALCSIPPRG LKMSSTFIGN STSIQELFKR VGDQFTAMFR RKAFLHWYTG
A. p  TALCSIPPRG LKMSSTFIGN STSIQELFKR VGDQFTAMFR RKAFLHWYTG
S. n  TALCSVPPRG LKMSATFVGN STSIQELFKR IGDQFTAMFR RKAFLHWYTG
T. v  TALCAIPPRG LKMSSTFIGN STSIQELFKR VGEQFSAMFR RKAFLHWYTG

      401                                                       449
A. f  EGMDEMEFTE AESNMNDLVS EYQQYQDASI SEGEEEYLEE EEPLEHEE*
A. p  EGMDEMEFTE AESNMNDLVS EYQQYQDASI SEGEEEYLEE EEPLEHEE*
S. n  EGMDEMEFTE AESNMNDLVS EYQQYQEASI SEGEEEY.DE EAPLEAEE*
T. v  EGMDEMEFTE AESNMNDLVS EYQQYQEAGI DEEEE..YED EAPMEAEE*
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due to the difficulty in generating corresponding mutant strains. For the cloning of genes from a multilocus biosynthetic pathway such as that of aflatoxigenic biosynthesis, it is required to generate the corresponding auxotrophic mutation in each of the pathway-blocked mutant strains. However, a dominant marker for selection of transformation offers the distinct advantage of not requiring the presence of a particular mutation in the recipients. The latter selection system also allows the introduction of directed mutations in aflatoxin associated genes in a wild type genetic background by gene disruption to study the identity of these genes and the potential function of these secondary metabolites in cell growth and development.

Benomyl is a benzenimidazole fungicide that inhibits polymerization of tubulin into heterodimers and thus prohibits fungal growth (Neff et al., 1983; May, 1989; MacRae, 1992; Iwasaki, 1993). Resistance of several microorganisms to benomyl has been determined to reside in ß-tubulin genes (Thomas et al., 1985; Orbach et al., 1986; Cooley and Caten, 1993; Goldman et al., 1993). These mutant alleles of ß-tubulin genes have been cloned and used to transform the corresponding benomyl sensitive strains to resistance in a homologous or heterologous system. In this report, a homologous benomyl resistant β-tubulin gene was successfully used to transform a benomyl sensitive strain of *A. parasiticus* to benomyl resistance. The homologous ß-tubulin gene (*ben*^{r}) more recently has been used as a dominant selectable marker in the transformation of a VA accumulating blocked mutant strain of *A. parasiticus* to aflatoxin production to confirm gene function (Shun-Shin Liang, personal communication).

In this report, a DNA fragment with sequence similarity to the cloned *N. crassa* ß-tubulin gene (*tub*-2$^+$) was isolated from a benomyl resistant mutant strain of *A. parasiticus*. The ability of this DNA fragment to confer benomyl resistance in *A. parasiticus* CS10 suggested that it encodes a benomyl resistant ß-tubulin protein in *A. parasiticus* and is functionally homologous to the *N. crassa* benomyl resistant ß-tubulin protein. The fact that the *N. crassa* ß-tubulin gene hybridized to this *A. parasiticus ben*^{r} gene under high stringency conditions supports this hypothesis. The efficiency of transformation of *A. parasiticus* observed with pYTPYRG or pYT1 (approximately 5 transformants per µg of DNA) makes the use of the *ben*^{r} gene as a selectable marker in screening of genomic DNA library by complementation less practical than the use of the homologous *nia*D gene (Horng et al., 1990) or *pyr*G gene (Skory et al., 1990). However, dominant markers, like the *ben*^{r} gene, will be especially important in developing constructs used in analysis of gene function in *A. parasiticus* because they allow generation of directed mutations or complementation of blocked mutants in a wild type genetic background. The observation that the transforming Ben^R phenotype remained relatively stable in *A. parasiticus* transformants is consistent with the stability of chlorate resistant transformants of *A. parasiticus* derived by integrative disruption or a gene replacement at the homologous *nia*D locus (Wu and Linz, 1992). Although differences in mitotic stability can be partially attributed to the location and the size of the integrated vector, the *ben*^{r} gene as a selectable marker in this gene replacement experiment was observed to be stable.

Transformation of *A. parasiticus* with constructs containing the *ben*^{r} gene

consistently resulted in clones with the Ben^R phenotype. However, analysis of the relative levels of resistance to benomyl allowed a further rough subdivision into low (1.0 μg/ml) and high level resistance (2 to 4 μg/ml). A logical extrapolation of the complex Southern hybridization data of Ben^S and Ben^R transformants leads to an interesting hypothesis which must be further tested in the future. That is, a direct correlation exists between copy number of the *ben*^{r} gene in transformants clones and the level of resistance to benomyl. The simplest case is a single *ben*^{s} allele (*A. parasiticus*, or cells transformed with pPG3J). Similarly, cells having multiple copies of the *ben*^{r} gene (isolates 49, 136, 95, and 178) and all transformants with linear pYT1 appear to express a high level of benomyl resistance. The data become more complicated when looking at the phenotypes of transformants 155, 156, 80 and 219. The pattern of hybridization to the *ben*^{r} probe is identical but three phenotypes are represented; transformants 155 and 156 are Ben^S, transformants 80 and 219 express low and high benomyl resistance, respectively. Our hypothesis to explain these data is that gene conversion results in two copies of the *ben*^{s} allele in transformants 155 and 156, and two copies of the *ben*^{r} allele in transformant 219. Transformant 80 has apparently one copy of *ben*^{s} and one copy of *ben*^{r} (lack of gene conversion) resulting in low level of benomyl resistance. However, the RIP (repeat-induced point mutation) observed in *N. crassa* during the introduction of exogenous sequence (Selker et al., 1987) may occur in transformants 155 and 156 and result in inactivation of the introduced *ben*^{r} gene. Gene conversion may also play a significant role in transformation with circular pYT1. Southern hybridization analysis of transformants

showed an identical hybridization pattern to nontransformed cells, yet these strains expressed high level benomyl resistance. We interpret this to mean that the single *ben*^{s} allele in the recipient strain was converted to a single *ben*^{r} allele. Since the only ß-tubulin in these cells would be the *ben*^{r} variety, the cells express a high level of resistance. In support of this argument the constructs containing the *ben*^{r} gene integrated at a high frequency at the *ben*^{s} locus allowing a close association between the two *ben* alleles and potential for a gene conversion event in either direction. The use of PCR for direct sequencing of the *ben*. alleles in transformants and nontransformed cells may shed some light on these hypotheses.

The sequences of ß-tubulin genes and the mutations conferring benomyl resistance have been determined in several filamentous fungi (Thomas et al., 1985; Orbach et al.,1986; Schwatz, 1987; Seip et al., 1990; Cooley and Caten, 1993; Goldman et al.,1993). Mutations conferring benomyl resistance usually mapped to a single amino acid change in the ß-tubulin polypeptide. The sequence of the mutant ß-tubulin gene (*ben*^{r}) potentially responsible for benomyl resistance in *A. parasiticus* is determined in this report. Unlike the mutant allele of the ß-tubulin genes in *N. crassa* (*tub*-2^r) and *A. flavus* in which a phenylalanine to tyrosine change at amino acid 167 appears to be responsible for the conferment of benomyl resistance, position 167 of the deduced amino acid sequence of *A. parasiticus ben*^{r} is a phenylalanine. The substitutions of amino acids in β-tubulin which lead to the benzimidazole fungicide resistance in fungi are not restricted to position 167 in β-tubulin. Amino acid position 198 in β-tubulin also plays an important role in the interaction with

fungicides. Substitution of amino acids in β-tubulin in the region of 237-250, a ^{241}His from Arg in *S. cerevisiae* (Thomas et al., 1985), a ^{237}Thr to Ala, and a ^{250}Leu to Phe in *N. crassa* also increases the resistance of fungi to carbendazim fungicide (Fujimura et al., 1994). When a comparison was made with respect to the sequences of the mutant allele of β-tubulin gene in *A. flavus* and *A. parasiticus ben*^{r} gene, a discrepancy at position 6 was observed. A tyrosine at position 6 was identified in *A. parasiticus ben*^{r} polypeptide whereas a histidine was at the corresponding position in the benomyl resistance β-tubulin gene of *A. flavus* (Seip et al., 1992). Sequences for the benomyl resistant β-tubulin genes in *Septoria nodorum* (*tub*A^{R}) and *Trichoderma viride (tub*-2) also show a histidine to tyrosine change at position 6. This indicates that the amino acid change at position 6 in *A. parasiticus ben*^{r} may play a role in the conferment of benomyl resistance. The use of the polymerase chain reaction (PCR) to amplify the wild-type β-tubulin gene and a sequence comparison between the wild type β-tubulin gene and *ben*^{r} may result in the definitive identification of the mutation conferring benomyl resistance.

CHAPTER III

RECOMBINATIONAL INACTIVATION OF THE GENE ENCODING NITRATE REDUCTASE IN *ASPERGILLUS PARASITICUS*

INTRODUCTION

Gene disruption is a procedure in which a nonfunctional allele of a cloned gene is introduced into cells to disrupt or replace its wild-type chromosomal counterpart by homologous recombination (Scherer and Davis, 1979; O'Hara and Timberlake, 1989; Timberlake and Marshall, 1989; Chevalet et al., 1992). Recent data on transformation of *A. parasiticus* with the homologous genes encoding nitrate reductase (*niaD*) or orotidine monophosphate decarboxylase (*pyrG*) suggested that integration of transforming DNA can occur at homologous sequences in the genome at a relatively high frequency (Horng et al., 1990; Skory et al., 1990).

The hypothesis proposed for this work is that homologous recombination between transforming DNA and genomic DNA can be utilized to disrupt the function of a model gene (*niaD*) cloned from *A. parasiticus*. The *niaD* gene was chosen because simple positive (chlorate resistance) and negative (inability to utilize nitrate as the sole nitrogen source) selection protocols have been developed to analyze nitrate reductase function (Cove, 1976a; Cove, 1976b). The strategy used for disruption of the *niaD* gene described here has now been applied to generate aflatoxin nonproducing strains for potential use as biocontrol agents. Disruption of genes associated with aflatoxin biosynthesis may also make it possible to establish if aflatoxin or intermediates in the biosynthetic pathway play a role in fungal growth, morphogenesis, or survival in the environment.

MATERIALS AND METHODS

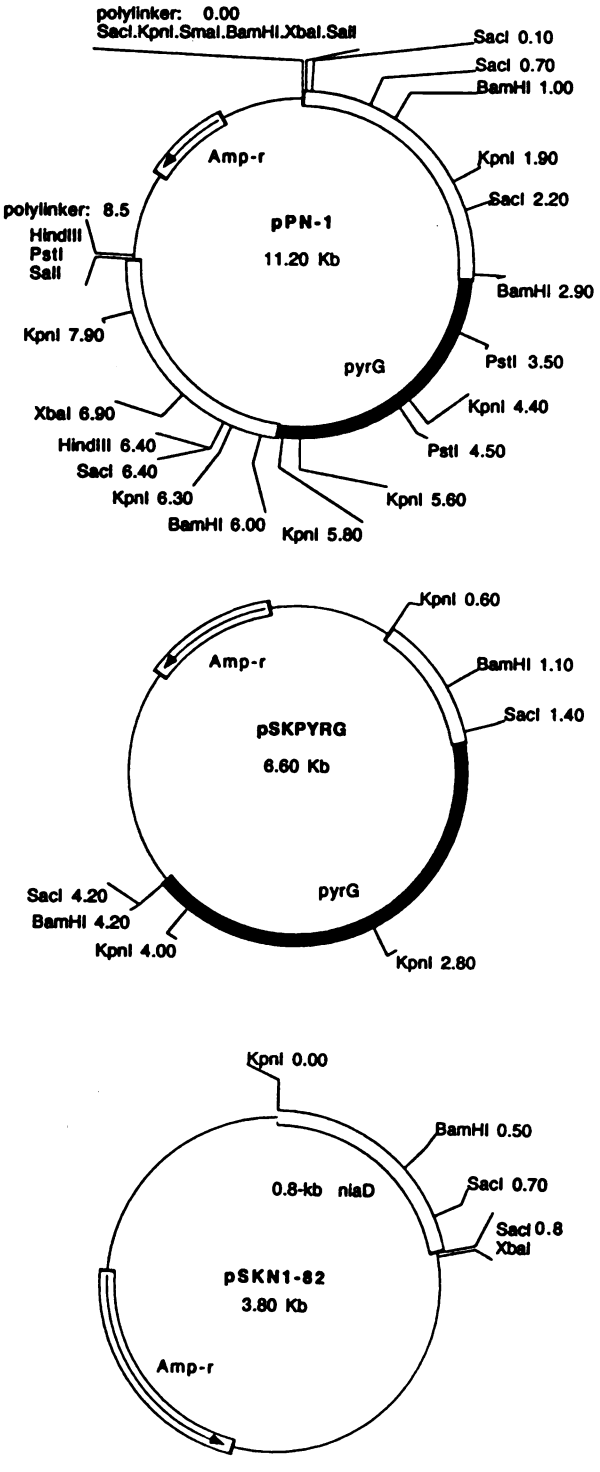
Strains and plasmids

Plasmid DNA used for transformation of *Aspergillus parasiticus* was propagated in *E. coli* DH5α (Gibco BRL, Life Technologies, Inc. Gaithersburg, MD) and purified by CsCl gradient centrifugation of cell extracts prepared by alkaline lysis (Maniatis et al., 1989). The fungal strain used as the recipient in transformation, *A. parasiticus* CS10 (*ver*-1,*wh*-1, *pyr*G) (Skory et al., 1990), was a *pyr*G mutant strain derived from *A. parasiticus* ATCC36537 (*ver*-1,*wh*-1) (Bennett and Goldblatt, 1973) which accumulates versicolorin A.

The *Eco*RI restriction site in pUC19 was deleted by digesting the vector with *Eco*RI followed by blunt-ended ligation to yield pUC19-1. An 8.2-kb *Sal*I restriction fragment from pSL82 (Horng et al., 1990), which contains a complete copy of the *nia*D gene plus 5' and 3' flanking regions, was subcloned into the *Sal*I restriction site of pUC19-1 to produce pSL82-1. pPN-1, the plasmid construct used in gene replacement (Figure 14) was constructed by deleting a 2.5-kb *Eco*RI internal fragment of *nia*D from pSL82-1 and replacing it by blunt end ligation with the functional *pyr*G gene contained on a 2.9-kb *Bam*HI-*Sal*I restriction fragment obtained from plasmid pPG3J. pSKN1-82 (Figure 14) was constructed by cloning a small internal restriction fragment of *nia*D (0.8-kb *Kpn*I-*Bgl*II) into the *Kpn*I-*Bam*HI restriction sites in pBluescriptII SK- (Stratagene, La Jolla, Calif.). pSKPYRG, used in integrative disruption (Figure 14), was constructed by subcloning a 2.9-kb *Bam*HI-*Sal*I fragment containing the *pyr*G gene into pSKN1-82 at the *Xba*I site by blunt-ended ligation.

Figue 14. Construction and restriction maps of pPN-1, pSKPYRG, and pSKN1-82. A 2.5-kb *Eco*RI fragment, which contains the majority of the *nia*D gene (open box) of *A. parasiticus*, was deleted from pSL82-1, a derivative of pSL82 (Horng et al., 1990), and replaced by a 2.9-kb *Bam*HI-*Sal*I fragment (filled box) of pPG3J containing the functional *pyr*G gene (Skory et al., 1990) to generate pPN1. Plasmid pSKN1-82 was constructed by subcloning a 0.8-kb *Kpn*I-*Bgl*II internal fragment of the *nia*D gene into *Kpn*I-*Bam*HI sites of pBLUESCRIPTII SK-. Plasmid pSKPYRG was then generated by subcloning the 2.9-kb *Bam*HI-*Sal*I *pyr*G fragment into the *Xba*I site (blunt-ended) of pSKN1-82.

Figure 14.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SacI 0.10
SacI 0.70
BamHI 1.00
KpnI 1.90
SacI 2.20
BamHI 2.90
PstI 3.50
KpnI 4.40
PstI 4.50
KpnI 5.60
KpnI 5.80
BamHI 6.00
KpnI 6.30
SacI 6.40
HindIII 6.40
XbaI 6.90
KpnI 7.90
polylinker: 8.5
HindIII
PstI
SalI
Amp-r
pPN-1
11.20 Kb
pyrG
KpnI 0.60
BamHI 1.10
SacI 1.40
KpnI 2.80
KpnI 4.00
BamHI 4.20
SacI 4.20
Amp-r
pSKPYRG
6.60 Kb
pyrG
KpnI 0.00
BamHI 0.50
SacI 0.70
SacI 0.8
XbaI
0.8-kb niaD
pSKN1-82
3.80 Kb
Amp-r

Transformation of fungal protoplasts/analysis of transformant clones

Transformation of fungal protoplasts was performed essentially as described by Oakley (1987), with minor modification (Skory et al., 1990). Fungal strains were maintained on potato dextrose agar (Difco, Detroit, MI). Frozen spore stocks of *A. parasiticus* CS10 were incubated to generate mycelia in yeast extract-sucrose liquid media (YES, 2% yeast extract, 6% sucrose, pH 5.5) supplemented with 100 µg/ml of uridine. Prototrophic transformants (*pyr*G$^+$) of *A. parasiticus* CS10 were isolated on Czapek-Dox agar (CZ agar, Difco, Detroit, MI) containing 1.69 g/l sodium glutamate (Sigma Chemical CO, MO) as the alternative nitrogen source (CZ agar also contains nitrate) and 0.6 M KCl as the osmotic stabilizer.

Transformants, defective in nitrate assimilation, could be identified by growth after 3 days (positive selection) on CZ agar supplemented with 470 mM potassium chlorate (Horng, 1989) as the selective agent and sodium L-glutamate (1.69 g/l) as the alternative nitrogen source. Chlorate is an analog of nitrate and is thought to be reduced by nitrate reductase to the toxic compounds chlorite or hypochlorite (Åberg, 1947; LaBrie, 1991). Cove (1976a) however, reports that chlorate resistance in *A. nidulans* is a complex phenomenon and can arise from mutations in several different genes. Not every loss of function mutation at these loci confers chlorate resistance suggesting that factors other than reduction of chlorate may lead to chlorate resistance (Cove, 1976a; Cove, 1976b; Cove, 1979). The resistance of *A. nidulans* to chlorate is reported to depend on defects in the nitrate permease gene (*crn*A), nitrate reductase structural gene (*nia*D), two genes involved in regulation of nitrogen metabolism (*nir*A

or *are*A) or genes (*cnx*) involved in the assembly of a molybdenum cofactor (Cove, 1976a; Cove, 1976b; Cove, 1979; Tomsett and Cove, 1979; Kinghorn, 1989). Mutations in *nia*D can be distinguished from mutations at these other loci by growth on minimal salt medium (MM) (Pontecorvo, 1953; Barratt et al., 1965) supplemented with 470 mM $KClO_3$ plus one of the following nitrogen sources: $NaNO_3$ (0.85 g/l), $NaNO_2$ (0.69 g/l), ammonium tartrate (1.84 g/l), hypoxanthine (0.1 g/l). *nia*D mutants are unable to use nitrate but can grow on medium containing each of the other nitrogen sources whereas mutations in the other genes involved in nitrogen metabolism have different growth patterns on these same nitrogen sources (Horng, 1989; Kinghorn, 1989).

In *A. parasiticus*, it is important to select for Chl^r after 3 days growth because some colonies, appearing after 6 days growth on chlorate medium, could also utilize nitrate as the sole nitrogen source. The explanation for these late appearing colonies is not clear at this time. They may represent mutants deficient in nitrate uptake, which are moderately chlorate-resistant but capable of growth on nitrate. A similar phenotype has been reported for some *A. nidulans* permease mutants (Tomsett and Cove, 1979; Kinghorn, 1989).

Mitotic stability of disrupted transformant isolates

For the study of mitotic stability, five *pyr*G^+, Chl^r transformants were single-spore isolated. After completing the full vegetative growth cycle on nonselective medium (PDA supplemented with 100 µg/ml uridine and 1.69 g/l sodium glutamate) asexual spores were harvested. Appropriate dilutions of these spore preparations were

inoculated again onto nonselective medium (CZ/glu/uri) and allowed to grow for 3 days. Individual colonies were then transferred onto selective growth media (CZ/glu/chlorate) and scored for growth and sporulation after 3 days. As a control, spores from 100 colonies of *A. parasiticus* CS10 were patched onto selective (plus chlorate) and nonselective growth media both containing uridine.

Chemicals and general procedures

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN.). Enzyme digestion, agarose gel electrophoresis, and Southern hybridization analyses were performed according to standard procedures (Maniatis et al., 1989). Radiolabeled DNA probes were generated with a Random Primed DNA Labeling kit (Boehringer Mannheim Biochemicals) with [α-^{32}p] dCTP (DuPont). High-molecular-weight genomic DNA was isolated from mycelia using the phenol/chloroform procedure of Cihlar and Sypherd (1980) as modified by Horng (1990).

RESULTS

One step gene replacement

Plasmid pPN-1 was digested with *Sma*I to generate a linear construct and used to transform *A. parasiticus* CS10. The frequency of transformation of *A. parasiticus* CS10 to *pyr*G$^+$ with linear pPN-1 (approximately 8 transformants/µg of DNA/10^6 protoplasts) was similar to that of circular pPG3J which contains the *pyr*G gene only (10 transformants/µg of DNA/10^6 protoplasts). *pyr*G$^+$ transformants were then

screened for defects in nitrate assimilation. Fifteen of 66 *pyr*G$^+$ transformants (23%) were Chl^r and none of these 15 transformants could grow on CZ agar which contains nitrate as the sole nitrogen source. This result indicated that these 15 isolates were nitrate nonutilizing mutants. This experiment was repeated with similar results. Eight of 32 (25%) *pyr*G$^+$ transformants of *A. parasiticus* CS10 were Chl^r and could not grow on CZ agar. The 23 Chl^r transformants from two experiments were further characterized by replica plating to MM media containing various nitrogen sources (see media and culture conditions). None of 23 Chl^r transformants could grow on $NaNO_3$ as sole nitrogen source but could grow on $NaNO_2$, ammonium tartrate, or hypoxanthine consistent with a mutation in *nia*D.

Nine independent clones (designated 6, 13, 15, 17, 51, 57, 64, 67, and 69) defective in nitrate reductase function, were analyzed by Southern hybridization analysis (see below).

Integrative disruption

Circular pSKPYRG was used to transform *A. parasiticus* CS10. In three independent experiments, the frequency of transformation of *A. parasiticus* CS10 with pSKPYRG to *pyr*G$^+$ was approximately 6 transformants/μg of DNA/10^6 protoplasts. Only 4 of 238 *pyr*G$^+$ transformants (< 2%) were Chl^r and none of the 4 grew on CZ agar. These 4 Chl^r transformants could grow on $NaNO_2$, ammonium tartrate, or hypoxanthine. These results suggested that the *nia*D gene had been disrupted in these 4 isolates. Two of these four transformants (designated 8 and 174) plus two *pyr*G$^+$, Chl^s transformants (2 and 28) were selected for Southern hybridization analysis.

Transformation of *A. parasiticus* CS10 protoplasts with no DNA or with pPG3J were performed as controls. In the absence of transforming DNA, control protoplasts could not grow on CZ agar, indicating that no *pyrG*$^+$ revertants were generated. In addition, of 200 *pyrG*$^+$ clones transformed with pPG3J, no Chl^r colonies were observed after 3 days of growth, indicating that no spontaneous mutations occurred at the *niaD* locus.

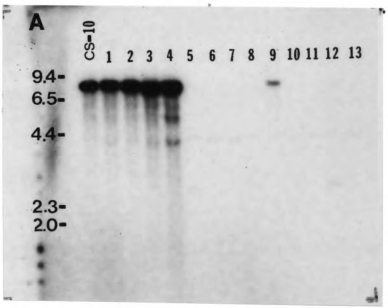
Southern hybridization analysis

One step gene replacement. Southern hybridization analysis was performed on genomic DNAs isolated from *A. parasiticus* CS10 (control), *pyrG*$^+$ isolates transformed with pPG3J (control) or *pyrG*$^+$, *niaD*$^-$ isolates transformed with linear pPN-1. Genomic DNA samples, digested with *SalI*, were probed with a radiolabeled 0.9-kb *SstII* fragment located within the region of the *niaD* fragment which was deleted in pPN-1. A single 8.2-kb DNA fragment hybridized to the *niaD* probe in genomic DNA purified from untransformed cells and *pyrG*$^+$ cells transformed with pPG3J, suggesting that the genomic copy of the *niaD* gene was unaffected by transformation. The *niaD* internal probe did not hybridize to any *SalI* fragment in 8 of 9 randomly selected *pyrG*$^+$, *niaD*$^-$ isolates transformed with pPN-1, indicating that this *niaD* fragment was deleted from the genome. A weakly hybridizing 8.2-kb DNA fragment was observed in the other isolate (designated 51 in Figure 15A), suggesting that it was a heterokaryon which had not been successfully resolved during clone isolation.

An identical set of *SalI* digested genomic DNAs was also hybridized to a 2.9-

Figure 15. Southern hybridization analysis of genomic DNAs from *A. parasiticus* CS10 (recipient) and isolates transformed with pPG3J and linear pPN-1. (A) Genomic DNAs were digested to completion with *Sal*I and hybridized with a radiolabeled internal *nia*D fragment. (B) Identical genomic DNA samples were hybridized with a radiolabeled 2.9-kb *Sal*I-*Bam*HI *pyr*G fragment from pPG3J. Lane CS-10; genomic DNA from the untransformed *A. parasiticus* CS-10. Lanes 1-4; four *pyr*G$^+$, Chl^s transformants (isolates 0, 1, 2 and 6, respectively) of *A. parasiticus* CS-10 transformed with pPG3J. Lanes 5-13; 9 *pyr*G$^+$, Chl^r transformants transformed with linear pPN-1 (isolates 6, 13, 15, 17, 51, 57, 64, 67 and 69 respectively). DNA size standards at left: *Hin*dIII-digested lambda DNA.

Figure 15.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CS-10
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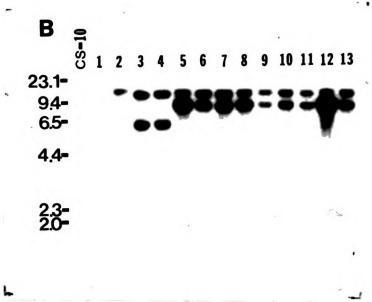
9.4-
6.5-
4.4-
2.3-
2.0-


B
CS-10
1 2 3 4 5 6 7 8 9 10 11 12 13
23.1-
9.4-
6.5-
4.4-
2.3-
2.0-

kb *Bam*HI-*Sal*I fragment containing the *pyr*G gene from pPG3J (Figure 15B). One DNA fragment, approximately 10.0-kb in size hybridized to the *pyr*G probe in all DNA samples, indicating that the chromosomal *pyr*G gene lay on this genomic DNA fragment. An additional 6.3-kb *Sal*I fragment hybridized to the *pyr*G probe in 3 of 4 *pyr*G$^+$ isolates transformed with pPG3J, suggesting that a single crossover between circular pPG3J and the chromosomal *pyr*G locus occurred, resulting in two copies of *pyr*G separated by vector sequences. Although plasmid integration clearly did not occur at the *nia*D locus in these 3 transformants, we can not conclusively rule out the possibility that the integration might have occurred at a location other than the *pyr*G gene. Only the 10.0-kb *Sal*I fragment was observed in the fourth pPG3J-transformant suggesting that the functional *pyr*G gene in pPG3J replaced the nonfunctional chromosomal *pyr*G allele of *A. parasiticus* CS10. In clones transformed with the gene replacement vector pPN-1 the *pyr*G probe hybridized to an additional 8.2-kb *Sal*I DNA fragment suggesting that the nonfunctional *nia*D gene construct containing the *pyr*G gene replaced the functional chromosomal *nia*D gene by a double-crossover event in the flanking regions of *nia*D adjacent to the cloned *pyr*G. Since the *pyr*G gene fragment (2.9-kb) used to replace the *nia*D internal fragment (2.5-kb) in pPN-1 was of approximately the same size, gene replacement by this vector would result in the generation of a *Sal*I restriction fragment of approximately 8.2-kb which now hybridized to *pyr*G and not *nia*D. Southern hybridization analysis was repeated on these same genomic DNA samples (pPN-1 transformants) which had been digested with *Sal*I plus *Sac*I and probed with *pyr*G. The hybridization patterns observed were

also consistent with a gene replacement event (Figure 16A). The 6.4-kb band observed for transformants represents the genomic copy of *pyr*G gene in *A. parasiticus*. An additional 4.1-kb fragment hybridized to the *pyr*G probe in pPN-1 transformants, indicating that the a gene replacement event occurred betweem the *nia*D region of pPN-1 and the chromosomal counterpart in the recipient. When genomic DNAs from pPN-1 transformants were probed with pUC19 (Figure 16B), 4 of 9 isolates (6, 17, 51, and 67) contained different sized DNA fragments which hybridized to the pUC19 probe (but not to an *nia*D or *pyr*G probe) suggesting that pUC19 was capable of heterologous recombination independent of the remaining vector. The other five isolates did not hybridize to the pUC19 probe indicating that pUC19 sequences were deleted during the double crossover gene replacement event.

Integrative gene disruption. Genomic DNAs of *A. parasiticus* NRRL5862 (SU-1), the recipient strain *A. parasiticus* CS10, and several clones transformed with pPG3J or the integrative vector pSKPYRG were digested with *Sac*I and hybridized to a 2.9-kb *pyr*G gene probe (Figure 17). A single *Sac*I DNA fragment hybridized to the *pyr*G probe suggesting that gene replacement or gene conversion between the *pyr*G gene in plasmid pSKPYRG and the nonfunctional genomic *pyr*G allele occurred in one of two *pyr*G$^+$, Chl^s transformants. In the other clone, homologous integration of pSKPYRG occurred at the genomic *pyr*G locus resulting in tandem copies of *pyr*G carried on 7.4-kb and 4.6-kb *Sac*I DNA fragments. In the two *pyr*G$^+$, Chl^r transformants (8 and 174) analyzed, an 8.2-kb *Sac*I DNA fragment and a 3.2-kb *Sac*I DNA hybridized to the *pyr*G probe. The sizes of these *Sac*I fragments were consistent with homologous

Figure 16. Southern hybridization analysis of genomic DNAs from *A. parasiticus* CS10 (recipient) and isolates transformed with pPG3J and linear pPN-1. (A) Genomic DNAs were digested to completion with *Sal*I in combination with *Sac*I and hybridized with a radiolabeled 2.9-kb *Sal*I/*Bam*HI *pyr*G fragment from pPG3J. (B) Identical genomic DNA samples were digested with *Sal*I and hybridized with radiolabeled pUC19. Lane CS-10; genomic DNA from the untransformed *A. parasiticus* CS-10. Lanes 1-4; four *pyr*G$^+$, Chl^s transformants (isolates 0, 1, 2, and 6 respectively) of *A. parasiticus* CS-10 transformed with pPG3J. Lanes 5-13; 9 *pyr*G$^+$, Chl^r transformants transformed with linear pPN-1 (isolates 6, 13, 15, 17, 51, 57, 64, 67, and 69 respectively). DNA size standards at left: *Hin*dIII-digested lambda DNA.

Figure 16.

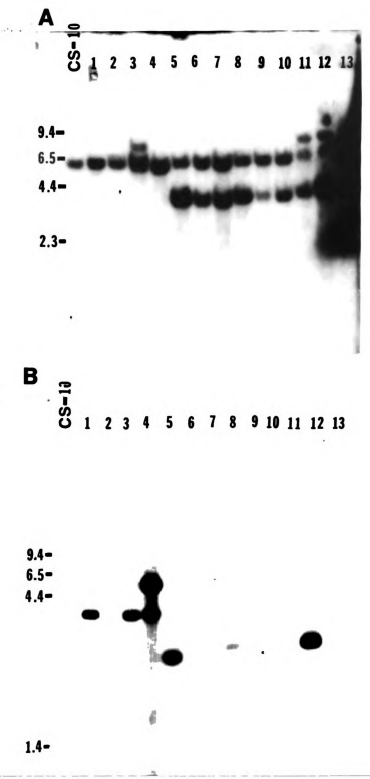
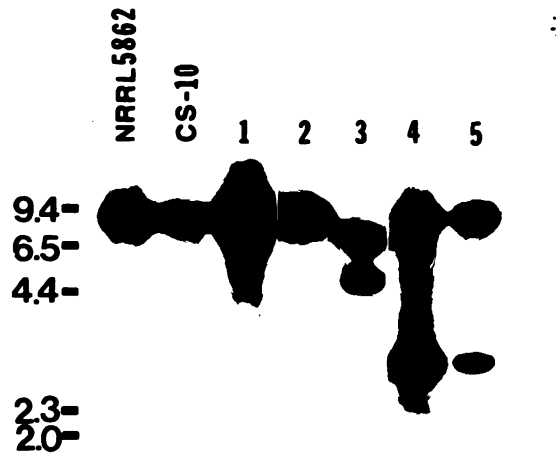

A
CS-10
1 2 3 4 5 6 7 8 9 10 11 12 13
9.4-
6.5-
4.4-
2.3-
B
CS-13
1 2 3 4 5 6 7 8 9 10 11 12 13
9.4-
6.5-
4.4-
1.4-

Figure 17. Southern hybridization analysis of genomic DNAs from *A. parasiticus* NRRL5862, *A. parasiticus* CS10 (recipient), and isolates transformed with pPG3J and pSKPYRG. Genomic DNAs were digested to completion with *Sac*I and hybridized with a radiolabeled 2.9-kb *pyr*G probe. Lane NRRL5862; genomic DNA from *A. parasiticus* NRRL5862. Lane CS-10; genomic DNA from untransformed *A. parasiticus* CS-10. Lane 1; *pyr*G$^+$, Chl^s transformant of *A. parasiticus* CS-10 transformed with pPG3J. Lane 2 and 3; two *pyr*G$^+$, Chl^s transformants transformed with pSKPYRG (isolates 2 and 28, respectively). Lane 4 and 5; two *pyr*G$^+$, Chl^r transformants transformed with pSKPYRG (isolates 8 and 174 respectively). DNA size standards at left: *Hin*dIII-digested lambda DNA.

Figure 17.



integration of pSKPYRG at the chromosomal *nia*D allele in these clones.

Mitotic stability of *nia*D disrupted strains

Chl^r clones transformed with pPN-1 or pSKPYRG were tested for mitotic stability by allowing them to complete the full vegetative cycle on nonselective growth medium. Appropriate dilutions of the resultant spore stocks were inoculated onto nonselective medium. Individual colonies grown on nonselective medium were then transferred onto selective growth media (see materials and methods) to test for chlorate resistance. In two separate experiments all 700 individual clones from each of the three gene replacement transformants and two integrative disruption transformants tested stably retained the chl^r phenotype. None of the 100 control *nia*D$^+$ *A. parasiticus* CS10 colonies were Chl^r after 3 days growth.

DISCUSSION

One step gene replacement of the *nia*D gene was performed at a high frequency (25%) by introducing a linear plasmid construct, pPN-1, into *A. parasiticus* CS10. A circular plasmid, pSKPYRG, containing a truncated *nia*D gene fragment, also disrupted the genomic *nia*D gene by a single cross-over integrative recombination event but at a lower frequency (< 2%). The observed difference in disruption frequency is likely due in part to a difference in the sizes of DNA fragments which undergo homologous recombination. Since plasmid pSKPYRG carries a small (0.8-kb) *nia*D internal fragment, a single crossover or gene replacement at the 2.9-kb *pyr*G gene on pSKPYRG appeared to be the preferred event. In addition, the mitotic

stability data suggest that genetically stable transformants can be generated effectively using either gene replacement or integrative disruption. Using linearized pPN-1, 4 of 9 *niaD*$^-$ gene replacement transformants carried vector sequences in the genome suggesting that pUC19 was capable of heterologous recombination. Since the absence of vector sequences (foreign DNA) would be highly desirable in potential biocontrol strains, deletion of vector sequence from the disruption constructs before transformation is therefore strongly suggested.

Four genes, *nor*-1, *ver*-1, *uvm*8, and *pks*A, which are directly involved in the aflatoxin biosynthetic pathway of *A. parasiticus* have been cloned (Chang et al., 1992; Skory et al., 1992; Trail et al., 1995a, Mahanti et al., 1995 in press). The recombinational inactivation strategies used in this report have been applied to disrupt each of these genes to establish their functional role in the pathway (Liang and Linz, 1994; Trail et al., 1994; Trail et al., 1995a). Strains disrupted in *uvm*8 and *pks*A are of particular interest because these mutants do not accumulate any known pathway intermediate and have been demonstrated to be blocked at a very early step in the aflatoxin pathway (Trail et al., 1995a; Mahanti et al., 1995 in press). Cloning of *uvm*8 and disruption of the genomic copy of *uvm*8 gene have been performed. The ability of these nontoxigenic strains to be used as a biocontrol agent is currently under investigation.

CHAPTER IV

ANALYSIS OF THE REGULATION OF THE EXPRESSION OF THE *VER-1* GENE ASSOCIATED WITH THE CONVERSION OF VERSICOLORIN A TO DEMETHYLSTERIGMATOCYSTIN IN *ASPERGILLUS PARASITICUS*

INTRODUCTION

The long term goal of this research is to eliminate aflatoxins from the food chain. A short term goal which will aid in the accomplishment of the long term goal is the generation of nontoxigenic and genetically stable biocontrol agents. This task requires an understanding of the aflatoxin biosynthetic pathway at the molecular level but also the regulatory mechanisms governing aflatoxin production. Previous studies demonstrated that the appearance of several aflatoxin metabolic enzymes (Cleveland et al., 1987; Lin and Anderson, 1992; Anderson and Green, 1994; Liang and Linz, 1994) and the accumulation of *nor*-1, *ver*-1, and *omt*-1 transcripts coincides with the cessation of exponential growth of the fungus and the onset of aflatoxin production (Chang et al., 1992; Skory et al., 1992; Skory et al., 1993; Yu et al., 1993). These observations lead to the generation of a hypothesis that the regulation of aflatoxin biosynthesis is in part at the transcriptional level. In order to test this hypothesis, a system for systematic study of the transcriptional regulatory mechanisms governing aflatoxin production had to be developed. This system also needed to be able to identify the genetic elements involved in the transcriptional regulation of aflatoxin biosynthesis, including the cis-acting DNA elements in the promoter region and the transacting regulatory proteins. Analyses of transcriptional regulation in filamentous fungi have been performed on promoters involved in primary metabolism (Hamer and Timberlake, 1987; Hata et al., 1992). Recently, transcriptional analysis of a promoter region of a gene involved in production of a secondary metabolite (penicillin) in *A. nidulans* has also been reported (Gómez-Pardo and Peñalva, 1990;

Pérez-Esteban et al.,1993). These studies shared a common feature in that "reporter genes" were used for the analyses of functional promoters. Reporter genes have been intensively used in the investigation of gene regulation in bacteria, fungi, plants and mammals (Hamer and Timberlake, 1987; Robert et al., 1989; Alam and Cook, 1990; Quattrocchio et al., 1990; Thomas and Flavell, 1990; Zhou and Thiele, 1991; Hata et al.,1992). One of the most commonly used reporter genes is the *uid*A gene (GUS) encoding ß-glucuronidase in *E. coli* (Jefferson, 1989; Schlaman et al., 1994).

The objective of this study was to validate the use of GUS as a reporter gene in a system to test the hypothesis that the regulation of aflatoxin biosynthesis is in part at the transcriptional level (using the *ver*-1 promoter). In this report, we confirm that (1) aflatoxin production is regulated in part at the transcriptional level; (2) GUS can be used as a reporter gene in the transcriptional analysis of genes involved in aflatoxin biosynthesis. The data also suggest that insertion of the *ver*-1 promoter and transcription terminator at a heterologous chromosomal location does not affect its temporal expression pattern but may alter the promoter's ability to consistently regulate the level of expression. Based on these studies, the GUS expression vector can be used in future transcriptional studies which include identification of the transcription cis-acting sites and trans-acting regulatory factors, which regulate gene expression.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli DH5α F'^{q} [F'/*endA1 hsdR17* ($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1*Δ (*lacZYA-argF*)$_{u169}$:(m80 *lacZ*Δ *M15*)] (Gibco BRL, Life Technologies, Inc. Gaithersburg, MD) was used as the recipient strain to propagate plasmid DNA. All plasmid DNA used for transformation was prepared by CsCl gradient centrifugation of cell extracts prepared by alkaline lysis (Maniatis et al., 1989). A *nia*D mutant strain (NR-2) derived from wild-type *A. parasiticus* NRRL5862 (SU-1, Bennett, 1979) was used as the recipient strain throughout the experiments.

A vector, pGL2-basic (Figure 18) (GeneLight™ Plasmids Technical Manual, Promega, Madison, WI), was selected for construction of the plasmids used in this experiment. pGL2-basic provides an advantage in that a DNA fragment containing the SV40 polyadenylation region has been placed just upstream of the promoter insertion site to reduce background transcription. The reporter gene selected for transcriptional analyses was the *E. coli uid*A gene (GUS), which encodes β-glucuronidase.

In order to construct the final plasmids for transcriptional analyses, plasmid pGLGUS-2-1 was constructed (Figure 18) by deleting the *Nco*I site in pGLGUS-2 to remove the translation initiation codon (ATG) in the GUS gene. This deletion allowed the generation of a translational fusion between the opening reading frame of GUS with the first few amino acids of *ver*-1A. pGLGUS-2 contains a 3.7-kb *Sma*I/*Eco*RI fragment representing the putative transcription termination signal in *ver*-

Figure 18. Schematic representation of the construction of plasmid pGLGUS-2-1.

Figure 18.

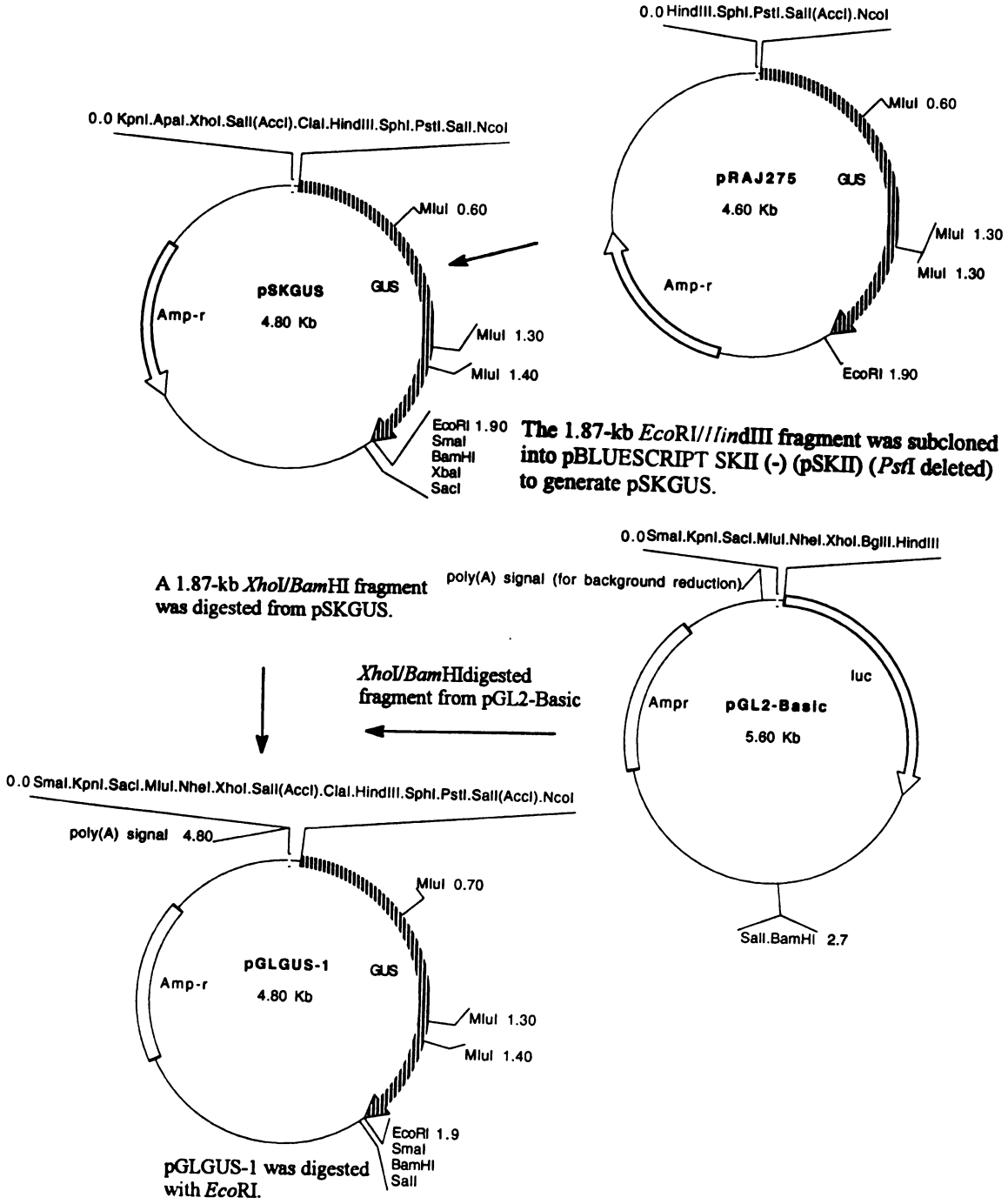

0.0 KpnI.ApaI.XhoI.SalI(AccI).ClaI.HindIII.SphI.PstI.SalI.NcoI
MluI 0.60
pSKGUS
GUS
Amp-r
4.80 Kb
MluI 1.30
MluI 1.40
EcoRI 1.90
SmaI
BamHI
XbaI
SacI
0.0 HindIII.SphI.PstI.SalI(AccI).NcoI
MluI 0.60
pRAJ275
GUS
4.60 Kb
MluI 1.30
MluI 1.30
Amp-r
EcoRI 1.90
The 1.87-kb EcoRI/HindIII fragment was subcloned into pBLUESCRIPT SKII (-) (pSKII) (PstI deleted) to generate pSKGUS.
A 1.87-kb XhoI/BamHI fragment was digested from pSKGUS.
0.0 SmaI.KpnI.SacI.MluI.NheI.XhoI.BglII.HindIII
poly(A) signal (for background reduction)
luc
Ampr
pGL2-Basic
5.60 Kb
SalI.BamHI 2.7
XhoI/BamHIdigested fragment from pGL2-Basic
0.0 SmaI.KpnI.SacI.MluI.NheI.XhoI.SalI(AccI).ClaI.HindIII.SphI.PstI.SalI(AccI).NcoI
poly(A) signal 4.80
MluI 0.70
pGLGUS-1
GUS
Amp-r
4.80 Kb
MluI 1.30
MluI 1.40
EcoRI 1.9
SmaI
BamHI
SalI
pGLGUS-1 was digested with EcoRI.

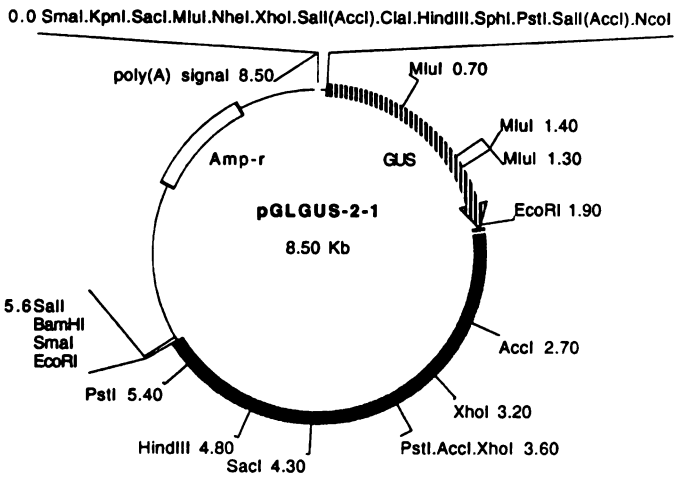
Figure 18. (Cont'd)

pGLGUS-1

A 3.7-kb *Eco*RI/*Eco*RI DNA fragment was generated from the 3.7-kb *Sma*I/*Eco*RI DNA fragment isolated from NorA cosmid and subcloned into *Eco*RI site of pGLGUS-1 to generate pGLGUS-2.

pGLGUS-2

The *Nco*I site in pGLGUS-2 was deleted by using Mung-Bean Nuclease to generate pGLGUS-1.



1B (Figure 19) and a 1.87-kb *Hin*dIII/*Eco*RI *uid*A gene (GUS) isolated from pRAJ275. Plasmid pHD1 (Figure 20) was constructed by subcloning a 1.1-kb *Cla*I/*Nar*I fragment (Figure 19), representing the 5'-promoter region in *ver*-1A, into the *Sph*I site in the polylinker of pGLGUS-2-1. pHD1 has the *ver*-1A promoter region (including the first 15 amino acids of the *ver*-1A protein) fused in frame to the GUS reporter gene (Figure 20). A selectable marker, a 6.6-kb *Xba*I fragment containing a functional *nia*D gene in pSL82 (Horng et al., 1990) was inserted at a unique *Nhe*I site in pHD1 to generate the plasmid pHD2. This construct was used in transformation of an *nia*D strain of *A. parasiticus* NRRL5862 (NR-2) for preliminary analysis to correlate the expression of GUS with aflatoxin production. However, the absence of convenient restriction endonuclease cutting sites made plasmid pHD2 unsuitable for further deletion analyses of the 1.1-kb *ver*-1A promoter region.

In order to develop a more convenient system for evaluation of the GUS reporter gene, plasmid pHD4-4, pHD7-3, and pHD6-6 (Figure 22) were constructed. Plasmid pHD6-6 contains the 1.1-kb *ver*-1A promoter region subcloned so that deletion analysis could be performed from the 5' end. A schematic representation of the construction of plasmid pHD6-6 is shown in Figure 22. Plasmid pHD6-6 was constructed by replacing the 3.7-kb 3'-transcription termination signal in pHD2 with a 1.8-kb *Sma*I/*Pst*I fragment derived from *ver*-1A (Figure 19). Plasmid pHD4-4 was constructed by deleting the 1.1-kb promoter from pHD6-6 (Figure 22 & Figure 23). A 51 bp *Msc*I/*Bst*EII fragment which contains the putative TATA box and transcription initiation site in the *ver*-1A promoter region was deleted from pHD6-6

Figure 19. Restriction endonuclease map of the *A. parasiticus* genomic DNA in the regions containing the *ver*-1A and *ver*-1B genes. The filled box is a 1.1-kb *Cla*I/*Nar*I fragment which represents the promoter region of the *ver*-1A gene subcloned in pHD2 and pHD6-6. The putative translational start codon, which is located 86 bp 3' to the transcription initiation site (+1), is indicated as ATG. The *Nar*I restriction endonuclease site is located 45 bp downstream from the ATG. The 1.8-kb *Sma*I/*Pst*I DNA fragment isolated from *ver*-1A was used as the transcription termination signal in pHD4-4, pHD7-3, and pHD6-6 whereas the 3.7-kb *Sma*I/*Eco*RI fragment from *ver*-1B was used to construct pHD2. The *Sma*I cutting site is located 87 bp 5' to the translation stop (TAA) codon in *ver*-1A. The translation stop codon (TAA) and the putative transcription termination signal sequence (TAATATAA) are also indicated.

Figure 19.

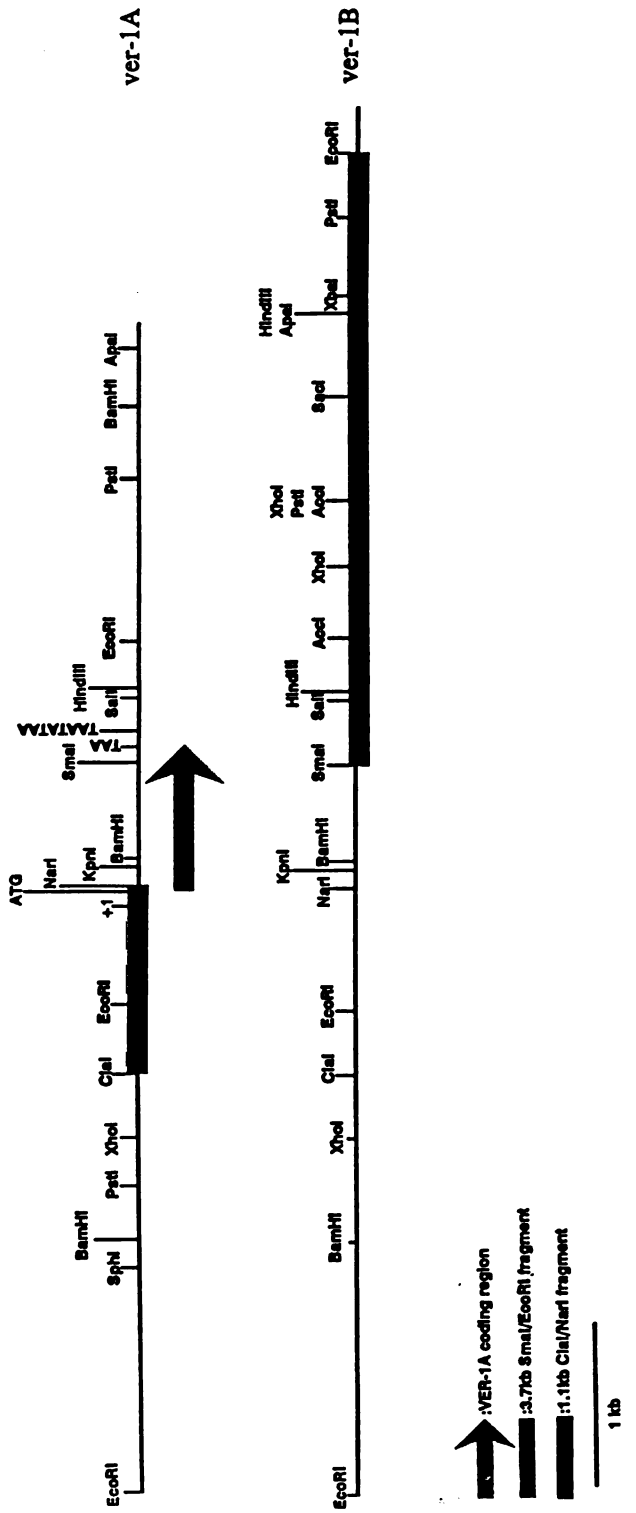

ver-1A
ver-1B
ATG
NarI
KpnI
BamHI
+1
SmaI
TAATATAA
TAA
HindIII
SalI
EcoRI
PstI
BamHI
ApaI
SphI
XhoI
ClaI
EcoRI
BamHI
KpnI
NarI
BamHI
SmaI
HindIII
SalI
AccI
XhoI
XhoI
PstI
AccI
SpeI
HindIII
ApaI
XbaI
PstI
EcoRI
EcoRI
BamHI
XhoI
ClaI
EcoRI
EcoRI
: VER-1A coding region
: 3.7kb SmaI/EcoRI fragment
: 1.1kb ClaI/NarI fragment
1 kb

Figure 20. Schematic representation of the construction of plasmids pHD1 and pHD2.

Figure 20.

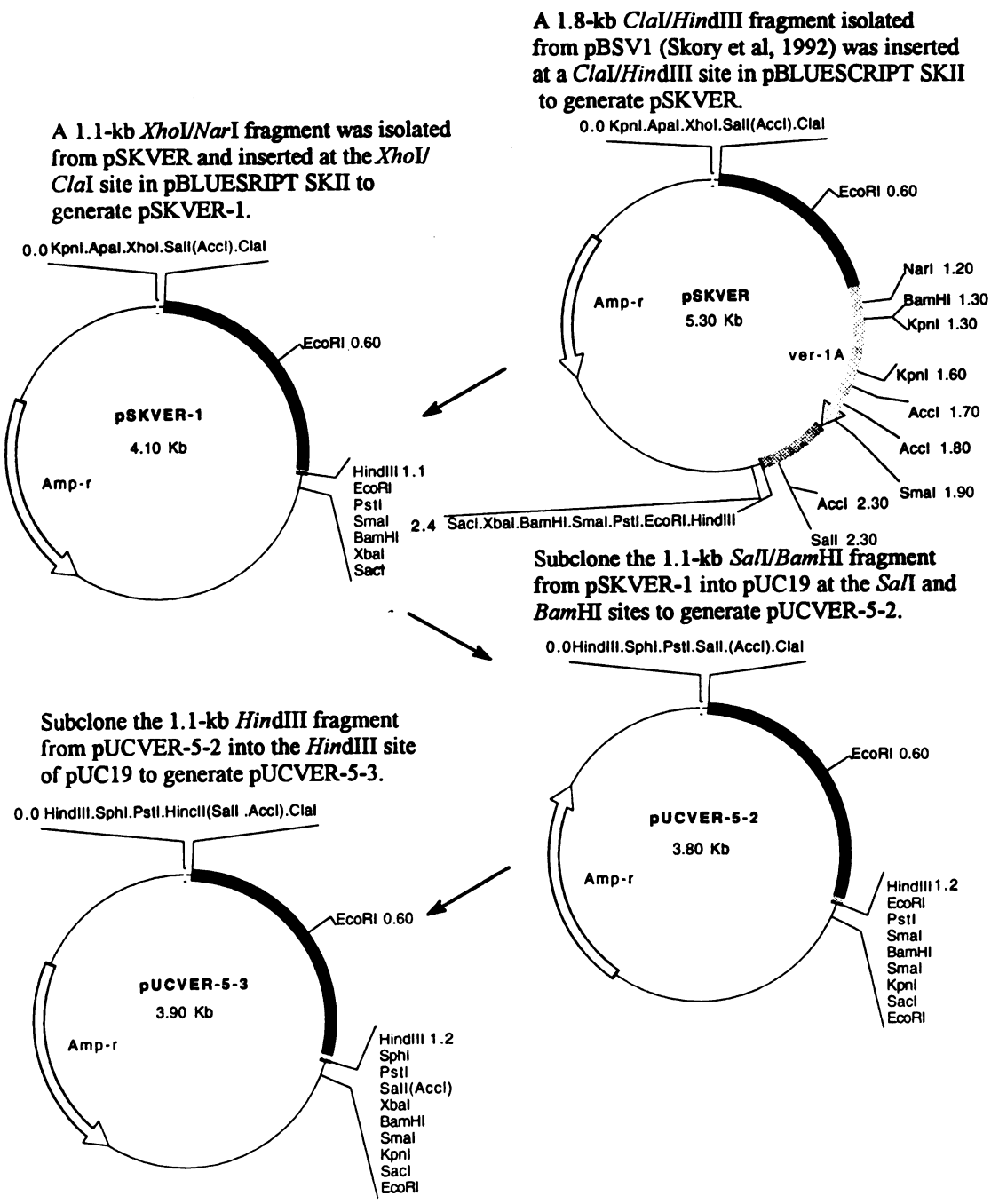
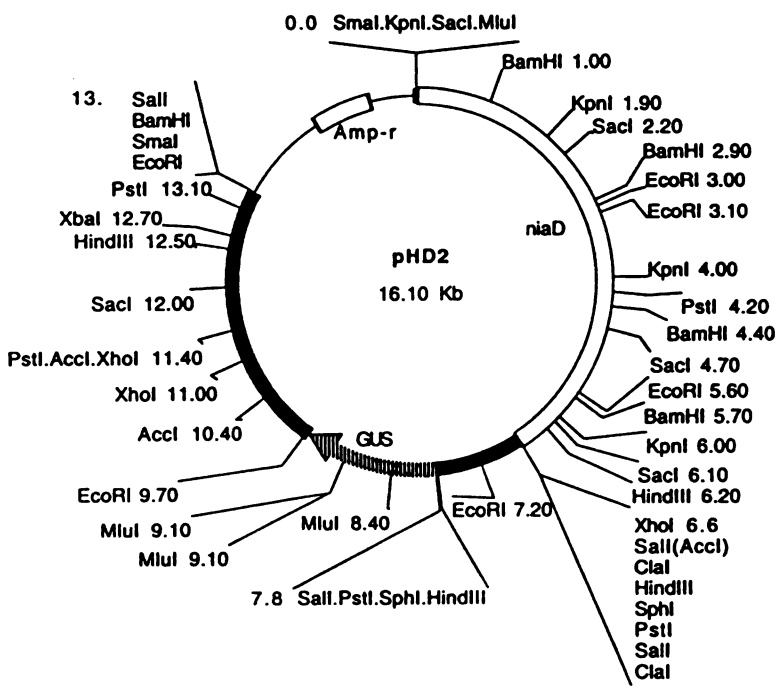

A 1.8-kb ClaI/HindIII fragment isolated from pBSV1 (Skory et al, 1992) was inserted at a ClaI/HindIII site in pBLUESCRIPT SKII to generate pSKVER.
A 1.1-kb XhoI/NarI fragment was isolated from pSKVER and inserted at the XhoI/ClaI site in pBLUESRIPT SKII to generate pSKVER-1.
0.0 KpnI.ApaI.XhoI.SalI(AccI).ClaI
EcoRI 0.60
NarI 1.20
BamHI 1.30
KpnI 1.30
KpnI 1.60
AccI 1.70
AccI 1.80
SmaI 1.90
AccI 2.30
SalI 2.30
pSKVER
5.30 Kb
Amp-r
ver-1A
pSKVER-1
4.10 Kb
HindIII 1.1
EcoRI
PstI
SmaI
BamHI
XbaI
SacI
2.4 SacI.XbaI.BamHI.SmaI.PstI.EcoRI.HindIII
Subclone the 1.1-kb SalI/BamHI fragment from pSKVER-1 into pUC19 at the SalI and BamHI sites to generate pUCVER-5-2.
0.0HindIII.SphI.PstI.SalI.(AccI).ClaI
pUCVER-5-2
3.80 Kb
HindIII 1.2
EcoRI
PstI
SmaI
BamHI
SmaI
KpnI
SacI
EcoRI
Subclone the 1.1-kb HindIII fragment from pUCVER-5-2 into the HindIII site of pUC19 to generate pUCVER-5-3.
0.0 HindIII.SphI.PstI.HincII(SalI .AccI).ClaI
pUCVER-5-3
3.90 Kb
HindIII 1.2
SphI
PstI
SalI(AccI)
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI

Figure 20. (Cont'd)

pUCVER-5-3

Subclone the 1.1-kb *Sph*I DNA fragment of pUCver-5-3 into pGLGUS-2-1 to generate pHD1.

pHD2 was constructed by subcloning a 6.6-kb *Xba*I DNA fragment containing a functional *nia*D gene in pSL82 (Horng et al., 1990) into the *Nhe*I site of pHD1.



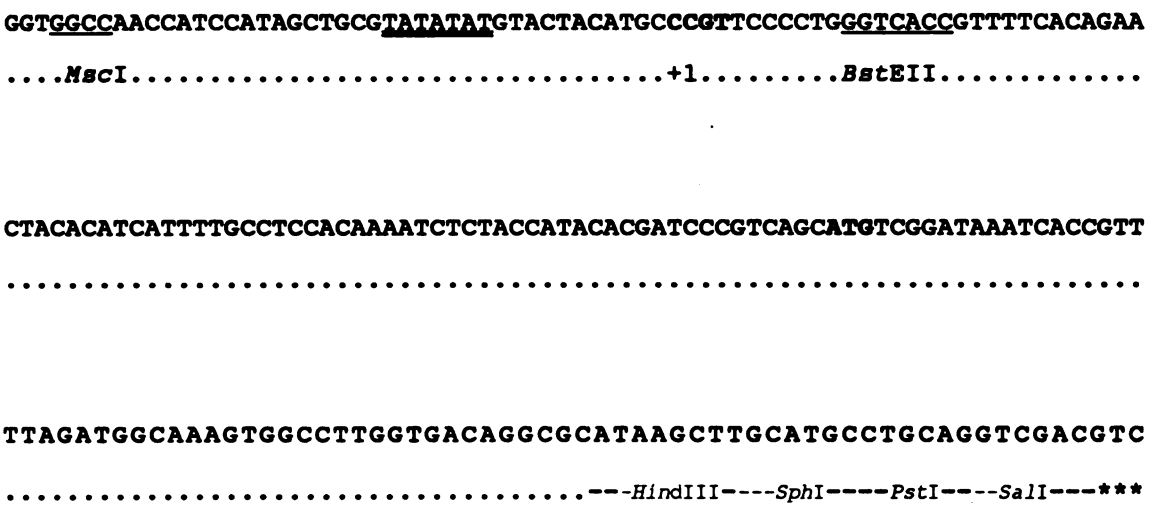
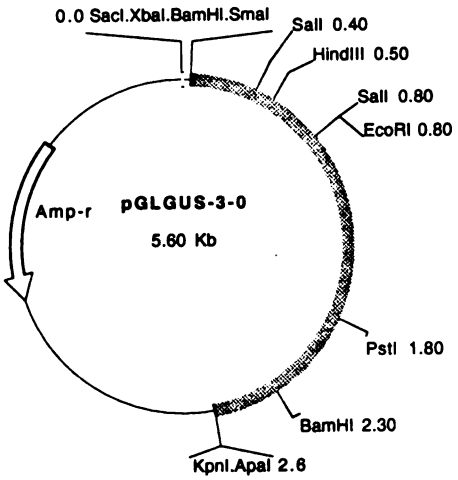


Figure 21. The nucleotide sequence of the *ver*-1 promoter region containing the TATA box and the transcription initiation site. The dotted line (...) represents the *ver*-1A sequences; the dashed line (---) represents sequences derived from pBLUECRIPT SKII as a result of plasmid subcloning; the stars (***) represent the first amino acid in the *uid*A (GUS) gene. The boldface type ATG is the *ver*-1 translation initiation codon. A 51 bp fragment between *Msc*I and *Bst*EII cutting sites was deleted in pHD7-3. The *ver*-1A promoter sequences upstream from the indicated *Hin*dIII site were deleted in pHD4-4. The putative TATA box (doubleunderlined) and the transcription initiation site (+1, boldface type) are also shown.

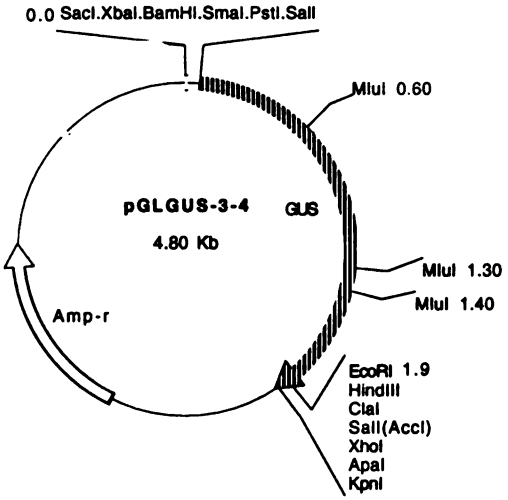
Figure 22. Schematic representation of construction of plasmid pHD4-4 and pHD6-6.

Figure 22.

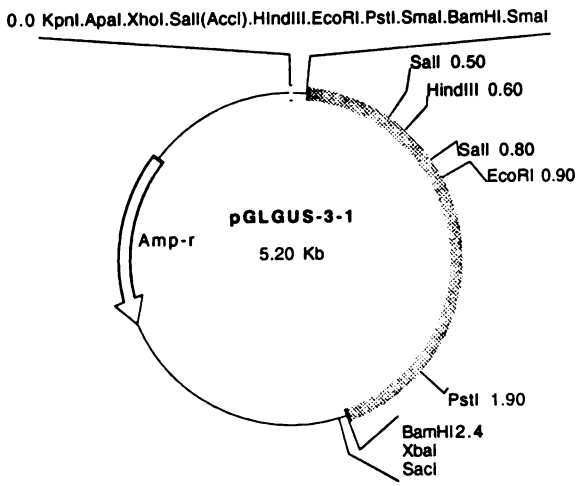
A 2.6-kb *SmaI/ApaI* fragment of *ver*-1 was subcloned and inserted into the *SmaI* and *ApaI* sites of pBLUESCRIPT SKII to generate pGLGUS-3-0.



A 1.87-kb *PstI/EcoRI* fragment from pGLGUS-2-1 was subcloned into the *PstI* and *EcoRI* sites in pBLUESCRIPT SKII to generate pGLGUS-3-4.



A 2.3-kb *BamHI* fragment from pGLGUS-3-0 was subcloned into the *BamHI* site in pBLUESCRIPT SKII to generate pGLGUS-3-1.



A 1.8-kb *KpnI/SacI* fragment in pGLGUS-3-4 was inserted into *KpnI* and *SacI* sites in pUC19 to generate pGLGUS-3-5.

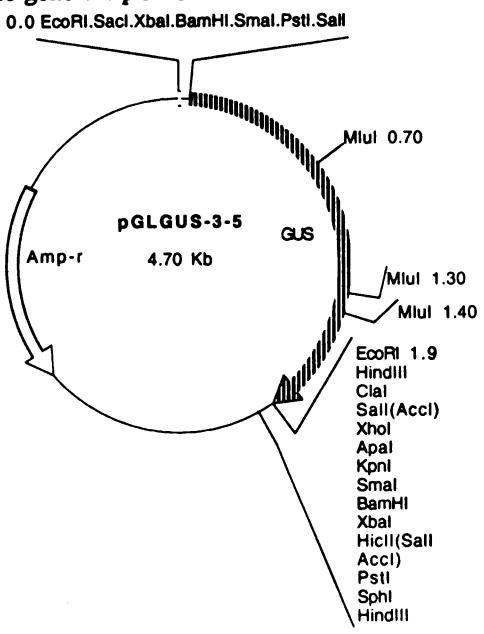
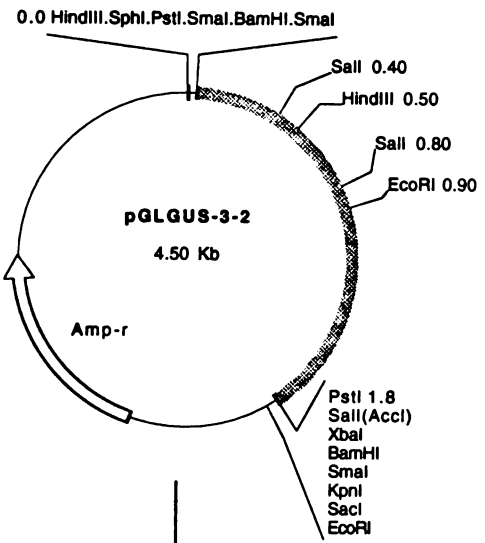


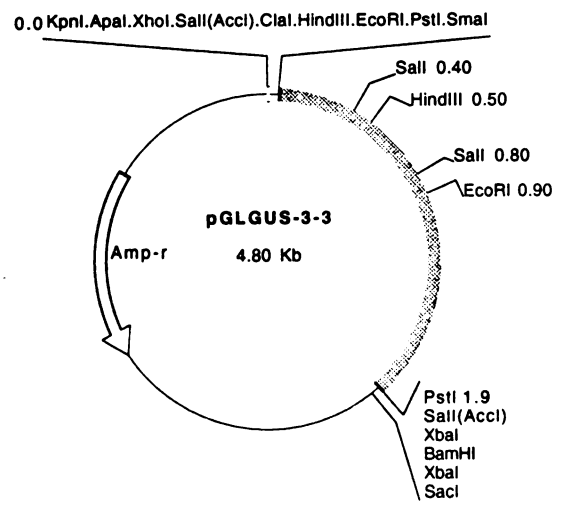
Figure 22. (cont'd).

pGLGUS-3-1

A 1.8-kb *Pst*I fragment from pGLGUS-3-1 was subcloned into the *Pst*I site in pUC19 to generate pGLGUS-3-2.



Insert the 1.8-kb *SmaI/Bam*HI fragment in pGLGUS-3-2 into the *SmaI/Bam*HI site in pBLUESCRIPT SKII to generate pGLGUS-3-3.



pGLGUS-3-5

A 1.8-kb *PstI/Bam*HI fragment from pGLGUS-3-5 was subcloned into the *Pst /Bam*HI site in pGLGUS-2-1 to generate pGLGUS-3-6.

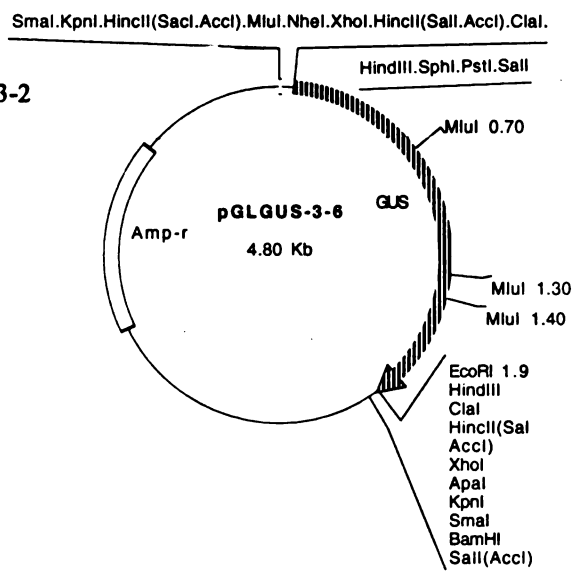
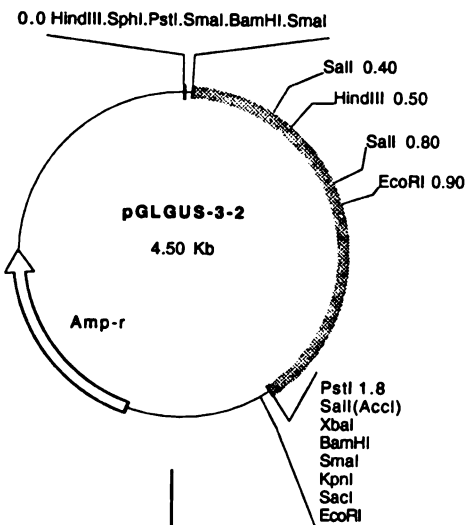


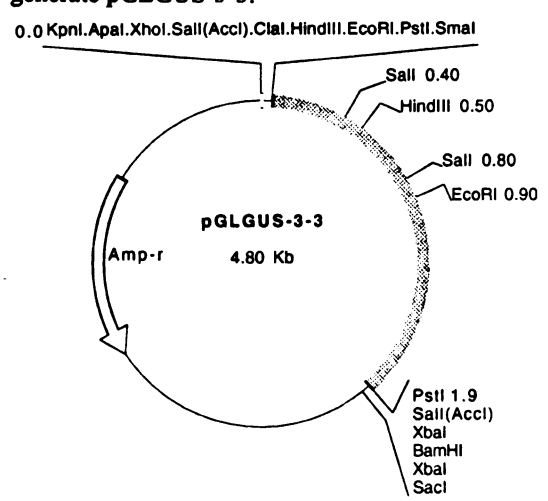
Figure 22. (cont'd).

pGLGUS-3-1

A 1.8-kb *Pst*I fragment from pGLGUS-3-1 was subcloned into the *Pst*I site in pUC19 to generate pGLGUS-3-2.



Insert the 1.8-kb *Sma*I/*Bam*HI fragment in pGLGUS-3-2 into the *Sma*I/*Bam*HI site in pBLUESCRIPT SKII to generate pGLGUS-3-3.



pGLGUS-3-5

A 1.8-kb *Pst*I/*Bam*HI fragment from pGLGUS-3-5 was subcloned into the *Pst* /*Bam*HI site in pGLGUS-2-1 to generate pGLGUS-3-6.

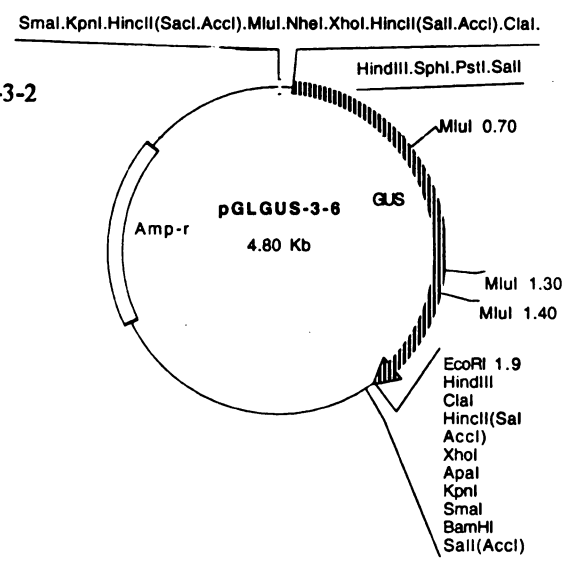
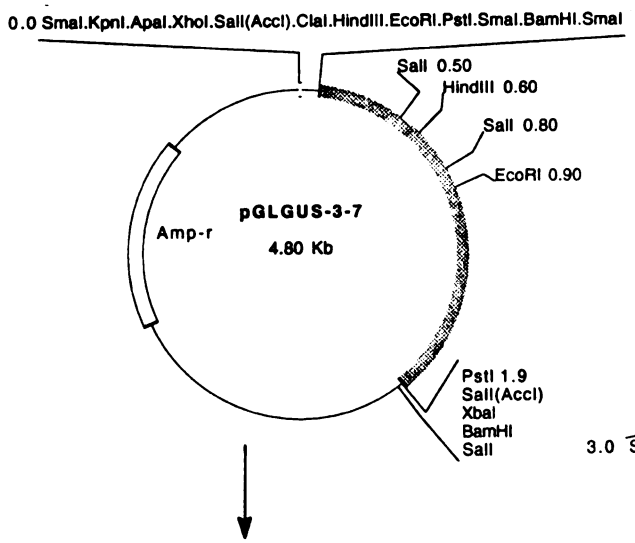
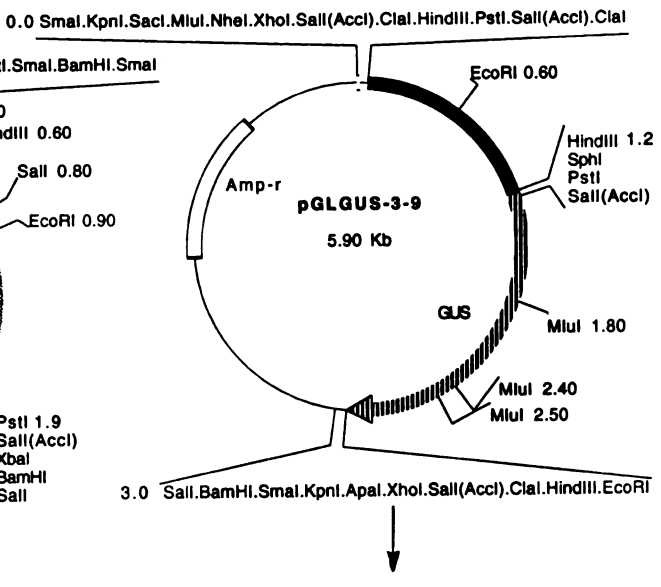


Figure 22. (Cont'd)

The 1.8-kb *KpnI/Bam*HI fragment in pGLGUS-3-3 was inserted into pGLGUS-3-6 digested with *Kpn*I and *Bam*HI to generate pGLGUS-3-7.

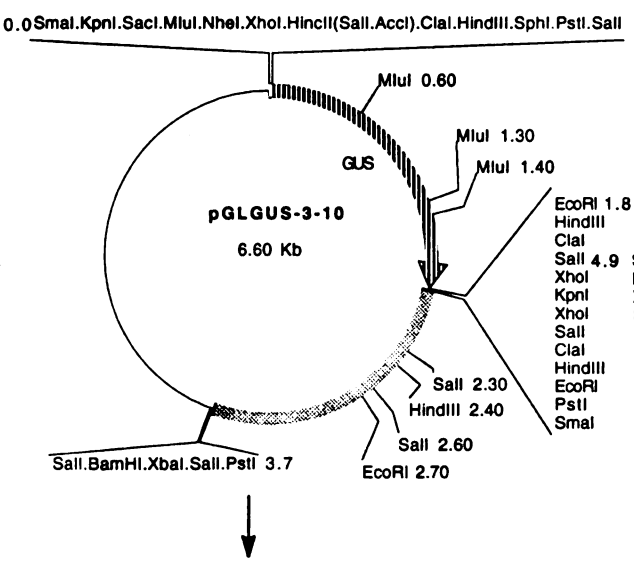


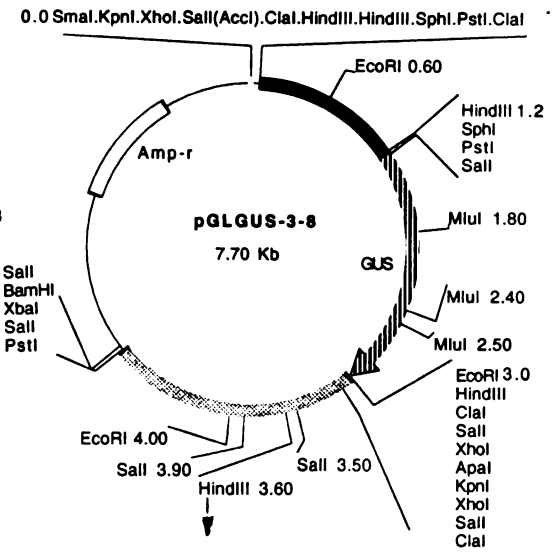
The 1.1-kb *Sph*I fragment of pGLGUS-2-1 was inserted into the *Sph*I site in pGLGUS-3-6 to generate pGLGUS-3-9.



Subclone the 1.87-kb *Kpn*I fragment from pGLGUS-3-6 into the *Kpn*I site of pGLGUS-3-7 to generate pGLGUS-3-10.

Insert 3.0-kb *Kpn*I fragment from pGLGUS-3-9 to the *Kpn*I site of pGLGUS-3-7 to generate pGLGUS-3-8.





Insert the 6.6-kb *nia*D containing *Xba*I fragment from pSL82 into the *Xba*I site in pGLGUS-3-10 to generate pHD4-4.

Subclone a 6.6-kb *nia*D containing *Xba*I fragment from pSL82 into the *Xba*I site of pGLGUS-3-8 to create pHD6-6.

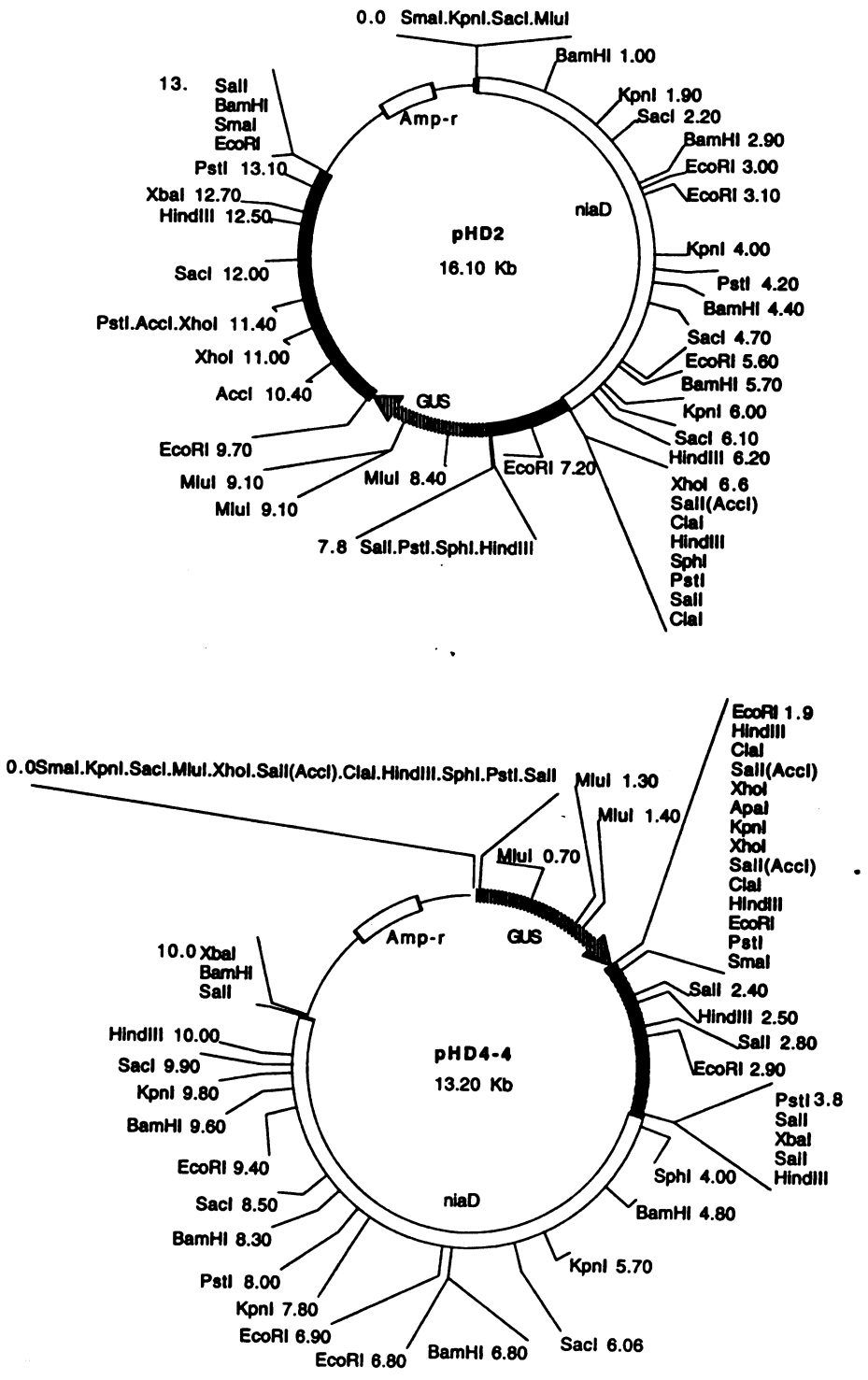
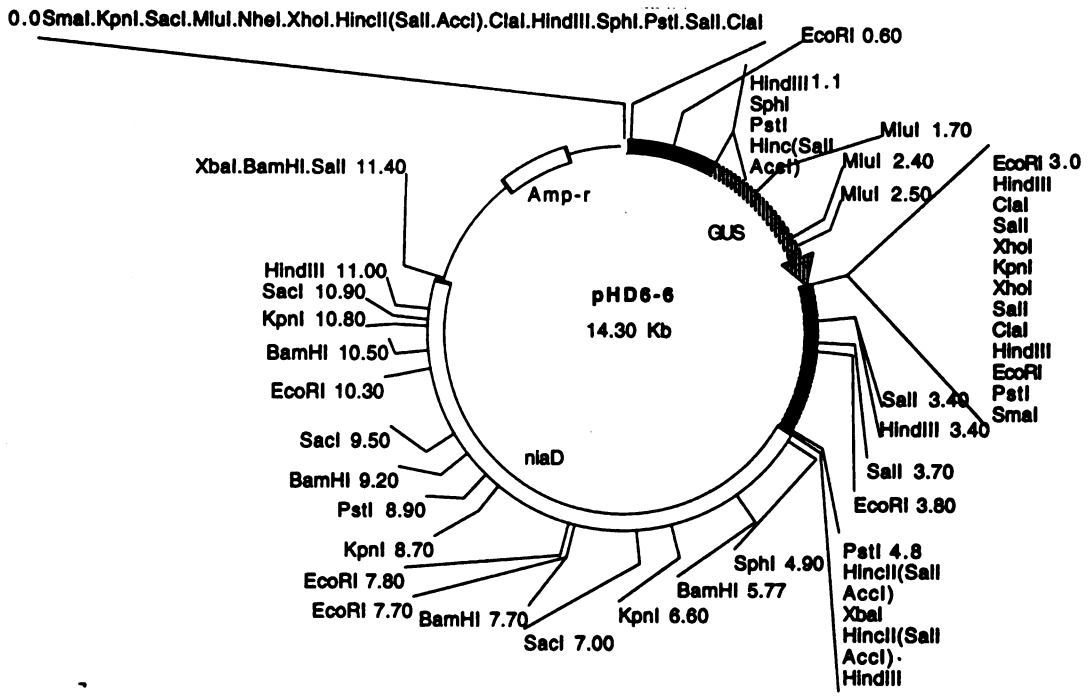
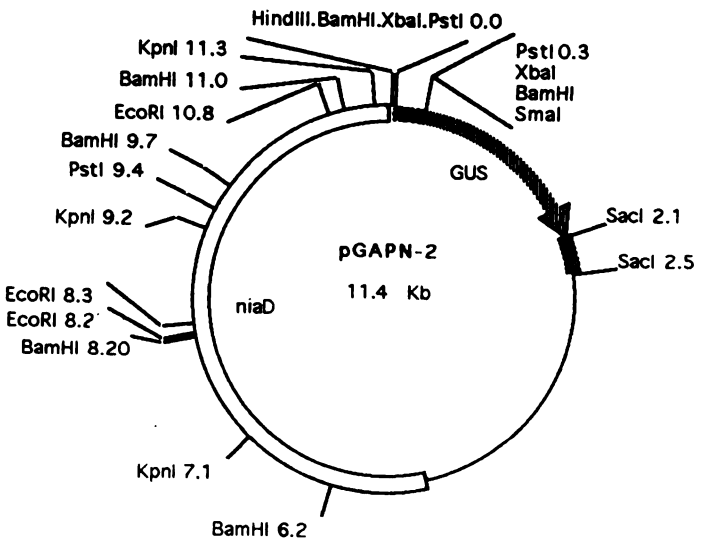


Figure 23. Restriction maps of plasmids pHD2, pHD4-4, pHD6-6, and pGAPN-2.

Figure 23. (cont'd).





to generate pHD7-3. Plasmid pGAPN-2 (Figure 23) was constructed by inserting a 6.0-kb *Hin*dIII fragment containing the functional *nia*D gene from pSL82 into the unique *Hin*dIII site of pGAP-2 (Woloshuk and Payne, 1994). pGAP-2 contains an *A. flavus* β-tubulin gene promoter and a transcription termination signal derived from the *A. nidulans* glutamine amidotransferase gene (*trp*C) fused to the GUS gene. Restriction maps of plasmid pHD2, pHD4-4, pHD6-6 and pGAPN-2 are shown in Figure 23.

Chemicals and general procedures

Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Enzyme digestion, agarose gel electrophoresis, and Southern hybridization analyses were performed according to standard procedures (Ausubel et al., 1987). High-molecular-weight genomic DNA was isolated from mycelia by the phenol-chloroform procedure of Cihlar and Sypherd (1980) as modified by Horng et al (1990). Radiolabeled DNA probes were generated with a Random-Primed DNA Labeling kit (Boehringer Mannheim Biochemicals) with [α-32P]dCTP (DuPont).

Transformation of fungal protoplasts and culture conditions

Fungal protoplasts were transformed essentially as described by Oakley et al. (1987), with minor modifications (Skory et al., 1990). Cells transformed with plasmid pSL82, pHD2, pHD4-4, pHD7-3, pHD6-6, or pGAPN-2 were screened on Czapek-dox agar (CZ agar, Difco) for the utilization of nitrate as the sole nitrogen source (*nia*D$^+$). Fungal spore stocks were prepared from 10 day old mycelia by scraping the surface of potato dextrose agar (PDA, Difco, Detroit, Mich.) inoculated with a spore

suspension. Spore cultures were grown in 150 cm^2 canted neck polystyrene tissue culture flasks (Corning, New York, N.Y.). Fungal strains were maintained as frozen spore stocks in 20% glycerol at -70°C.

For genomic DNA isolation, 100 ml of yeast extract-sucrose liquid medium (YES; 2% yeast extract, 6% sucrose [pH 5.5]) in a 250 ml erlenmeyer flask was inoculated with $2x10^6$ frozen spores of individual fungal isolates. Cultures were incubated on a rotary shaker at 160 rpm at 30°C in the dark for 2 days. Mycelia used for the determination of mycelial dry weight, total RNA isolation, protein extraction and GUS activity assay were harvested at specific time points by filtration through miracloth (Behring Diagnostics, La Jolla, Calif.). The culture medium was analyzed for aflatoxin production by ELISA. For the nutritional shift assay (Figure 24), $1x10^7$ spores from each isolate were inoculated in two 500 ml erlenmeyer flasks containing 200 ml PMS media (Buchanan et al., 1983; Buchanan and Lewis, 1984), and incubated at 30°C with shaking at 200 rpm in the dark. Mycelia were collected at 65 h and 0.5 g aliquots (wet weight) were transferred into 30 ml PMS or GMS, and incubated for another 3, 7, 10, and 24 h. The mycelia and culture medium obtained from these four time points were subject to total RNA isolation, protein isolation and GUS activity analysis as well as aflatoxin detection.

Analysis of mycelial dry weight and aflatoxin production

Fungal mycelia were harvested at specified times by filtration through miracloth (Behring Diagnostics, La Jolla, Calif.). A quarter of the collected mycelia was dried completely at 70°C prior to weighing. The production of aflatoxins was analyzed by

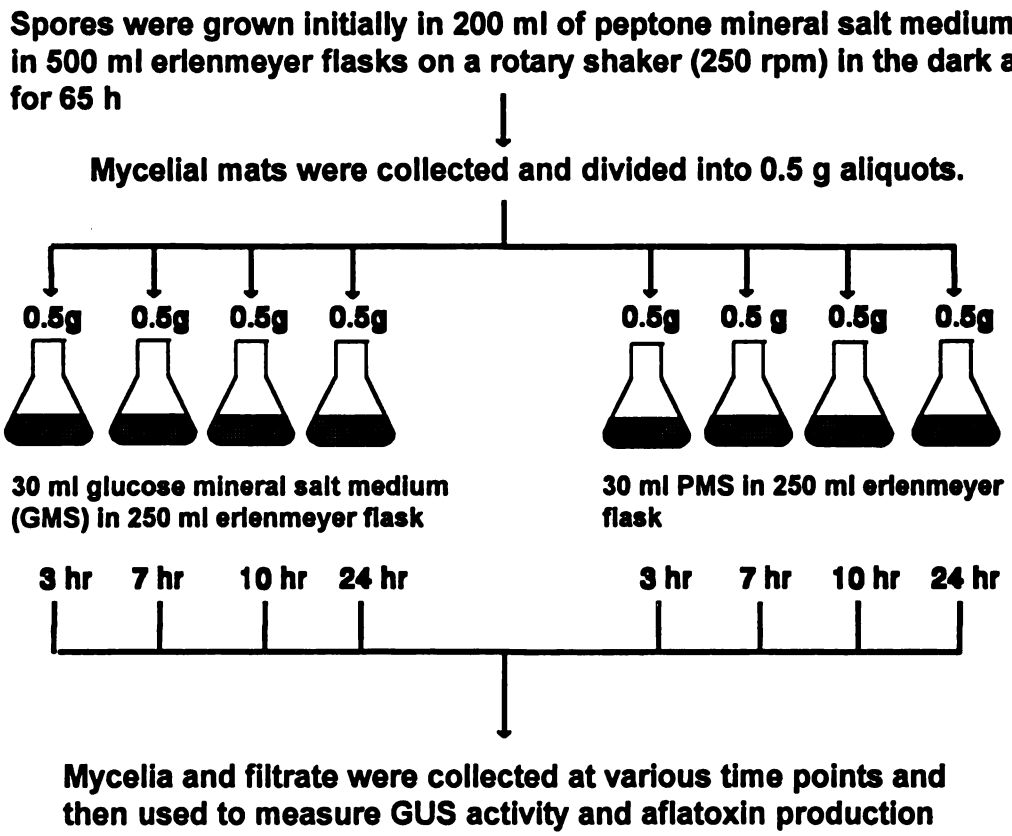


Figure 24. Diagrammatic representation of the nutritional shift assay. An *niaD* strain of *A. parasiticus* NRRL5862 (recipient strain), transformant 8 (control transformant), transformants 2 and 6 were used in the nutritional shift assay to correlate GUS activity with aflatoxin production. PMS: peptone mineral salt medium, an aflatoxin-noninducing medium. GMS: glucose mineral salt medium, an aflatoxin-supporting medium.

direct competitive enzyme-linked immunosorbent assay (ELISA). ELISA analyses were preformed on the culture medium according to the procedure developed by Pestka (Pestka, 1988) using AFB1 monoclonal antibodies and AFB1-horseradish peroxidase conjugate (kindly provided by Dr. J. Pestka, Michigan State University).

Isolation and analysis of RNA

Fungal mycelia were collected as described in the general procedures. Total RNA was purified using a hot-phenol protocol previously described by Maramatsu (1973). Northern analysis was performed on total fungal RNA as described by Ausubel et al. (1987). Approximately 30 μg of total RNA from each sample was separated on a 1.0% denaturing formaldehyde agarose gel. Electrophoresis was performed until the marker dye (bromphenol blue) migrated 2/3 of the way through the gel. RNA was transferred onto Nytran membrane (Schleicher and Schuell, Inc., Keene, N.H.). Northern hybridization analyses were performed using radiolabeled DNA probes.

Protein extraction and Western blot analyses

A cell extract was prepared using a method described for GUS protein analysis (Jefferson, 1987). Total protein was determined by the dye-binding method of Bradford (1976) using a kit provided by Bio-Rad Laboratories (Hercules, CA). Western blot analysis was performed using denaturing SDS-PAGE according to standard procedures (Ausubel et al., 1987). For Western blot analysis, 150 μg of total protein was denatured in sodium dodecyl sulfate (SDS) gel loading buffer (50 mM Tris [pH6.8], 100 mM Dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 2% β-mercaptoethanol) and resolved on a 10% polyacrylamide gel. After

electrophoresis, the separated proteins were transferred to PVDF membrane (Du Pont). The separated proteins were detected using polyclonal antibody raised against a *ver*-1 maltose binding fusion protein (Liang and Linz, 1994) or antiserum against β-glucuronidase (Clontech Laboratories, Inc.) using a RAD-FREE chemiluminescent detection kit (Schleicher & Schuell) .

GUS activity analysis

Preliminary screening of transformants for GUS activity was performed qualitatively by fluorometry using 4-methylumbelliferyl glucuronide (MUG, Sigma) as the substrate. A spore suspension (spore amount undetermined) of each isolate was inoculated into YES in individual wells of a 48-well microtiter plate and incubated under stationary conditions at 30°C in the dark. Mycelia were harvested after 48 hr growth, macerated, and approximately 500 mg was used for a qualitative GUS assay. The harvested mycelia were pulverized in 500 µl lysis buffer (50 mM NaH_2PO_4 [pH7.0], 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mM beta-mercaptoethanol) with 40 mg of glass beads (75 micron) using a mortar and pestle. 20 µl of cell extract were then mixed with 200 µl of lysis buffer containing 2 mM MUG (substrate for β-glucuronidase). The reaction was incubated at 37°C, and terminated with the addition of 0.8 ml of 0.2 M Na_2CO_3. GUS activity was determined by the appearance of fluorescence under long U.V. light (345 nm) in a transilluminator.

To determine GUS activity quantitatively, mycelia from batch fermentation or from a nutritional shift were harvested at specific time points and pulverized in liquid

nitrogen using a mortar and pestle. Five mm^3 of the mycelial powder was suspended in 500 µl lysis buffer and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was used to determine total protein by the method of Bradford. GUS activity was determined by mixing 10 µg of total protein from each lysate with 200 µl lysis buffer containing 2 mM MUG. The reaction was incubated at 37°C and terminated with the addition of 0.8 ml stop buffer at 0, 15, and 30 min intervals. The GUS activity at each time point was determined using a spectroflurometer (excitation at 365 nm, emission at 455 nm) .

RESULTS

Plasmid pHD2 was constructed in order to generate preliminary data to verify that the GUS gene could be used as a reporter gene for analysis of transcription regulation of aflatoxin pathway genes in *A. parasiticus*. However, pHD2 could not be used to identify cis-acting DNA elements by deletion analysis. Therefore, plasmids pHD4-4, pHD7-3, and pHD6-6 were constructed in subsequent experiments to validate the use of GUS as the reporter gene.

Transformation

The results derived from five independent transformation experiments using *ver*/GUS plasmid constructs are summarized in Table 2. The average transformation efficiency was 30-40 *niaD*$^+$ transformants per µg of transforming DNA per 10^6 protoplasts.

Table 2. Five independent transformation experiments of *A. parasiticus* NR2 transformed with *ver*-1/GUS plasmids and control plasmids.

	pSL82	pHD2	pHD4-4	pHD7-3	pHD6-6	pGAPN-2
Total *niaD*$^+$ transformant	48	60	212	61	173	35
individual transformants tested for GUS activity[a]	30	7	16	61	15	11
GUS^{+b}	0	4	0	7	7	6
GUS^{-b}	30	3	16	54	8	5
DNA isolation[c]	0	7	6	13	15	8

[a]GUS assays were performed initially by the qualitative GUS assay described in the text.
[b]GUS activity was monitored under long U.V. light (345 nm) using transilluminator.
[c]Number of transformants selected for Southern hybridization analyses.

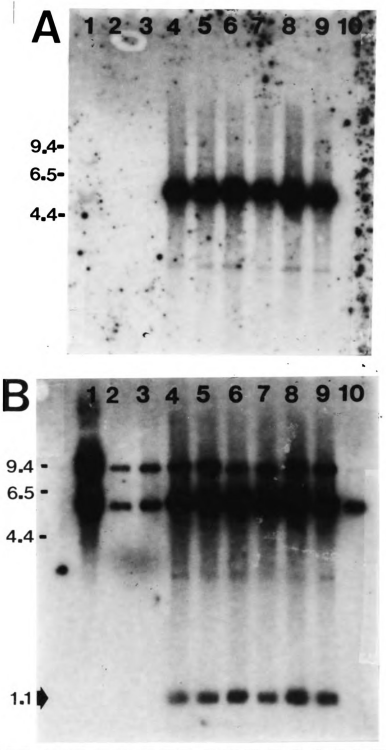
Preliminary evaluation of the GUS reporter system using pHD2

Southern hybridization analysis. After transformation of *A. parasiticus* NRRL5862 (SU-1, NR2) with pHD2, 60 *niaD*$^+$ transformants were identified. Southern hybridization analysis was performed on genomic DNAs isolated from the recipient strain (NR2) and 7 (designated isolates 2, 3, 4, 5, 6, 7, and 8) *niaD*$^+$ transformants. Genomic DNA samples were digested with *Sal*I and probed with a radiolabeled 1.87-kb *Eco*RI/*Bam*HI fragment containing the β-glucuronidase coding sequence (GUS probe) isolated from pRAJ275 (Figure 25A) (Jefferson, 1987) or a radiolabeled 0.6-kb *Cla*I/*Eco*RI fragment (*ver*-1 probe) containing the 5' untranslated region of *ver*-1A (Figure 25B). A 5.6-kb DNA fragment hybridized to the GUS probe in genomic DNA isolated from transformants 2, 3, 4, 5, 6, and 7, indicating that pHD2 integrated into the fungal genome by a single crossover at the *niaD* locus (Figure 25A). When hybridized to the 0.6-kb *Cla*I/*Eco*RI *ver*-1A promoter fragment, 9.0-kb and 6.0-kb DNA fragments (Figure 25B), which represent the *ver*-1A and *ver*-1B loci, were observed. A 1.1-kb *Sal*I fragment observed for transformants 2, 3, 4, 5, 6, and 7 resulted from the integration of pHD2 sequences. These results indicated that pHD2 integrated into the genome at the *niaD* locus in transformants 2, 3, 4, 5, 6, and 7 and a gene conversion event may have occurred in transformant 8 (no GUS).

GUS activity analysis. NR2 and the same 7 *niaD*$^+$ transformants were analyzed for GUS expression by a qualitative GUS assay. GUS activity (fluorescence) was observed for transformants 2, 3, 6, and 7 whereas NR2 and transformants 4, 5, and 8 did not appear to have GUS activity. Two transformants, 2 and 6, which expressed

Figure 25. Southern hybridization analyses of genomic DNA isolated from *A. parasiticus* NR2 (recipient) and 7 *nia*D$^+$ isolates transformed with pHD2 or pSL82. (A) Genomic DNA was digested with *Sal*I and hybridized with a radiolabeled 1.87-kb *Sal*I/*Eco*RI *uid*A gene fragment (GUS probe). (B) Identical genomic DNA samples were hybridized with a radiolabeled 0.6-kb *Cla*I/*Eco*RI *ver*-1A promoter probe. Lane 1, genomic DNA isolated from untransformed *A. parasiticus* NRRL5862 (SU-1); lane 2, genomic DNA from untransformed *A. parasiticus* ATCC36537 (*ver*-1, *wh*-1); lane 3, *nia*D$^+$ transformant of *A. parasiticus* NR2 transformed with pSL82; Lane 4 to 10, 7 *nia*D$^+$ transformants (isolates 2 to 8, respectively) transformed with pHD2. *Hin*dIII digested Lambda DNA molecular weight markers are indicated at left.

Figure 25.



GUS activity in the qualitative assay, plus transformant 8, and NR2 were tested in the quantitative GUS assay. These four strains were incubated for 17, 24, and 36 h in batch fermentation and 3, 7, 10, and 24 h after the shift in a nutritional shift assay, respectively. A rapid increase in GUS activity was observed in transformants 2 and 6 during 36 h growth in YES (Table 3). No GUS activity was detected in NR2 or transformant 8 (*niaD* gene conversion) at the three time points indicating that there is no endogenous GUS activity in *A. parasiticus*. The pattern of GUS expression (Table 3) in transformants 2 and 6 correlated well with the pattern of aflatoxin production in which high levels of aflatoxins were not detected until 36 h of growth in YES.

Table 3. Aflatoxin accumulation and GUS activity in aflatoxin supporting medium

	AFB_1 conc. (ng/mL filtrate)[a]				GUS activity (pmol 4-MU/min/mg)			
Hrs	No. 2	No. 6	control	wt	No. 2	No. 6	control	wt
17	155	180	150	285	8.76	3.6	nd	nd
24	155	6500	200	540	3.96	8.04	nd	nd
36	6100	7200	1350	6450	66	50	nd	nd

[a]Determined by direct competitive ELISA
nd, none detected; na, not available; wt, wild type strain of *A. parasiticus* (SU-1); control, transformant 8 indicated in the text.

In order to confirm the correlation between the pattern of GUS expression and aflatoxin production, a nutritional shift assay was performed on NR2 and three pHD2 transformants (isolates 2, 6, and 8). This assay was employed to minimize the effects of fungal growth stage and fungal mass on gene expression and aflatoxin production. The timing of GUS expression in transformants 2 and 6 correlated well with the timing of aflatoxin production in these isolates (Table 4 and Figure 26) whereas no GUS activity was observed in NR2 or transformant 8 (control). However, a 27 fold higher level of GUS activity was observed in transformant 6 compared to transformant 2 whereas no significant difference in GUS activity was observed between these two transformants after 17, 24, and 36 h growth in YES (Table 3). The data obtained from batch fermentation and nutritional shift indicate that the timing of GUS expression in transformants 2 and 6 is similar to the timing of the production of aflatoxins in these transformants. The results also suggest that integration of a gene involved in secondary metabolism at a heterologous chromosomal location (*niaD* locus) does not appear to affect its temporal expression pattern. However, we still can not rule out the possibility that the GUS gene may be subject to post-transcriptional or translational modification.

The experimental evidence obtained from the preliminary evaluation of GUS reporter system by using pHD2 transformants indicates that the GUS gene is a valuable tool in the transcriptional analysis of aflatoxin biosynthesis. Therefore, experiments were performed to thoroughly evaluate the GUS reporter system.

Table 4. Aflatoxin accumulation and GUS activity following nutritional shift

AFB_1 concentration (ng/mL filtrate)[a]								
	No. 2		No. 6		control		wt	
Hrs[b]	PMS	GMS	PMS	GMS	PMS	GMS	PMS	GMS
0	28	28	34	34	7.1	7.1	24	24
3	11	12	160	nd	16	5	14	nd
7	35	250	100	330	nd	1900	nd	315
10	22	510	nd	590	24	na	62	650
24	240	2050	420	2000	na	6900	66	2500

GUS Activity (pmol 4-MU/min/mg)								
	No. 2		No. 6		control		wt	
Hrs[b]	PMS	GMS	PMS	GMS	PMS	GMS	PMS	GMS
0	nd	nd	nd	nd	nd	nd	nd	nd
3	nd	nd	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd	nd	nd
10	nd	nd	nd	15.6	nd	nd	nd	nd
24	nd	3	nd	84	nd	nd	nd	nd

[a]Determined by direct competitive ELISA

[b]Hours after shift from initial PMS medium to either fresh PMS or GMS.

PMS, peptone mineral salt medium;

GMS, glucose mineral salt medium;

nd, none detected; na, not available; wt, wild type strain of *A. parasiticus* (SU-1);

control, transformant 8 indicated in the text.

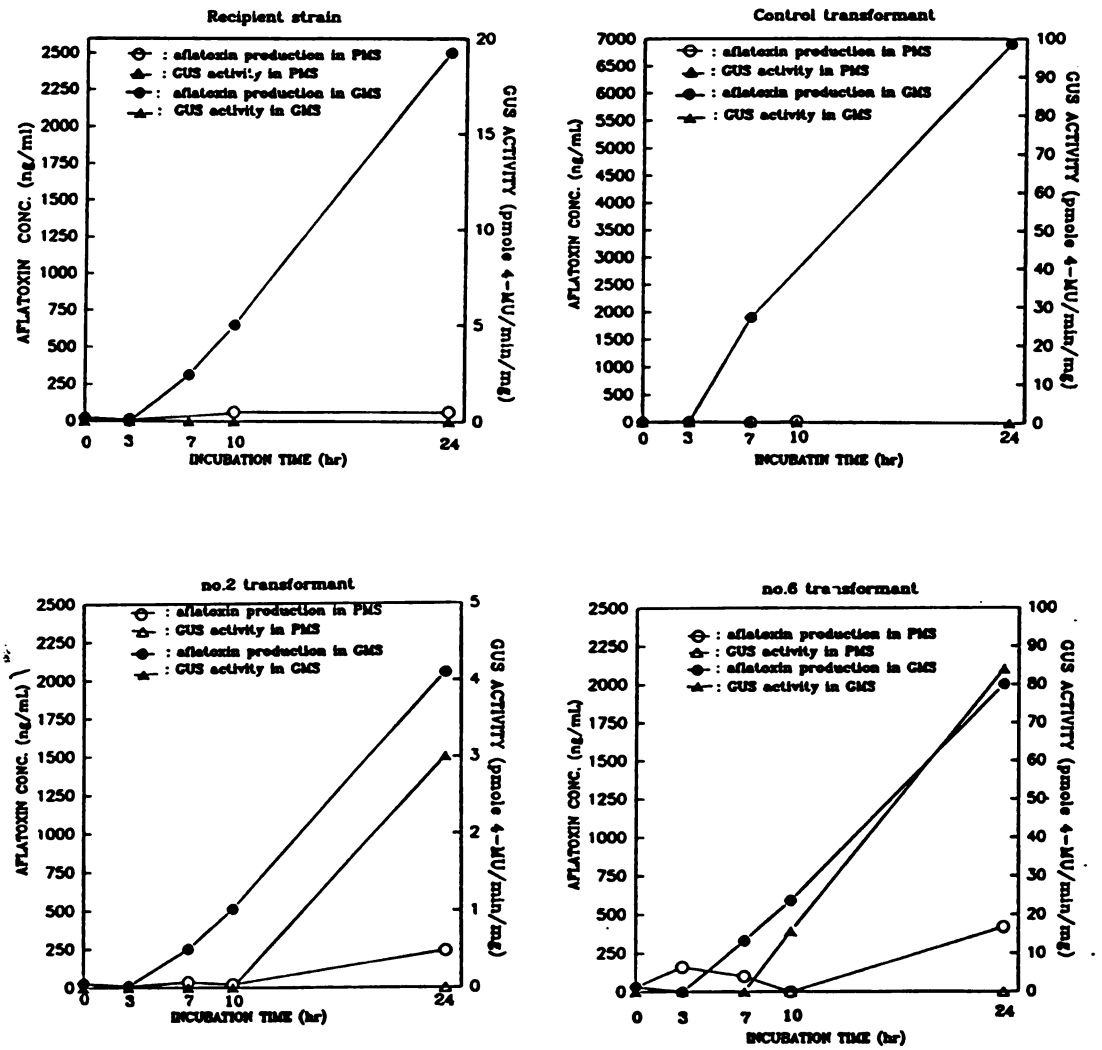


Figure 26. A comparison of the timing of appearance of aflatoxin and GUS activity of *A. parasiticus* NR2 (recipient), transformants 8 (control), 2, and 6 during nutritional shift.

Development of a convenient system for evaluation of aflatoxin gene expression using a GUS reporter gene

Plasmids pHD4-4, pHD7-3, pHD6-6, pGAPN-2, and pSL82 were used as the transforming vectors in the thorough evaluation analyses. After transformation of *A. parasiticus* NR2, *nia*D$^+$ transformants were identified on CZ medium (Table 2).

Qualitative GUS assay. *nia*D$^+$ transformants of *A. parasiticus* NR2 transformed with pSL82, pHD4-4, pHD7-3, pHD6-6, and pGAPN-2 were analyzed for GUS activity by the qualitative GUS assay prior to DNA isolation and subsequent Southern hybridization analysis (Table 2). The identification of GUS$^+$ transformants among the population of *nia*D$^+$ transformants served the following purposes: (1) to screen out the *nia*D$^+$ transformants with a gene conversion (gene replacement) event at the *nia*D locus; (2) to select for *nia*D$^+$ and GUS$^+$ pHD7-3 transformants to establish the role of the putative TATA box in expression of the *ver*-1A gene; (3) to identify *nia*D$^+$, pHD6-6 transformants lacking the ability to express GUS.

Southern hybridization analysis. Southern hybridization analyses were performed on genomic DNAs isolated from six GUS$^-$ pHD4-4 transformants (isolates 3, 4, 5, 14, 15, and 16), 8 GUS$^-$ pHD7-3 transformants (isolates 1, 2, 5, 15, 16, 17, and 51) and 5 GUS$^+$ pHD7-3 transformants (isolates 9, 14, 30, 42, and 57), 8 GUS$^-$ pHD6-6 (isolates 5, 7, 8, 9, 12, 16, 17, and 20) and 7 GUS$^+$ pHD6-6 transformants (isolates 1, 2, 4, 10, 13, and 18), and 4 GUS$^-$ pGAPN-2 (isolates 3, 4, 8, and 10) and 4 GUS$^+$ pGAPN-2 transformants (isolates 1, 6, 7, and 12) (Table 2 and Figure 27A and 27B). Genomic DNAs were digested to completion with *Xba*I and hybridized with a

radiolabeled 1.87 kb-*SalI*/*Eco*RI *uid*A fragment (GUS gene) (Figure 27A). A 18.0-kb DNA fragment observed in pHD6-6 transformants 1 and 4 suggested that a single integration event occurred between the 3' transcription termination signal of the vector and its chromosomal counterpart. The single 7.8-kb DNA fragment observed for pHD6-6 transformants 2, 5, 8, 9, 10, and 18, respectively, suggested that a single integration event occurred between the subcloned *nia*D gene in pHD6-6 and the genomic *nia*D locus in the recipient strain. pHD6-6 transformants 6 and 13 resulted from multiple integrations at the *nia*D locus. Gene conversion most likely occurred in pHD6-6 transformants 7, 16, and 20, generating *nia*D$^+$, GUS$^-$ transformants. The more complicated DNA hybridization pattern observed in transformants 12 and 17 cannot be explained by homologous integration events. This nonhomologous integration event likely occurred at a location other than *nia*D or *ver*-1. A 6.7-kb DNA fragment was observed in pHD4-4 transformants 3, 5, and 14, indicating a single crossover between the subcloned *nia*D in pHD4-4 and its genomic counterpart. The lack of an additional *nia*D fragment suggests that a gene conversion (gene replacement) occurred in pHD4-4 transformants 4, 15, and 16 while in pHD7-3 transformants 5, 15, 17, and 51 a single crossover at *nia*D locus likely generated the 7.8-kb *nia*D DNA fragment. The analysis of pHD7-3 transformants is similar. A 18.0-kb DNA fragment observed in pHD7-3 transformant 14 indicates that integration of pHD7-3 may have occurred at the *ver*-1A 3'-region and a gene conversion may have occurred in pHD7-3 transformants 2 and 52. pHD7-3 transformants 42 and 57 appear to have multiple integrations at the *nia*D locus while nonhomologous

Figure 27. Southern hybridization analysis of genomic DNAs isolated from *A. parasiticus* NR2 transformed with pHD4-4, pHD7-3, and pHD6-6. (A) Genomic DNAs were digested with *Xba*I and hybridized with a radiolabeled 1.87-kb *PstI/Eco*RI fragment containing the GUS gene from pRAJ275 (Figure 18). (B) Identical genomic DNAs were digested with *Xho*I and hybridized with a radiolabeled 1.8-kb *SmaI/Pst*I fragment containing the *ver*-1A 3'-end. Lane NR2, genomic DNA from untransformed *A. parasiticus* NR2 (recipient); lanes 1, 2 and 3, three *nia*D$^+$, pHD4-4 transformants (isolates 3, 4, and 5, respectively); lane 4 to 9, six *nia*D$^+$, pHD7-3 transformants (isolates 9, 14, 16, 30, 42, and 57, respectively); lane 10 to 24, fifteen *nia*D$^+$, pHD6-6 transformants (isolates 1, 2, 5, 6, 7, 8, 9, 10, 12, 4, 13, 16, 17, 18, and 20, respectively); lane 25 to 27, three *nia*D$^+$, pHD4-4 transformants (isolates 14, 15, and 16, respectively); lane 28 to 34, seven pHD7-3 transformants (isolates 1, 2, 5, 15, 17, 51, and 52, respectively). DNA size markers included at the left for each corresponding panel: *Hin*dIII-digested lambda DNA (in kilobases).

Figure 27.

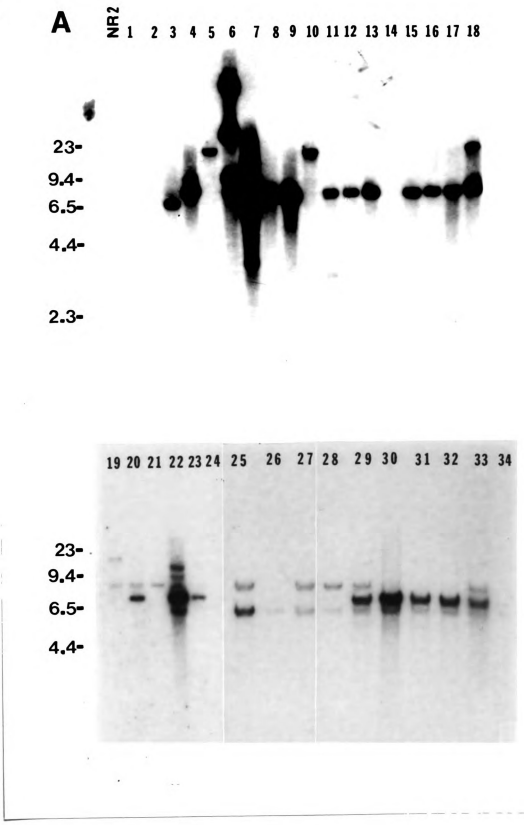
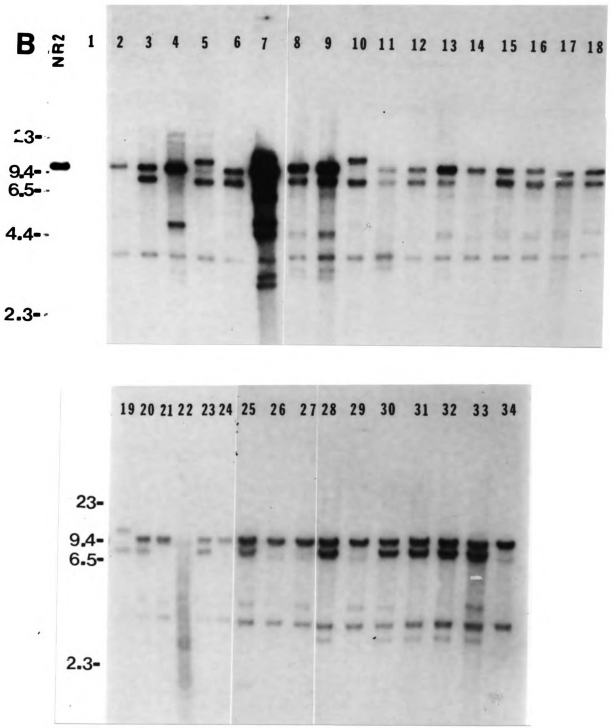

A
NR2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
23-
9.4-
6.5-
4.4-
2.3-
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
23-
9.4-
6.5-
4.4-

Figure 27. (cont'd)



integration may have occurred in transformants 1, 9, 16, and 30.

The identical DNAs were digested with *Xho*I and hybridized with a radiolabeled 1.8-kb *Sma*I/*Pst*I fragment (Figure 19) containing the *ver*-1A 3'-end (Figure 27B). Eleven kb and 3.8-kb DNA fragments, representing part of the endogenous *ver*-1A and *ver*-1B sequences, respectively, were observed in the recipient strain and all of the pHD6-6, pHD4-4, and pHD7-3 transformants. However, 13.5-kb and 3.8-kb DNA fragments were observed for two pHD6-6 transformants (isolates 1 and 4) and one pHD7-3 transformant (isolate 14), confirming that a single crossover occurred in the *ver*-1A 3' region in pHD6-6 transformants 1 and 4 and pHD7-3 transformant 14. A 9.3-kb DNA fragment observed in pHD4-4 transformants 3, 5, and 14, pHD7-3 transformants 5, 15, 17, and 51, and pHD6-6 transformants 5, 8, 9, 2, 10, and 18 confirmed that an integration event occurred between the subcloned *nia*D gene in the vector and its genomic counterpart. The absence of this fragment in pHD4-4 transformants 4, 15, and 16, pHD7-3 transformant 52, and pHD6-6 transformants 7, 16, and 20 confirmed that a gene conversion event occurred at the *nia*D locus. The increased intensity observed for the 11.0-kb DNA fragment in pHD6-6 transformants 6 and 13 and pHD7-3 transformants 42 and 57 was consistent with a multiple integration event at the *nia*D locus. The distinct banding patterns in pHD7-3 transformants 9, 16, and 30 suggest that a nonhomologous integration event may have occurred. However, we cannot rule out the possibility of a deletion or genetic rearrangement in the genomic DNA for these three transformants. A pSL82 transformant, three *nia*D$^+$, GUS$^-$ pHD4-4 transformants (isolates 3, 5, and 14), three

*nia*D$^+$, GUS$^-$ pHD7-3 transformants (isolates 5, 15, and 17), five *nia*D$^+$, GUS$^+$ (transformants 1, 2, 4, 10, and 18), one *nia*D$^+$, GUS$^-$ pHD6-6 transformants (isolate 8), as well as three *nia*D$^+$, GUS$^+$ pGAPN-2 transformants (isolates 6, 7, and 12) were selected for further analyses.

Determination of mycelial dry weight and aflatoxin production. Mycelial dry weight and aflatoxin production were determined on the selected transformants grown for 24, 48, and 72 h in batch fermentation (Table 5). A significant variation in total aflatoxin production (ng/ml medium) was observed among the transformants (Table 5). However, this variation was significantly decreased when aflatoxin production was normalized to the dry weight of the producing mycelia (Table 5).

Quantitative GUS activity analysis. The selected pSL82, pHD4-4, pHD7-3, pHD6-6 and pGAPN-2 transformants were subjected to a quantitative GUS assay. The results from the quantitative GUS assay (Table 6) were consistent with those obtained in the qualitative assay. The *nia*D$^+$, GUS$^-$ transformants determined by qualitative assay, a pSL82 transformant, three pHD4-4 transformants (isolates 3, 5, and 15), three pHD7-3 transformants (isolates 5, 15, and 17), and one pHD6-6 transformant (isolate 8) did not express detectable levels of GUS activity or expressed low levels of GUS activity in the quantitative assay. The quantitative assay for five pHD6-6 transformants (isolates 1, 2, 4, 10, and 18) also indicated that the temporal expression pattern of GUS activities in these transformants correlated well with the pattern of aflatoxin production (Figure 28). However, 500 to 1,000 fold higher GUS activities were observed in pHD6-6 transformants 1 and 4 (Table 6). The three pGAPN-2

Table 5. Mycelial dry weight and aflatoxin production in YES after 24, 48, and 72 h growth of *A. parasiticus* NR2 transformed with pSL82, pHD4-4, pHD7-3, pHD6-6 and pGAPN-2.

	mycelial dry weight (g)			aflatoxin (ng/ml)			aflatoxin/dry weight (μg/g)*		
	24h	48h	72h	24h	48h x10^{-3}	72h x10^{-3}	24h	48h x10^{-3}	72h x10^{-3}
pSL82	0.19	0.84	1.58	12.5	40.8	153	6.60	4.90	9.70
pGAPN-2, #6	0.16	0.75	1.06	11.8	54.0	107	7.40	7.20	10.1
#7	0.16	0.76	0.90	11.5	36.6	47.2	7.20	4.80	5.20
#12	0.39	0.61	1.18	31.8	40.6	113	8.20	6.60	9.60
pHD4-4, #3	0.14	0.75	0.96	10.5	22.1	55.9	7.50	3.00	5.80
#5	0.17	0.85	0.86	19.4	23.0	40.8	11.4	2.70	4.70
#14	0.12	0.43	0.95	1.95	14.2	75.0	1.60	3.30	7.90
pHD7-3, #5	0.11	0.58	0.73	4.90	22.0	43.0	4.50	3.80	5.90
#15	0.15	0.68	0.98	21.7	40.4	62.2	14.4	5.90	6.30
#17	0.17	0.63	1.45	23.6	28.2	109	13.9	4.50	7.50
pHD6-6, #2	0.18	0.62	0.88	3.00	32.0	37.2	1.70	5.20	4.20
#10	0.10	0.76	0.82	7.40	26.6	41.7	7.40	3.50	5.10
#18	0.16	0.58	1.04	7.50	29.4	48.8	4.70	5.10	4.70
#4	0.19	1.00	1.50	5.80	42.1	65.0	3.10	4.20	4.30
#8	0.17	0.73	0.82	6.60	27.7	47.4	3.90	3.80	5.80

*aflatoxin/dry weight (μg/g): total aflatoxin production in 100 ml YES medium divided by the mycelial dry weight.

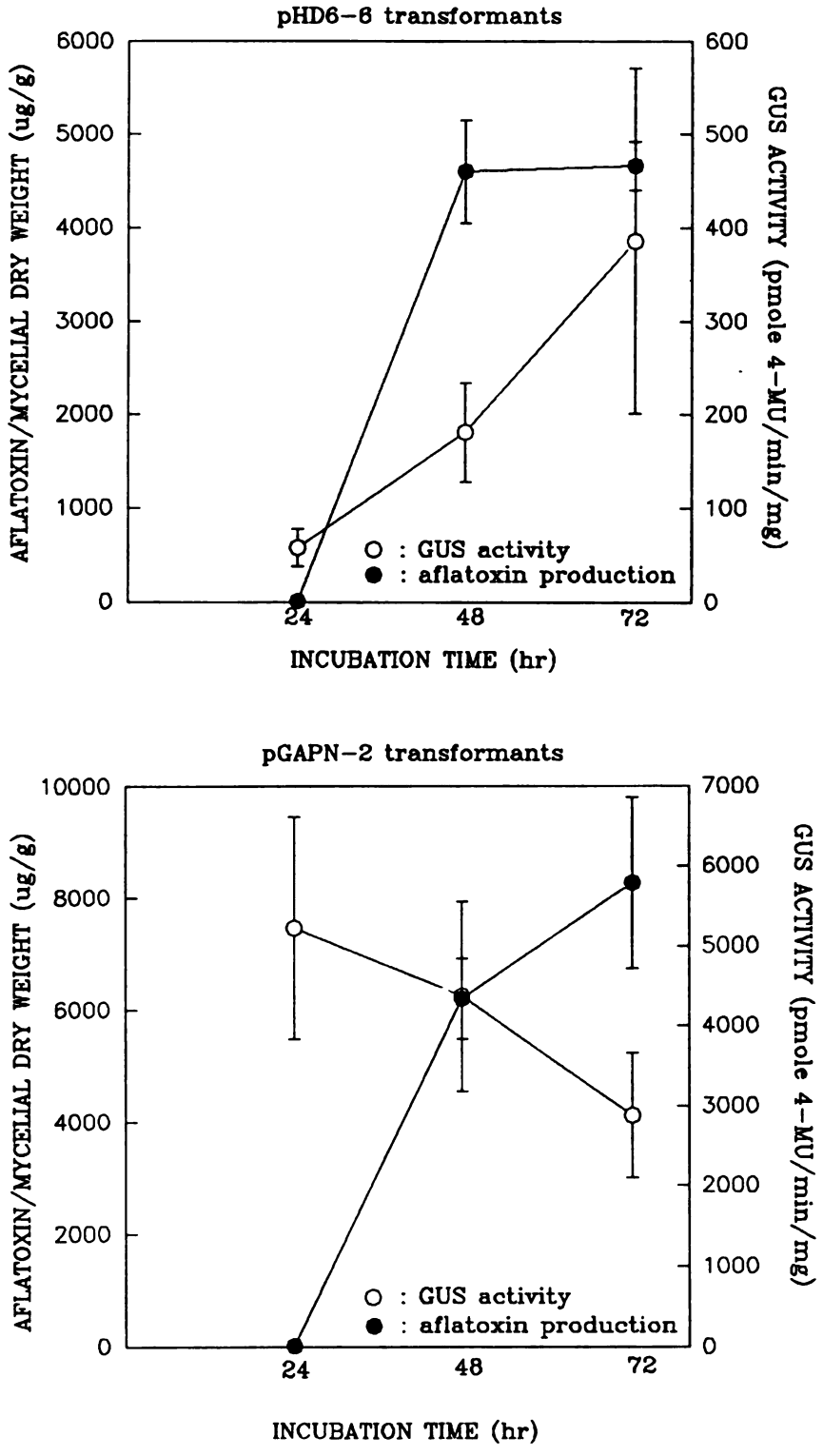
Table 6. GUS specific activities in cell lysates isolated from *A. parasiticus* NR2 transformed with pSL82, pHD4-4, pHD7-3, pHD6-6, and pGAPN-2 after growth in YES for 24, 48, and 72 h.

		relative GUS specific activity per mg of total protein(p-mole 4-MU/ min/ mg)		
		24h	48h	72h
pSL82		40	53	12
pGAPN-2,	#6	7910	6480	4450
	#7	3250	2370	1990
	#12	4540	4260	2210
pHD4-4,	#3	nd	13	26
	#5	nd	14	42
	#14	20	30	nd
pHD7-3,	#5	18	7	24
	#15	18	nd	nd
	#17	9	9	5
pHD6-6,	#2	53	100	185
	#10	25	161	217
	#18	96	281	757
	#1	24000	6.8×10^5	9.4×10^5
	#4	3600	3.5×10^5	4.7×10^5
	#8	10	18	20

1.nd: not detectable
2.GUS activity was determined at excitation 365 nm and emission 455 nm by using a spectroflurometer.

Figure 28. GUS expression and aflatoxin production of *A. parasiticus* NR2 transformed with pHD6-6 or pGAPN-2 after 24, 48, and 72 h growth in YES medium. The GUS activities and aflatoxin production represent the mean values obtained from three pHD6-6 transformants (isolates 2, 6, and 18) or three pGAPN-2 transformants (isolates 6, 7, and 12). Standard errors are also indicated. The hollow circle indicates GUS activity whereas the filled circle indicates aflatoxin production.

Figure 28.


pHD6-6 transformants
AFLATOXIN/MYCELIAL DRY WEIGHT (ug/g)
GUS ACTIVITY (pmole 4-MU/min/mg)
6000
5000
4000
3000
2000
1000
0
600
500
400
300
200
100
0
○ : GUS activity
● : aflatoxin production
24
48
72
INCUBATION TIME (hr)
pGAPN-2 transformants
AFLATOXIN/MYCELIAL DRY WEIGHT (ug/g)
GUS ACTIVITY (pmole 4-MU/min/mg)
10000
8000
6000
4000
2000
0
7000
6000
5000
4000
3000
2000
1000
0
○ : GUS activity
● : aflatoxin production
24
48
72
INCUBATION TIME (hr)

transformants showed decreasing GUS activity over a 72 h growth range, consistent with the general expression pattern of house-keeping genes (Table 6 and Figure 28). These results indicate that the expression of GUS in *A. parasiticus* NR2 transformed with pHD6-6 and pGAPN-2 is determined by the subcloned *ver*-1A and β-tubulin gene promoters, respectively. In addition, no GUS expression was observed for the pHD4-4 transformants or 89% of the pHD7-3 transformants in which the promoter or the TATA box plus transcriptional initiation site were deleted, respectively. The GUS assay (Table 2) together with the Southern hybridization analyses (Table 7) suggest that: (1) in the transformants with a gene conversion event at the *nia*D locus, no GUS activity was observed; (2) deletion of the TATA box together with the transcription initiation site in plasmid pHD7-3 resulted in no GUS activity in the transformants in which pHD7-3 integrated at the genomic *nia*D locus. Integration of pHD7-3 at the *ver*-1A locus (e.g. isolate 14 in Table 7), at a heterologous locus other than *nia*D or *ver*-1A (e.g. isolates 9 and 30), or by multiple integration at the *nia*D locus (e.g. isolates 42 and 57) gave rise to GUS activity, indicating that alternative transcription machinery may have been used in these GUS^+ transformants; (3) approximately 50% of the pHD6-6 transformants in which the vector integrated at the *nia*D locus did not express GUS activity, consistent with the observation obtained from pHD2 transformants; (4) integration of pHD6-6 at the *ver*-1A locus gave rise to higher GUS activity. This result indicates that a potential positional effect may have occurred or that the 1.1-kb *ver*-1A promoter selected for studies is missing critical DNA elements which are supplied in the transfromants with plasmid integrated at *ver*-1A locus but

Table 7. Summary of qualitative GUS assay and Southern hybridization analyses of *A. parasiticus* NR2 transformed with pHD4-4, pHD7-3, pHD6-6, and pGAPN-2.

plasmid used GUS activity*	pHD4-4		pHD7-3		pHD6-6		pGAPN-2	
	-	+	-	+	-	+	-	+
single integration at *niaD*	3 5 14	-	5 15 17 51	-	5 8 9	2 10 18	-	1 6 7 12
single integration at *ver*-1A	-	-	-	14	-	1 4	-	-
multiple integration at *niaD*	-	-	-	42 57	-	6 13	-	-
gene conversion at *niaD*	4 15 16	-	52	-	7 16 20	-	3 4 8 10	-
illegitimate integration	-	-	1 2 16	9 30	12 17	-	-	-

* The GUS activity indicated in this table is determined by the qualitative GUS assay.

missing in the transformants with plasmid integrated at *nia*D locus.

Northern hybridization analysis. Northern hybridization analyses were performed on total RNA isolated from transformants grown for 24, 48, and 72 h in YES using *ver*-1A, GUS, or *pyr*G as the probe. A 1.1-kb transcript was observed 48 and 72 h after inoculation (Figure 29A and Figure 29B) using the *ver*-1A promoter as a probe (a 0.6-kb *ClaI*/*Eco*RI fragment) indicating that the accumulation of the *ver*-1A transcript was low in the total RNA isolated from 24 h cultures, accumulated to a high level by 48 h and then decreased by 72 h. The appearance of the *ver*-1A transcript was detected during the transition from trophophase to idiophase indicating that the expression of the aflatoxin pathway gene, *ver*-1A, is regulated in part at the transcriptional level (Cleveland, 1987; Skory et al., 1993; Trail et al., 1995a). Northern analysis was also performed using a 1.87-kb *SalI*/*Eco*RI GUS fragment as a probe. The temporal expression pattern of GUS transcripts in pHD6-6 transformants 18, 1, and 4 was consistent with that of the *ver*-1A gene (Fig 29A), indicating that the GUS gene in these transformants was under similar regulatory control as *ver*-1A. The expression of the GUS transcript in three pGAPN-2 transformants (isolates 6, 7, and 12) was observed early in the mycelia (24 h) (Fig 29A), indicating that GUS gene expression is regulated by the ß-tubulin promoter. The levels of GUS transcript in pHD6-6 transformants 1, 4, and 18 were higher than the other transformants, indicating that transcription efficiency may play a key role in the high GUS activities observed for the corresponding transformants (Table 6). Northern hybridization analysis using *pyr*G probe indicated that similar amounts of total RNAs were applied

Figure 29. Northern hybridization analysis of total RNAs isolated from *A. parasiticus* NR2 transformed with pSL82, pHD4-4, pHD6-6, pHD7-3, and pGAPN-2. Total RNAs were isolated from mycelia harvested at 24, 48, and 72 h after inoculation in YES. (A) The accumulation of transcripts was detected by using *ver*-1A, GUS, or *pyr*G probes. Lanes 1 to 5, pHD6-6 transformants 2, 10, 18, 8, and 4; lanes 6 to 8, pHD4-4 transformants 3, 5, and 14; lanes 9 to 11, pHD7-3 transformants 5, 15, and 17; lanes 12 to 14, pGAPN-2 transformants 6, 7, and 12; and lane 15, a pSL82 transformant. (B) The accumulation of *ver*-1A, GUS, and *pyr*G transcripts in pHD6-6 transformant 1 after 24, 28, and 72 h growth in YES was shown. The first, second, and third strips were total RNAs hybridized to a *ver*-1A probe, a *pyr*G probe, and a GUS probe. Transcripts hybridizing to the 1.1-kb *ver*-1A probe, the 1.8- kb GUS probe, and the 1.4-kb *pyr*G probe are indicated by a hollow arrow, a filled arrow, and a circle, respectively.

Figure 29.

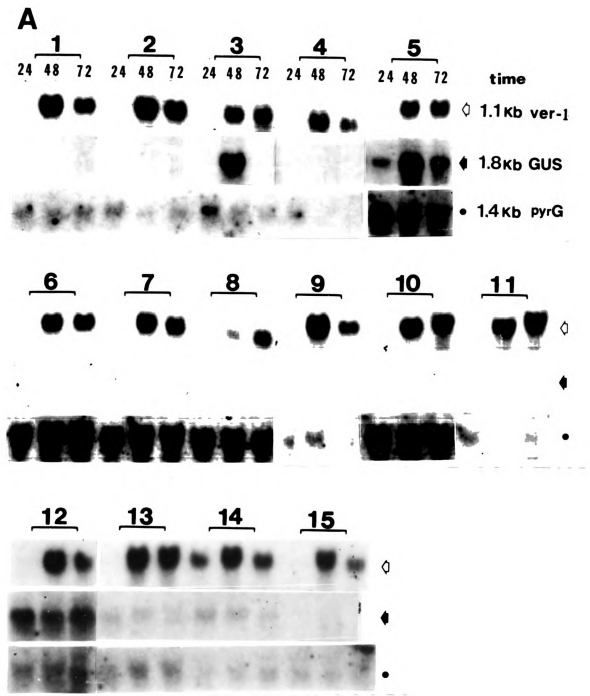
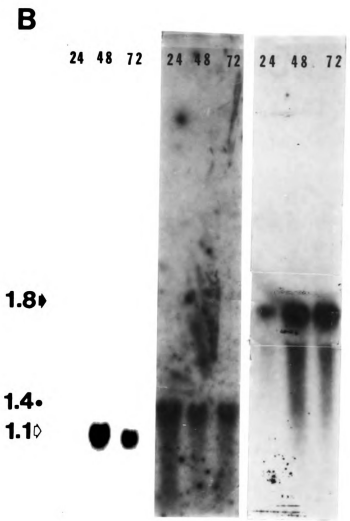

A
1
2
3
4
5
24 48 72 24 48 72 24 48 72 24 48 72 24 48 72
time
1.1Kb ver-1
1.8Kb GUS
1.4Kb pyrG
6
7
8
9
10
11
12
13
14
15

Figure 29. (Cont'd)

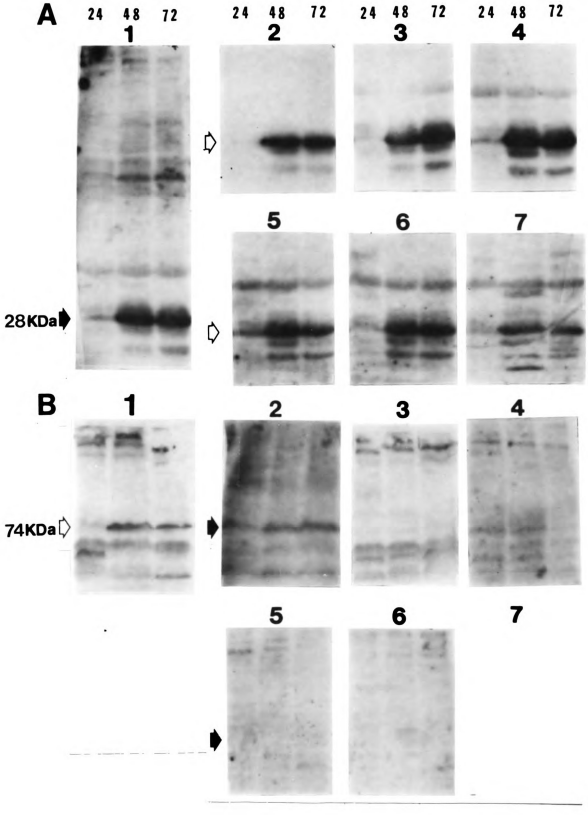


and that the RNAs were subject to little degradation (Fig 29A). The difference in signal intensities of *pyr*G transcripts may be a result of different specific activities of the probe used in Northern hybridization analyses.

Western blot analyses. Western blot analyses were performed on cell extracts prepared 24, 48, and 72 h after inoculation from three pHD6-6 transformants (isolates 1, 4, and 18), one pGAPN-2 transformants (isolate 6), one pHD7-3 transformant (isolate 5), one pHD4-4 transformant (isolate 3), and one pSL82 transformant (control), respectively. A major protein at the expected molecular mass of about 28 kDa was observed in the transformants using antibody raised against a *ver*-1A/ maltose binding fusion protein (Figure 30). A significant increase in this 28 kDa protein was observed at 48 h and the level remained high until 72 h. No GUS protein was detected by β-glucuronidase antiserum in cell extracts from pHD7-3, pHD4-4, and pSL82 transformants at the three time points. A major protein with a molecular mass of about 74 kDa was detected in 48 and 72 h samples in pHD6-6 transformants 1 and 4, consistent with the timing and quantity observed for *ver*-1A protein. A protein with similar mobility was also observed in pGAPN-2 transformant 6. The 74 kDa protein detected in pGAPN-2 transformant 6 had maximum accumulation at 24 and 48 h and decreased at 72 h. The observation that the putative GUS protein in pHD6-6 transformants was not detected until 48 h after inoculation coincided with the onset of aflatoxin production, GUS and *ver*-1 transcript accumulation, and GUS activity, strongly indicating that the expression of the reporter GUS is under the control of the fused promoter confirming that expression is regulated at the transcriptional level.

Figure 30. Western blot analyses of proteins isolated from *A. parasiticus* NR2 transformed with pSL82, pHD4-4, pHD7-3, pHD6-6, and pGAPN-2. (A) Western blot analysis was performed using an antibody against a *ver*-1A/maltose binding fusion protein (Liang and Linz, 1994). (B) Identical cell extracts were analyzed by GUS antibody. Lane 1, pHD6-6 transformant 4; lanes 2 and 3; pHD6-6 transformants 1 and 18; lane 4, pGAPN-2 transformant 6; lanes 5, 6, and 7; pHD4-4 transformant 3, pHD7-3 transformant 5, and pSL82 transformant, respectively.

Figure 30.


A
24 48 72
1
24 48 72
2
24 48 72
3
24 48 72
4
28KDa
5
6
7
B
1
2
3
4
74KDa
5
6
7

This notion was also demonstrated in that in pHD6-6 transformants the levels of GUS transcript, portein, and GUS activity were positively correlated.

DISCUSSION

Reporter genes have been intensively used in the investigation of gene regulation. In this study, we used GUS (*uid*A) isolated from *E. coli* as the reporter gene. There are several major advantages for the use of GUS as the reporter gene in *A. parasiticus*: (1) the GUS protein can tolerate a large addition of amino acids at the N-terminus (Jefferson, 1989; Jefferson et al., 1987); (2) there is no detectable endogenous GUS activity in *Aspergillus* (Jefferson, 1989); and (3) qualitative and quantitative analyses of GUS activity have been developed (Jefferson et al., 1987).

Several lines of evidence validate the working hypotheses that aflatoxin production is regulated in part at the transcriptional level: (1) the TATA box has been reported to be essential for expression of many eukaryotic genes (Chen and Struhl, 1985; Carcamo et al., 1990; O'Shea-Greenfield and Smale, 1992; Rowlands et al., 1994). The absence of GUS expression following deletion of the TATA box and transcription initiation site in a high proportion of pHD7-3 transformants (89%) suggest that these elements of the *ver*-1A promoter are important for normal gene expression to occur. (2) The maximum accumulation of *ver*-1A transcript and its protein coincided with the onset of aflatoxin production. (3) The pattern of accumulation of the *uid*A transcript in pGAPN-2 transformants as well as pHD6-6 transformants 2, 10, 18, 1, and 4 coincided with the expression of GUS protein and

activity, suggesting that expression of the GUS reporter gene was mainly under transcriptional control and that control at the post-transcriptional level is unlikely or has minimal effects on the expression of GUS. (4) The similar temporal expression pattern of *ver*-1A transcript and protein and GUS transcript and protein suggests that they are under common regulatory control conferred by the *ver*-1A promoter. This also indicates that the GUS gene, subcloned in the transforming vector and subsequently integrated in the recipient genome, is not subject to other major transcriptional regulatory mechanisms. (5) The observation that high levels of GUS transcript give rise to high GUS activity suggests that measurement in changes in GUS activity accurately reflect efficiency of transcription of the reporter gene. Therefore, identification of the critical cis-acting elements involved in transcriptional regulation of the gene can be achieved by monitoring the change in GUS activity after generating mutations which affect *ver*-1A promoter function. (6) The high rate of transcription of the GUS gene at 48 h and 72 h in pHD6-6 transformants 1 and 4 corresponds well with GUS protein level (Western analysis) and GUS activity (GUS assay), indicating a possible "positional effect" on *ver*-1 promoter function (heterologous locus vs. homologous locus).

The fact that aflatoxin biosynthesis is in part regulated at the transcriptional level could lead us to generate strategies to control aflatoxin contamination. Identification and subsequent elimination of the cis-acting elements in the promoters of the pathway genes and/or manipulation of the level of or binding of regulatory proteins which interact with promoters of the biosynthetic genes may reduce or eliminate the

expression of the corresponding genes. Manipulation of the levels of regulators can be achieved through the generation of null mutations in the genes encoding them or through the use of specific inhibitors. The use of naturally occurring synthetic chemical inhibitors against these regulators may result in the reduction of aflatoxin production. The genetic engineering of host plants to produce appropriate inhibitors is an alternative way to eliminate toxin production by fungi. "Gene knockout" in conjunction with the manipulation of regulatory mechanisms could also result in the generation of a stable, nontoxigenic biocompetitive strain.

Transcriptional analyses of gene regulation have been performed by several different approaches. Gel shift analysis, DNA footprinting, or methylation interference provide a way to identify transcriptional regulators and their DNA binding sites. Recently, three separate regions in the *nor*-1 promoter which appear to bind specific transcriptional regulators have been identified in our laboratory by gel shift analyses (personal communication, Dr. Frances Trail). Deletion analysis to identify the essential cis-acting DNA elements in the promoter region has also been applied in transcriptional studies. In deletion analysis, a DNA fragment containing the putative promoter of the gene of interest is isolated and fused to a reporter gene. Successive deletions are then generated from one or both ends of the subcloned promoter. The deletion constructs are transformed into the appropriate host strain and the expression of the reporter gene monitored. Transformants with changes in the expression of the reporter gene indicate that critical cis-acting elements in the promoter region have been partially or completely deleted from the transforming vectors. In the GUS

reporter system developed in this report, deletion of critical cis-acting elements from the promoter of the *ver*-1 gene subcloned in pHD6-6 should result in distinct expression patterns of GUS activity which can be monitored. Preliminary data, for example, suggest that the TATA box and/or transcription initiation site is necessary for the promoter function. Deletion of this region resulted in a significant decrease in GUS^+ clones (50% to 11%).

Several additional interesting observations arose during the course of these studies. First, of the *nia*D^+ pHD6-6 transformants in which the vector integrated at the *nia*D locus, approximately 50% of the transformants did not express GUS activity. Studies in *N. crassa* showed that exogenous DNA may induce irreversible RIP (repeat-induced point mutation) mediated inactivation (Selker et al., 1987; Selker and Garrett, 1988) of the transformed duplicated genes through base substitutions, small insertion or deletions. Another phenomenon, transgeneic suppression, was observed when the expression of transgenes in petunia was affected by the degree of methylation of the inserted gene (Meyer and Heidmann, 1994). Quelling, in which inactivation takes place both in the endogenous and the sequences introduced by transformation was also observed in *N. crassa*, (Pandit and Russo, 1992; Cogoni et al., 1994). Quelling occurs during the vegetative stage of the life cycle and consists of a transient, reversible inactivation of gene expression by introducing homologous sequences. In most cases, quelling occurs after transformation. The introduction of homologous pigmentation albino gene into *N. crassa* (Cogoni et al.,1994), the chalcone synthase (*chs*) gene into *Petunia* plants (Napoli et al., 1990), a chimeric

petunia dihydroflavonol-4 reductase (*dfr*) gene (van der Krol et al., 1990) into petunia, and the nopaline synthase (Nos) transgene into tobacco (Goring et al., 1991), all resulted in the inactivation of the homologous genes. In addition to possible RIP and quelling effects, the lack of GUS activity in 50% of the pHD6-6 transformants may also be derived from a titration effect in which the introduction of multiple copies of the cis-acting sequences in *ver*-1 promoter results in titration of the regulating factors (Kelly and Hynes, 1987). However, we can not rule out the possibility that deletion of some crucial DNA elements in the subcloned 1.1-kb *ver*-1A promoter may play a role in the absence of GUS expression in 50% of the pHD6-6 transformants, although we see the same thing with *nor*-1/GUS constructs.

Second, transformants with pHD6-6 integrated at the *ver*-1 locus appeared to have higher levels of GUS expression compared to those with vectors integrated at the *nia*D locus. This observation may represent a possible "positional effect". The phenomenon of "positional effects" has occasionally been observed in attempts to integrate genes into a nonhomologous genomic environment (Miller et al., 1987; Stief et al., 1989). The transcriptional activity of certain genes integrated randomly into the genome of receiving organisms may vary with the chromosomal environment of the insertion site. This effect of the chromosomal position on transcriptional activity may be associated with the topological structure of the chromatin, regional cis-acting elements, or location-specific trans-acting mechanisms. However, we can not rule out the possibility that deletion of some crucial DNA elements in the subcloned 1.1-kb *ver*-1A promoter may play a role in the low level of GUS expression at the *nia*D

locus.

Third, deletion of the TATA box together with the transcription initiation site in the *ver*-1A promoter appears to affect GUS expression in pHD7-3 transformants in which the integration of vector occurred at the *nia*D locus. However, integration of pHD7-3 at the *ver*-1A locus, multiple integrations at *nia*D locus, or nonhomologous integration can generate low levels of GUS activity whereas single integration at *nia*D resulted in no GUS activity. Because of the low number of transformants analyzed, it is difficult to determine conclusively if TATA is functional in the *ver*-1A gene. Several lines of evidence indicate the absence of a TATA box in several genes in *A. parasiticus* and *A. flavus* (Seip et al., 1990; Woloshuk et al., 1994). Alternative transcription initiation sites were also observed in many genes (Skory et al., 1992; Prade and Timberlake, 1993; Trail et al., 1994). Therefore, the expression of GUS in pHD7-3 transformants may be derived from the use of an alternative transcription initiation site or entire promoter already present at the integration site.

In this report, we confirm that (1) GUS can be used as a reporter gene in the transcriptional analysis of genes involved in aflatoxin biosynthesis; (2) aflatoxin production is regulated in part at the transcriptional level. The data suggest that insertion of the *ver*-1 promoter and terminator at a heterologous chromosomal location does not affect its temporal expression pattern but may alter the promoter's ability to consistently regulate the level of expression. The GUS expression vectors developed in these studies can be used in future transcriptional studies which include identification of the transcriptional cis-acting sites and trans-acting regulatory factors.

CONCLUSIONS

A genetic approach to develop a detailed understanding of the regulation of aflatoxin biosynthesis was established in this report. This information may lead us to generate more efficient strategies to achieve the goal of controlling of aflatoxin contamination.

A transformation system for genetic analyses in *A. parasiticus* was developed based on a dominant selectable marker, *ben*^{r} gene, which was isolated from a benomyl-resistant mutant strain of *A. parasiticus*. This dominant selectable marker provides a valuable tool to study aflatoxin biosynthetic gene function by gene disruption or complementation in a wild type genetic background. For example, the *ben*^{r} gene has been used as a selectable marker to successfully transform a versicolorin A accumulating blocked mutant of *A. parasiticus* CS10 (*ver*-1, *wh*-1, *pyr*G) to aflatoxin production (Shun-Shin Liang, personal communication).

Recombinational inactivation strategies were established using nitrate reductase as the model gene. Recombinational inactivation could be achieved through gene replacement (gene conversion) or by integrative gene disruption. The resulting transformants have also been shown to remain mitotically stable. Similar strategies have been used to generate nontoxigenic, genetically stable biocontrol agents which may help achieve the control of aflatoxin production. The strategies developed in this

model system have also been used to disrupt several aflatoxin biosynthetic genes to study their functions (Trail et al., 1993; Trail et al., 1994; Liang and Linz, 1994).

Aflatoxin biosynthesis was confirmed to be regulated in part at the transcriptional level. A useful system for analyses of transcription of an aflatoxin biosynthetic gene (*ver*-1) was developed using a GUS reporter gene. The expression of GUS at the transcript level, the protein level, and the level of enzyme activity correlated with the levels of the *ver*-1 transcript and *ver*-1 protein as well as aflatoxin production. The results indicate that GUS can be used as a reporter gene in the analyses of the regulation of aflatoxin biosynthetic genes.

APPENDICES

APPENDIX A

DISRUPTION OF A GENE ASSOCIATED WITH POLYKETIDE BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS*

INTRODUCTION

The initial steps in aflatoxin production are part of a polyketide-derived biochemical pathway (Figure 3) (Dutton, 1988; Bhatnagar et al., 1992). The generation of nontoxic and genetical ly stable biocontrol agents can be achieved through disruption in the genes involved in polyketide biosynthesis. Therefore, a PKS provides a good target for disruption. Disruption of PKS gene may also lead to changes in fungal growth, morphogenesis or survival (Hopwood, 1988; Davis and Chater, 1990; Miyake et al., 1990). An understanding of the relationship between the production of secondary metabolites and fungal growth, development and survival may also lead to generation of alternative strategies for prevention of preharvest aflatoxigenesis. For example, the application of environmental or chemical inhibitors against the production of certain pathway intermediate(s) or pathway regulator(s) necessary for fungal development or survival could result in prevention of fungal growth and/or in reduction of aflatoxin production.

The hypothesis associated with this study is that disruption of aflatoxin biosynthetic genes can be achieved through a homologous recombination event at a

PKS gene involved in the early step of aflatoxin biosynthesis in *A. parasiticus*. In this report, a putative PKS-related gene in *A. parasiticus* which shares a high degree of similarity with the *P. patulum* 6-methylsalilcylic acid synthetase (MSAS) gene was the target for disruption. Preliminary results indicate that this gene is not directly related to aflatoxin production but appears to be involved in fungal growth and/or development.

PRELIMINARY RESULTS

Isolation of a PKS gene from *A. parasiticus* (by Reuven Rasooly)

A 1.2-kb cDNA fragment, which encodes the ß-ketoacyl synthase domain of the 6-methylsalicylic acid synthase (MSAS) in *P. patulum* (Beck et al., 1990), was used as a probe to screen an *A. parasiticus* genomic DNA library in phage lambda. Four lambda clones that hybridized to the 1.2-kb cDNA probe under high stringency were isolated and mapped by restriction digestion. Two of these clones contained approximately 4.0-kb and 6.0-kb *Bam*HI DNA fragments. The third clone contained a 9.0-kb *Sac*I insert. The two *Bam*HI fragments were found to be adjacent to each other. The 9.0-kb *Sac*I fragment was totally contained within this 10.0-kb region. A 680 bp *Hin*dIII/*Cla*I fragment, located at the junction between the 4.0-kb and 6.0-kb *Bam*HI subclones, was subcloned and sequenced. The predicted amino acid sequence showed a high degree of identity (75%) with the β-ketoacyl synthetase functional domain of *P. patulum* MSAS, which is isolated near N-terminus of MSAS. Northern analysis of *A. parasiticus* total RNA using this 680 bp fragment as a probe (performed

by Richard Zhou) detected a single transcript 5.8-kb in size, which is similar to the size of MSAS transcript in *P. patulum* (Beck et al., 1990). This indicated that the 9.0-kb *Sac*I DNA subclone may contain a PKS gene. The identity of this PKS gene is currently under investigation via gene disruption.

Disruption of a putative PKS gene

I aided in development of a strategy to disrupt the subcloned putative PKS gene based on the gene disruption experiment described previously. However, the actual experiments were performed by Richard Zhou.

A 6.6-kb *Sph*I subclone derived from the original 9.0-kb *Sac*I λ fragment was subcloned into pUC19 to generate pUC19-6 (Figure 31). A 6.0-kb *Hin*dIII fragment containing a functional *nia*D gene (selectable marker) in pSL82 (Horng et al., 1990) was inserted at a single *Sma*I site in pUC19-6 to generate pPKS (Figure 31). A *niaD* auxotrophic mutant strain of *A. parasiticus* NRRL5862 (NR-1) was used as the recipient strain and transformed with the linearized pPKS. After transformation of *A. parasiticus* NR-1 with pPKS, *nia*D$^+$ transformants were analyzed for PKS disruption via thin layer chromatography (TLC) and Southern hybridization analysis. TLC analysis on 60 of the 200 *nia*D$^+$ transformants indicated that none had diminished production of AFB1 or AFG1. Southern hybridization analysis was performed on genomic DNAs isolated from the 200 *nia*D$^+$ transformants using the 680 bp DNA fragment as a probe. Two transformants (1%) contained one disrupted PKS allele and one intact PKS allele (designated isolate 10) or one disrupted PKS allele (designated isolate 11). The other *nia*D$^+$ transformants either contained an intact PKS gene (85%),

indicating a gene conversion or a gene replacement at the *nia*D locus, or had heterologous integration at a locus other than *nia*D or PKS (14%). Morphological studies on transformant 10 and 11 indicated that these two transformants grew slowly and produced aerial hyphae without sporulation on CZ (a selective medium; contains nitrate as the sole nitrogen source) but returned to the wild type morphology after subsequent passages on coconut agar medium (CAM), a nonselective aflatoxin supporting medium. Southern hybridization analyses performed on DNAs isolated from the hyphae collected on CZ show a disrupted PKS whereas a wild type PKS gene was observed in hyphae from CAM. Cultures with intermediate levels of sporulation appeared to contain one copy of the wild type PKS and one disrupted copy. All cultures of isolate 10 and 11 grown on CAM produced aflatoxin at wild type levels. The morphological studies in combination with the Southern hybridization analyses suggest that the isolated PKS gene may be involved in fungal growth and/or development.

DISCUSSION

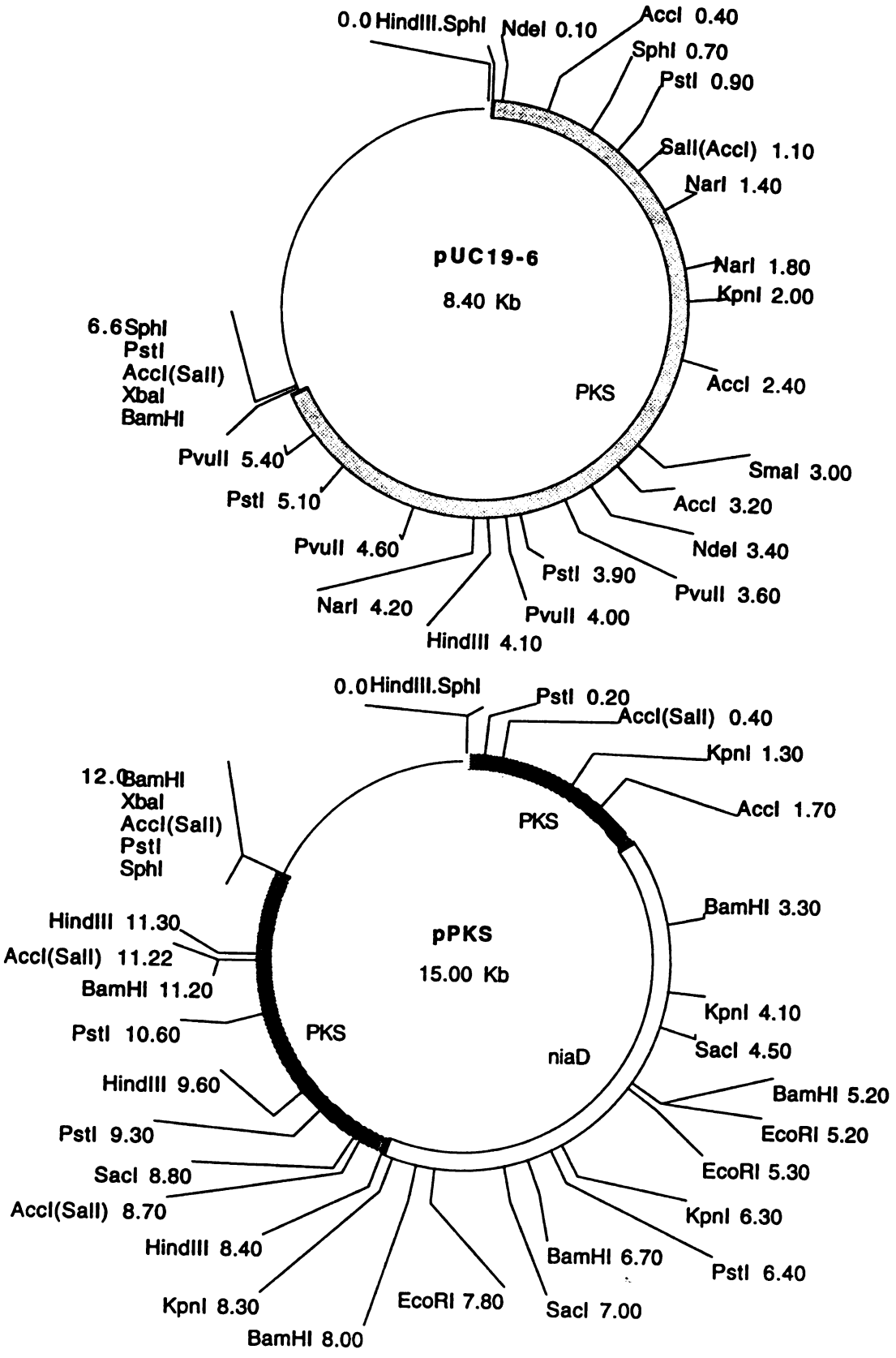
Experimental evidence collected to date suggest that the cloned PKS gene is not directly involved in aflatoxin biosynthesis but is involved in fungal growth and/or development of *A. parasiticus*. (1) Two *nia*D^{+} transformants, 10 and 11, in which at least one copy of PKS is disrupted, produce similar quantities of AFB1 as the wild type strain of *A. parasiticus*. (2) The 5.8-kb transcript isolated from mycelia collected after 24, 60, and 72 h growth in Adye-Mateles medium (an aflatoxin inducing

medium) showed a sequential increase in the abundance, indicating the involvement of the subcloned PKS in secondary metabolism or fungal development. (3) Two transformants containing a disrupted PKS survived as aerial hyphae without sporulation on CZ, indicating the direct or indirect involvement of the subcloned PKS in fungal sporulation.

Recently, the PKS gene involved in aflatoxin biosynthesis has been cloned (Trail et al., 1995a; Chang et al., submitted). This gene originally called gene-1 shares high degrees of similarity (80%) and identity (64%) over a 100 amino acid stretch with the acyl carrier protein (ACP) domain of *A. nidulans* *w*A (a PKS). A distinct region in the encoded protein also shows 20% to 32% identity with the acyltransferase domain in *A. nidulans* *w*A. This gene now designated *pks*A (Trail et al., 1995a; Chang et al., submitted) was found to be closely linked to numerous aflatoxin biosynthetic genes in the genome of *A. parasiticus* (Trail et al, 1995a). Recombinational inactivation of *pks*A through homologous recombination resulted in a strain which was unable to produce any known aflatoxin pathway intermediates. The genetic evidence indicate that *pks*A is a PKS gene involved in the formation of an anthrone decaketide intermediate in the aflatoxin biosynthetic pathway. Therefore, the two PKS genes isolated to date appear to play distinct roles in *Aspergillus* and do not conduct redundant functions.

Figure 31. Construction and restriction maps of pUC19-6 and pPKS.

Figure 31.


0.0 HindIII.SphI
NdeI 0.10
AccI 0.40
SphI 0.70
PstI 0.90
SalI(AccI) 1.10
NarI 1.40
NarI 1.80
KpnI 2.00
AccI 2.40
SmaI 3.00
AccI 3.20
NdeI 3.40
PvuII 3.60
PstI 3.90
PvuII 4.00
HindIII 4.10
NarI 4.20
PvuII 4.60
PstI 5.10
PvuII 5.40
6.6 SphI
PstI
AccI(SalI)
XbaI
BamHI
pUC19-6
8.40 Kb
PKS
0.0 HindIII.SphI
PstI 0.20
AccI(SalI) 0.40
KpnI 1.30
AccI 1.70
BamHI 3.30
KpnI 4.10
SacI 4.50
BamHI 5.20
EcoRI 5.20
EcoRI 5.30
KpnI 6.30
PstI 6.40
BamHI 6.70
SacI 7.00
EcoRI 7.80
BamHI 8.00
KpnI 8.30
HindIII 8.40
AccI(SalI) 8.70
SacI 8.80
PstI 9.30
HindIII 9.60
PstI 10.60
BamHI 11.20
AccI(SalI) 11.22
HindIII 11.30
12.0 BamHI
XbaI
AccI(SalI)
PstI
SphI
pPKS
15.00 Kb
PKS
niaD

APPENDIX B

THE PLASMIDS USED IN TRANSFORMATION

Table 8. The plasmids used in transformation

plasmid name	recipient strain	comments	page
pYT1	a *pyr*G mutant of *A. parasiticus* (*wh*-1, *ver*-1, *pyr*G)	A 7.4-kb *Nsi*I DNA fragment containing the *ben*^{r} gene isolated from a benomyl resistant mutant strain of *A. parasiticus* was subcloned into the *Pst*I site of pUC19. pYT1 was used in transformation of benomyl sensitive strain to benomyl resistance	p.77
pYTPYRG	a *pyr*G mutant of *A. parasiticus* (*wh*-1, *ver*-1, *pyr*G)	A 2.9-kb *Bam*HI/ *Sal*I DNA fragment containing a functional *pyr*G gene was isolated from pPG3J and inserted at the *Sma*I site of pYT1. pYTPYRG was used in transformation of a benomyl sensitive strain of *A. parasiticus* to benomyl resistance.	p.77
pPN-1	a *pyr*G mutant of *A. parasiticus* (*wh*-1, *ver*-1, *pyr*G)	A 2.5-kb *Eco*RI internal fragment of *nia*D in pSL82-1 was replaced by the 2.9-kb *pyr*G gene from pPG3J. pPN-1 was used in a system to disrupt the endogenous *nia*D gene by a one-step gene replacement.	p. 112
pSKN1-82		A 0.8-kb *KpnI*/*Bgl*II internal fragment of *nia*D from pSL82 was inserted into the *KpnI*/*Bam*HI site in pBLUESCRIPT SKII to generate pSKPYRG.	p. 112
pSKPYRG	a *pyr*G mutant of *A. parasiticus* (*wh*-1, *ver*-1, *pyr*G)	The 2.9-kb *pyr*G gene was subcloned into pSKN1-82 at the *Xba*I site. pSKPYRG was used in disruption of *nia*D gene by integrative disruption.	p. 112

pHD2	an *nia*D mutant of *A. parasiticus* (NR2)	A 1.1-kb promoter region from *ver*-1A and the 3.7-kb transcription termination signal from *ver*-1B were subcloned into pGL2-basic. A 1.87-kb GUS reporter gene was subcloned by a translational fusion. A 6.6-kb functional *nia*D gene was subcloned as the selectable marker. pHD2 was used in regulation analysis of the *ver*-1A gene.	p. 138 p. 143
pHD6-6	an *nia*D mutant of *A. parasiticus* (NR2)	The 3.7-kb transcription termination signal in pHD2 was replaced by a 1.8-kb DNA fragment from *ver*-1A. pHD6-6 was also used in regulation analysis of the *ver*-1A gene.	p. 142 p. 144
pHD4-4	an *nia*D mutant of *A. parasiticus* (NR2)	The 1.1-kb promoter region in pHD6-6 was deleted. pHD4-4 was used in the study of the functionality of the promoter region in *ver*-1A gene.	p. 142 p. 143
pHD7-3	an *nia*D mutant of *A. parasiticus* (NR2)	A 51 bp DNA fragment containing the putative TATA box and transcription initiation site in the *ver*-1A promoter region was deleted in pHD6-6. pHD7-3 was used in study of the functional role of the putative TATA box and the transcription initiation site of the *ver*-1A gene.	

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