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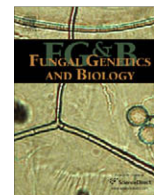

Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*

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ABSTRACT

Cyclopiazonic acid (CPA), an indole-tetramic acid mycotoxin, is produced by many species of *Aspergillus* and *Penicillium*. In addition to CPA *Aspergillus flavus* produces polyketide-derived carcinogenic aflatoxins. Aflatoxin biosynthesis genes form a gene cluster in a subtelomeric region. Isolates of *A. flavus* lacking aflatoxin production due to the loss of the entire aflatoxin gene cluster and portions of the subtelomeric region are often unable to produce CPA, which suggests a physical link of genes involved in CPA biosynthesis to the aflatoxin gene cluster. Examining the subtelomeric region in *A. flavus* isolates of different chemotypes revealed a region possibly associated with CPA production. Disruption of three of the four genes present in this region predicted to encode a monoamine oxidase, a dimethylallyl tryptophan synthase, and a hybrid polyketide non-ribosomal peptide synthase abolished CPA production in an aflatoxigenic *A. flavus* strain. Therefore, some of the CPA biosynthesis genes are organized in a mini-gene cluster that is next to the aflatoxin gene cluster in *A. flavus*.

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1. Introduction

Cyclopiazonic acid (α-cyclopiazonic acid; CPA) is an indole-tetramic acid mycotoxin. It was first isolated from *Penicillium cyclopium* (Holzapfel, 1968). Several species of *Penicillium* (*P. griseofulvum*, *P. camemberti*, *P. urticae*, and *P. commune*) and *Aspergillus* (*A. versicolor*, *A. flavus*, *A. oryzae*, *A. tamarii*, *A. fumigatus* and *A. phoenicis*) were subsequently found to produce CPA (Burdock and Flamm, 2000; Vinokurova et al., 2007). CPA-producing fungi can grow on many substrates, including cheese, meat products, and various grains and seeds. Co-contamination of food commodities by CPA and carcinogenic aflatoxins has been reported (Lansden and Davidson, 1983; Martins and Martins, 1999; Vaamonde et al., 2003). The aflatoxin-producing species mainly comprise members in *Aspergillus* section *Flavi* and include *A. flavus*, *A. parasiticus* and *A. nomius*. The ability of *A. flavus* isolates to produce aflatoxins B_1 and B_2 as well as CPA varies greatly. In the *A. flavus* soil populations of the southern United States, 71% of L-strain isolates and 99% of S-strain isolates produced both aflatoxins and CPA (Horn and Dorner, 1998). Comparable but also different results have been reported for isolates from other regions of the world (Barros et al., 2006; Blaney et al., 1989; Lisker et al., 1993; Razzaghi-Abyaneh et al., 2006). *A. parasiticus* and *A. nomius* isolates typically produce aflatoxins B_1, B_2, G_1 and G_2 but never CPA (Dorner et al., 1984; Ito et al., 1998; Takahashi et al., 2004; Vaamonde et al., 2003).

CPA is a specific inhibitor of calcium-dependent ATPase in the sarcoplasmic reticulum resulting in increased muscle contraction (Riley et al., 1992). In rats, CPA caused lesions of the liver, kidney, pancreas, spleen, and heart (Purchase, 1971). Broiler chickens fed 100 ppm CPA showed ulcerative proventriculitis, mucosal necrosis in the gizzard, hepatic and splenic necrosis, and slow weight gain with a significant mortality rate (Dorner et al., 1983). The combined toxic effects of CPA (50 mg/kg) and aflatoxin (3.5 mg/kg) on broiler chickens, in most cases, are additive (Smith et al., 1992). Postmortem examination of broilers fed CPA (34 mg/kg) and ochratoxin (2.5 mg/kg) revealed similar pathological lesions as those caused by CPA and aflatoxin (Gentles et al., 1999). CPA is not considered to be a potent acute toxin because its oral LD_{50} in rats is in the range of 30–70 mg/kg (Antony et al., 2003; Nishie et al., 1987). Surprisingly, among the large mammals, pigs appear to be quite sensitive to CPA with a non-observable-effect level (NOEL) of approximately 1.0 mg/kg (Lomax et al., 1984). Dogs administered CPA at 0.5 or 1.0 mg/kg died before the end of a 90-day study period (Nuehring et al., 1985), which suggests a much lower NOEL.

Field incidents of CPA mycotoxicoses have not been reported, but it has been speculated that CPA may be associated with 'kodo poisoning' characterized by nausea, vomiting, depression, intoxication and unconsciousness of men after consumption of CPA-contaminated Kodo millet in some parts of North India (Antony

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et al., 2003; Rao and Husain, 1985). CPA effects may be masked by concurrent aflatoxicosis: for example CPA and aflatoxins were isolated from peanut meal related to the turkey 'X' disease that caused the death of over 100,000 turkeys (Bradburn et al., 1994; Spensley, 1963). Although aflatoxins were regarded as the main culprit, CPA likely contributed to some of the observed pathological clinical signs (Cole, 1986).

Chemically, CPA belongs to the family of indole-derived ergot alkaloids. Radiolabelling experiments indicate that CPA is derived from tryptophan, a C5-unit formed from mevalonic acid and two molecules of acetic acid (Holzapfel and Wilkins, 1971). Up to now, little is known about the regulation of CPA biosynthesis although the *veA* gene has been implicated in its production (Duran et al., 2007). Biosynthesis of ergot alkaloids begins with the alkylation of tryptophan by dimethylallyl pyrophosphate. This reaction is catalyzed by dimethylallyl tryptophan synthase, which uses the indole ring of tryptophan as an acceptor (Gebler and Poulter, 1992). Genes involved in fungal ergot alkaloid synthesis are often clustered (Correia et al., 2003; Coyle and Panaccione, 2005; Fleetwood et al., 2007). In the *A. fumigatus* genome, 8 of the 22 gene clusters are located in subtelomeric regions (Perrin et al., 2007). Previous studies as well as the whole genome sequence from *A. flavus* and *A. oryzae* have indicated that the aflatoxin biosynthesis gene cluster is located in a subtelomeric region in chromosome III (Chang et al., 2005; Tominaga et al., 2006). Sequences in the subtelomeric regions beyond the aflatoxin gene cluster in closely related aflatoxin-producing *A. flavus*, an unnamed section *Flavi* taxon that produces B- and G-type aflatoxins, and *A. nomius* have been shown to exhibit marked differences (Ehrlich et al., 2005).

In this study, based on the 87-kb subtelomeric region next to the aflatoxin gene cluster in the *A. flavus* NRRL 3357 genome, we profiled the corresponding regions in other *A. flavus* isolates that produce both aflatoxins and CPA, aflatoxins only, CPA only, or neither as well as *A. parasiticus* isolates. Regions arbitrarily considered to be associated with CPA production were further analyzed. A putative gene encoding a dimethylallyl tryptophan synthase along with three adjoining genes, which encode a monoamine oxidase, a hybrid polyketide and non-ribosomal peptide synthase, and a Cys6 binuclear zinc-finger regulatory factor were disrupted in an *A. flavus* strain. Inactivation of any of the first three genes but not the regulator gene resulted in a loss of CPA production.

Table 1
Characteristics of *A. flavus* and *A. parasiticus* isolates.

Isolate[a]	AF/CPA[b]	*norB-cypA*[c]	Subtelomeric region (A to J)[d]
CA28s	+/+	I	+/+/+/+/+/–/–/–/+/–
CA42s	+/+	I	+/+/+/+/+/+/–/–/+/+
CA43s	+/+	I	+/+/+/+/+/+/+/–/+/–
GA10-18s	+/+	I	+/+/+/+/+/+/+/+/+/–
TX5-1s	+/+	I	+/+/+/–/–/–/–/–/+/+
VA4-36s	+/+	I	+/+/+/+/+/+/–/–/+/–
AF13	+/+	II	+/+/+/+/+/+/+/+/+/+
CA14	+/+	II	+/+/+/+/+/+/+/+/+/+
CA19	+/+	II	+/+/+/+/+/+/+/+/+/+
GA9-9	+/+	II	+/+/+/+/+/+/+/+/+/+
NM1-3	+/+	II	+/+/+/+/+/+/+/+/+/+
NRRL 3357	+/+	II	+/+/+/+/+/+/+/+/+/+
SC2-7	+/–	I	+/+/+/–/+/–/–/–/–/–
LA10-10	+/–	II	+/+/+/–/+/+/+/+/+/+
TX15-2	+/–	II	+/+/+/+/+/+/+/+/+/–
TX-9-39s	+/–	III	+/+/–/+/–/–/–/–/+/+
TX12-10-2s	+/–	III	+/+/–/–/–/–/–/–/+/+
TX13-21s	+/–	III	+/+/–/+/–/–/–/–/+/+
CA5	–/+	I	+/+/+/+/+/–/–/–/–/–
MS5-6	–/+	I	+/+/+/+/+/+/+/+/+/+
NM1-6	–/+	I	+/+/+/+/+/+/+/+/+/+
SC6-9	–/+	I	+/+/+/+/+/+/–/–/+/–
MS1-1	–/+	I	+/+/–/+/+/–/–/–/–/–
NC3-6	–/+	I	+/+/–/+/+/–/–/–/–/–
SC3-5	–/+	I	+/+/–/+/+/–/–/–/–/–
TX21-9	–/+	I	+/+/–/+/+/–/–/–/–/–
GA4-4	–/+	II	+/+/+/+/+/+/+/+/+/+
LA10-4	–/+	II	+/+/+/+/+/+/+/+/+/+
TX13-5	–/–	None	–/–/–/–/–/–/–/–/+/–
TX21-5	–/–	None	–/–/–/–/–/–/–/–/+/–
NC7-8	–/–	None	–/–/–/–/–/–/–/–/–/–
AL1-4	–/–	None	–/–/–/–/–/–/–/–/–/–
AL4-7	–/–	None	–/–/–/–/–/–/–/–/–/–
GA1-3	–/–	None	–/–/–/–/–/–/–/–/–/–
MS1-7	–/–	None	–/–/–/–/–/–/–/–/–/–
NC6-1	–/–	None	–/–/–/–/–/–/–/–/–/–
SC5-1	–/–	None	–/–/–/–/–/–/–/–/–/–
TX9-2	–/–	None	–/–/–/–/–/–/–/–/–/–
NRRL 21882	–/–	None	–/–/–/–/–/–/–/–/–/–
Ap SU-1	+/–	Intact	–/–/–/+/?/?/–/–/?/+
Ap SRRC 2043	–[e]/–	Intact	–/–/–/–/?/?/–/–/+/+
Ap BN009	+/–	Intact	–/–/–/–/?/?/–/–/?/+

[a] S indicates S strain *A. flavus*; Ap is *A. parasiticus*.
[b] AF, aflatoxins; CPA, cyclopiazonic acid.
[c] PCR fragments obtained by primers GTGCCCAGCATCTTGGTCCA and AAGGACTTGATGATTCCTC from this region are I, 0.3 kb; II, 0.8 kb; III, 0.7 kb; intact, 1.8 kb.
[d] See Fig. 1 for locations A to J. +, positive; –, negative; ?, non-specific PCR products (see Supplementary).
[e] SRRC 2043 accumulates OMST as the end product.

2. Materials and methods

2.1. Fungal strains

Aspergillus flavus and *A. parasiticus* isolates used in this study are listed in Table 1. The majority of the *A. flavus* isolates were collected from agricultural soils of the southern United States (Horn and Dorner, 1999) and Wolfskill Grant Experimental Farm (University of Davis, Winters, California, USA). The capital letter abbreviation of a strain designation indicates the state from which an isolate was collected. AF13 was collected from Arizona cotton fields (Cotty, 1989). NRRL 3357 is the strain used for the *A. flavus* genome sequencing project (Payne et al., 2006). NRRL 21882, the active ingredient of the afla-guard® biopesticide, was isolated from a Georgia peanut seed (Dorner, 2004). *A. parasiticus* SU-1 (NRRL 5862) was originally isolated from Uganda peanut. *A. parasiticus* SRRC 2043 accumulates *O*-methylsterigmatocystin as the end product due to a defect in the *ordA* gene involved in aflatoxin biosynthesis. *A. parasiticus* BN009 was isolated from Republic of Benin, West Africa (Cotty and Cardwell, 1999). The *A. flavus* strain CA14Δku70ΔpyrG was the fungal transformation recipient derived from aflatoxigenic *A. flavus* CA14, which phylogenetically belongs to the same clade as *A. flavus* NRRL 3357 (Chang et al., 2006). The *ku70* gene, a critical gene of the non-homologous end-joining pathway, had been deleted in this strain, which resulted in an extremely high gene-targeting frequency.

2.2. Culture conditions, isolation of genomic DNA, and PCR profiling of the subtelomeric region

Conidia were inoculated into 1 ml potato dextrose broth in a 2-ml microfuge tube. The tube was placed horizontally and incubated stationarily at 30 °C for 24 h. The harvested mycelia were disrupted using a Scientific Industries' Disruptor Genie™ (ZYMO RESEARCH, Orange County, California, USA). Genomic DNA was prepared using a ZR Fungal/Bacterial DNA Kit™ (ZYMO RESEARCH). The sequences of paired primers derived from *A. flavus* NRRL 3357 are summarized in Table 2. PCR was performed in a Perkin-Elmer GeneAmp PCR System 2400. Twenty-five picomoles of each primer and about 10 ng genomic DNA were added to 25 μl Platinum Blue PCR Supermix (Invitrogen, Carlsbad, California, USA). The PCR mix was heated at 94 °C for 5 min and then subjected to 30 cycles con-

Table 2
PCR primers for profiling the subtelomeric region beyond the aflatoxin biosynthesis gene cluster.

Location[a]	Forward primer	Reverse primer
A	GTCTCTGGATCGTTCCGTCG	GTATAGCACAGCTCCGATGT
B	CGTCCAGAGTCTGCGCAACG	TCTAATGCTTCGTTGCTCCT
C	ATCGATCCACTGGAATCTGC	CAACAACGATACTGACATTCG
D	CAGTCCGGACAGACCGCTCT	ATCAGAACTGCATAAGCGTG
E	TGCAGACGAGCATAGGATTC	ACTGTACCGCATGCACCGAG
F	GTAACGTTGATAGCGATCTC	ACCTTGACAGCTCGATCTGA
G	TCACTGATACACTCCTGTAC	TGGAGTACGTTGATGTTGGA
H	CAGACAAGATTTACGAGCAG	GATAATCAGCTCCATTGCAC
I	TAGTCATCTCCGTGATGAGA	TTATACGAGCGTTGAGCGCA
J	GATCTTCTATCAGTGCAGACG	GCGATGTGTGCCAAGCTACG

[a] See Fig. 1.

sisting of denaturation at 94 °C for 30 s, annelation at 55 °C for 30 s, and extension at 72 °C for 2.0 min. A final 4-min extension step at 72 °C was included. The expected sizes (bp) of the PCR products from locations in the subtelomeric region were A:765, B:915, C:714, D:881, E:998, F:924, G:995, H:936, I:930 and J:819.

2.3. Annotation of the subtelomeric region beyond the aflatoxin biosynthesis gene cluster in A. flavus NRRL 3357

The genome sequence of *A. flavus* NRRL 3357 is available at the Broad Institute (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html). A search shows that the aflatoxin gene cluster is located on Contig 7 (2.3 Mb). Compared to the genome organization of the closely related *A. oryzae* RIB40 (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao), the *A. flavus* aflatoxin gene cluster likely is located on chromosome III and 87 kb from one end (see Fig. 1). Although the telomeric sequence repeat has not been identified, the terminal 1.5-kb region is rich in TA (84%). Feature Map in the *Aspergillus* Comparative Database includes some basic annotations of the putative genes and encoded proteins in this subtelomeric region, but not all are sufficiently detailed to provide useful information. Therefore, further sequence comparison and analysis were carried out manually using BLAST tools at NCBI (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) and the DNAMAN software (Lynnon Soft, Vandreuil, Quebec, Canada).

2.4. Construction of knockout vectors and disruption of dmaT, ctfR1, maoA and pks–nrps in A. flavus

The disruption vectors were all based on pPG28 (Takahashi et al., 2002), a pUC18-derived vector that contains the *A. parasiticus* *pyrG* gene in a 2.7-kb BamHI–SalI fragment (GenBank Accession No. EU817656) as the selectable marker. The strategy used in the vector construction did not rely solely on the presence of intact restriction sites in the flanking genomic DNA of the gene to be targeted. In the cases that no suitable restriction sites were present a half site of SmaI (CCC or GGG), which are abundant in the genome, was used. As an example, the sequence, 5′-...ACAGCA C<u>GGG</u>TATT...-3′ is present in the 5′UTR of *maoA* and contains a half SmaI site. The 5maoA primer (5′-...ACAG<u>CCCGGG</u>TATT...-3′) was designed as such so that genomic C<u>A</u>C was replaced by C<u>C</u>C to give a SmaI site. Because the disruption vector was cut with SmaI (5′-CCC↓GGG-3′, the arrow indicates where SmaI cuts open the sequence) prior to transformation, the non-homologous sequence 5′-...ACAG<u>CCC</u>-3′ of the primer was removed leaving the homologous end, 5′-<u>GGG</u>TATT...-3′ for recombination.

The *dmaT* disruption vector was constructed as follows. A 1.3-kb 5′UTR plus coding region was generated by PCR using GCAACGGGAATTCGTGGACGACTAC (5dmaT) and GTTGAACG GATCCTGCTCAGTTCC that contain EcoRI and tagged BamHI, respectively. Another 1.1-kb coding region was generated with ATAGCATGCGTAGACATCTCCCTGGATCTAC (3dmaT) and TAT GTCGACCTCAAGGGCATTGTCC that contain tagged SphI and tagged SalI, respectively. The fragments were cloned sequentially into pPG28. The insertion of *pyrG* into the *dmaT* locus in the recipient genome would also delete the portion encoding amino acids 76–88. Similarly, PCR primer sets of TTCGAATTCATCATCGTTCACAAC (5ctfR, tagged EcoRI) and TCTAGCAGCCGCTCAGGATCCAGA (tagged BamHI) and ATAGTCGACTCCTCGCTCTGAGCACCA (tagged SalI) and TATAAGCTTCCCGGGAATGCGCTCCGGCTGCTCG (3ctfR, tagged SmaI) were used to generate a 1.3-kb and 1.1-kb fragments for the construction of the *ctfR1* disruption vector. The insertion of *pyrG* into the *ctfR1* locus would also delete the portion encoding amino acids 61–187. Primer sets of ACAGCCCGGGTATTGGAGGTC (5maoA, tagged SmaI) and GCATGGGGGATCCCGATGTA (BamHI) and TGGTGTCGACGTCCTCTTGAAGAC (tagged SalI) and TATA AGCTTTATCTAGACGTGGGTGTTG (3maoA, tagged HindIII) were used to generate 1.0-kb and 1.1-kb fragments for the construction of the *maoA* disruption vector. The insertion of *pyrG* into the *maoA* locus would also delete the portion encoding amino acids 208–238. Primer sets of CAGGCATTGGAGAGCTCGCT (5nrps, SacI) and AGGTGGATCCGTTGGTCCCTTG (tagged BamHI) and ACT CCGTCGACCAGGTTGGCATG (tagged SalI) and CGGATGTATCA GTTGCATGCAGAG (3nrps, SphI) were used to generate a 0.8-kb and 1.2-kb fragments for the construction of the *pks–nrps* disruption vector. The insertion of *pyrG* into the *pks–nrps* locus would also delete the portion encoding amino acids 1089–1104. The disruption vectors of *dmaT*, *ctfR1*, *maoA* and *pks–nrps* were linearized by HindIII/EcoRI, SmaI, EcoRI/SmaI and SacI/SphI, respectively, to generate DNA ends homologous to the targeted regions prior to

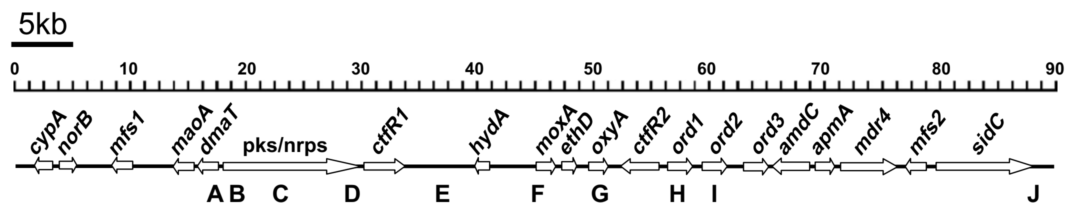

Fig. 1. Schematic representation of predicted genes located in the 87-kb subtelomeric region beyond the aflatoxin gene cluster in *A. flavus* NRRL 3357. The *norB* gene marks the end of the aflatoxin gene cluster. AFL2G indicates gene locus in the *A. flavus* genome annotated by the Broad Institute. *cypA* (AFL2G_07229.2; truncated P450 monooxygenase), *norB* (AFL2G_07230.2; truncated norsolorinic acid reductase), *mfs1*(AFL2G_07233.2; MFS transporter), *maoA* (AFL2G_07234.2; monoamine oxidase), *dmaT* (AFL2G_07235.2; dimethylallyl tryptophan synthase), *pks–nrps* (AFL2G_07236.2; hybrid polyketide and non-ribosomal peptide synthase), *ctfR1* (AFL2G_07237.2; C6-type transcription factor), *hydA* (AFL2G_07240.2; amidohydrolase), *moxA* (AFL2G_07242.2; P450 monooxygenase), *ethD* (AFL2G_07243.2; enzyme involved in ethyl tert-butyl ether degradation), *oxyA* (AFL2G_07244.2; oxygenase), *ctfR2* (AFL2G_07245.2; C6-type transcription factor), *ord1* (beyond AFL2G_07246.2 plus part of AFL2G_07247.2; P450 oxidoreductase), *ord2* (AFL2G_07248.2; FAD-linked oxidoreductase), *ord3* [AFL2G_07248.2; GMC (glucose–methanol–choline) oxidoreductase], *amdC* (AFL2G_07250.2; neutral/alkaline ceramidase), *apmA* (AFL2G_07251.2; amino acid permease), *mdr4* (AFL2G_07252.2; ABC multidrug transporter), *mfs2* (AFL2G_07253.2; MFS transporter), *sidC* (AFL2G_07254.2; non-ribosomal siderophore peptide synthase). The terminal 1.5-kb region is TA rich, which is about 84%.

transformation. Polyethylene glycol-calcium chloride mediated fungal transformation was performed as previously described (Chang, 2008). Regeneration plates were potato dextrose agar supplemented with 0.6 M potassium chloride. Colonies were transferred onto Czapek (CZ) and CZ supplemented with 10 mM ammonium salt for selection of genuine *pyrG*-positive transformants. The primer 3dmaT removed from the linearized vector by the restriction digestion and now located beyond the expected recombination site and the primer 5dmaT were used to confirm the disruption of *dmaT*. Likewise, 5ctfR also removed from the linearized vector and 3ctfR were used to confirm the disruption of *ctfR1*. For *maoA* and *pks–nrps*, 5maoA/3maoA and 5nrps/3nrps were used, respectively. PCR was performed using genomic DNA of *A. flavus* CA14 transformants as described above with an extension step at 72 °C for 5 min.

2.5. Thin layer chromatography analysis of CPA

The medium for CPA production contained 15% sucrose, 2% yeast extract, and 1% soytone, and the pH was adjusted to 6.0 with HCl prior to autoclaving. Approximately 10^5 conidia were inoculated into 1 ml liquid medium in a 4-ml glass vial. Stationary cultures were grown for 7 days at 30 °C in the dark for maximal CPA production. One millilitre of chloroform was added to each vial to extract metabolites. With this procedure, approximately 99% of the added CPA standard was recovered from the medium. The vial was vortexed for about 30 s, let sit for two hours and vortexed again. The liquid content was then transferred to a clean microfuge tube and spun at the maximum speed for 2 min to separate the aqueous and organic phases. Aliquots of 200 µl of the organic layer were transferred to clean microfuge tubes and air dried. CPA detection was performed using thin layer chromatography on Si250 silica gel plates (BAKER). Ethyl acetate/methanol/ammonium hydroxide (85:15:10) was the developing solvent system. The plates were sprayed with Ehrlich's reagent (1 g of 4-dimethylaminobenzaldehyde dissolved in 75 ml ethanol and 25 ml concentrated HCl), and CPA appeared as a blue-purple spot.

2.6. HPLC determination of CPA

Quantitation of CPA in liquid cultures was carried out according to an established method (Sobolev et al., 1998). Briefly, 100 µl of the chloroform extract of the stationary cultures grown for 7 days at 30 °C was evaporated to dryness under nitrogen and redissolved in 1 ml of HPLC mobile phase consisting of heptane/2-propanol/*n*-butanol/water/tetrabutylammonium hydroxide (2560:900:230:32:8). Twenty microlitres was injected onto a silica gel column, and CPA was detected with a UV diode array detector. The limit of quantitation was 1 µg/ml of culture medium.

3. Results

3.1. PCR profiling of subtelomeric regions of A. flavus and A. parasiticus isolates

A comparison of all CPA-producing *A. flavus* isolates showed that the majority of them contained the region A to E (Table 1) with the exception of TX5-1s and the isolates, MS1-1, NC3-6, TX21-9 and SC3-5, which all belong to the group B deletion pattern in the aflatoxin gene cluster (see Section 4). CPA non-producing *A. flavus* isolates, with the exception of TX15-2, had one or more defects or a complete deletion in this region. The region F to J was consistently present in *A. flavus* isolates that had type II deletion in *norB-cypA* and produced CPA regardless of aflatoxin production. In contrast, the majority of *A. flavus* isolates that had type I deletion in *norB-cypA* and produced CPA had more defects in the region F to J with the exception of MS5-6 and NM1-6. The three *A. parasiticus* isolates which do not produce CPA showed a much greater variation in the corresponding portions of the subtelomeric region (Table 1).

3.2. Identification of a dimethylallyl tryptophan synthase gene and annotation of the subtelomeric region

Although proof that the region A to E is involved in CPA production is not established by the PCR results, they suggested that this region could be important if genes for CPA biosynthesis are located in the subtelomeric region. The location of the *norB* gene, which marks one end of the aflatoxin biosynthesis gene cluster, is equivalent to AFL2G_07230.2. Therefore, the genomic portion from *norB* to AFL2G_07240.2 which covers region A to E was analyzed in detail. A search and comparison by BLASTX indicated the 2-kb region of AFL2G_07231.2 and AFL2G_07232.2 probably encoded only one predicted truncated conserved protein. The genes before location A, AFL2G_07233.2 and AFL2G_07234.2, encoding a major facilitator superfamily transporter and a monoamine oxidase, respectively, were previously reported for *A. flavus* AF13 (Ehrlich et al., 2005). The 3-kb region between AFL2G_07233.2 and AFL2G_07234.2 does not appear to contain any genes. The gene AFL2G_07235.2 (location A) encoded a predicted protein with about 45% amino acid identity to dimethylallyl tryptophan synthases from *Aspergillus clavatus*, *A. fumigatus*, *Balansia obtecta*, *Claviceps purpurea*, *Malbranchea aurantiaca*, and *Penicillium roquefortii*. AFL2G_07236.2 encompassing locations B, C and D encoded a hybrid polyketide and non-ribosomal peptide synthase. AFL2G_07237.2 encoded a predicted Cys6 type zinc-finger regulatory protein containing a CNGCRERKRRCVRRKRELPCLSCQAENRPC domain. Fig. 1 shows the annotated genes in the subtelomeric region.

3.3. Disruption of clustered maoA, dmaT, pks-nrps and ctfR1 in relation to CPA production

A dimethylallyl tryptophan synthase is critical for CPA biosynthesis. Therefore, the functions of the dimethylallyl tryptophan synthase gene (*dmaT*) and the three adjoining genes, the monoamine oxidase gene (*maoA*), the hybrid polyketide and non-ribosomal peptide synthase gene (*pks–nrps*) and the transcription factor gene (*ctfR1*), were examined. Disruption of the genes was achieved in an *A. flavus* recipient with high gene-targeting efficiency. The frequency of transformants with the correctly targeted insertion ranged from 85% to 100%. Formation of a large PCR product in transformants compared to a small PCR product in the parental strain indicated that the *pyrG* selectable marker was inserted correctly into respective genes through homologous recombination of flanking regions. The genomic pattern of the parental strain compared to that of a disruptant, as confirmed by PCR with specific primers encompassing the targeting region, was 2.4 kb vs. 5.0 kb for *dmaT*, 2.8 kb vs. 5.0 kb for *ctfR1*, 2.1 kb vs. 4.7 kb for *maoA*, and 2.0 kb vs. 4.7 kb for *pks–nrps* (Fig. 2). TLC analyses of four disruptants of each gene type indicated that except for the *ctfR1*disruptants other types of disruptants had lost the ability to produce CPA. HPLC quantification indicated that the *ctfR1*disruptants produced 44.9 ± 2.9 µg/ml CPA (mean ± SD; $n = 4$) within the range of 32.7 ± 2.5–57.9 ± 4.1 µg/ml produced by different parental strains. The *dmaT* disruptants did not produce any CPA.

4. Discussion

Aspergillus flavus isolates are generally grouped into two morphotypes (L and S strains) based on sclerotial size. L-strain isolates

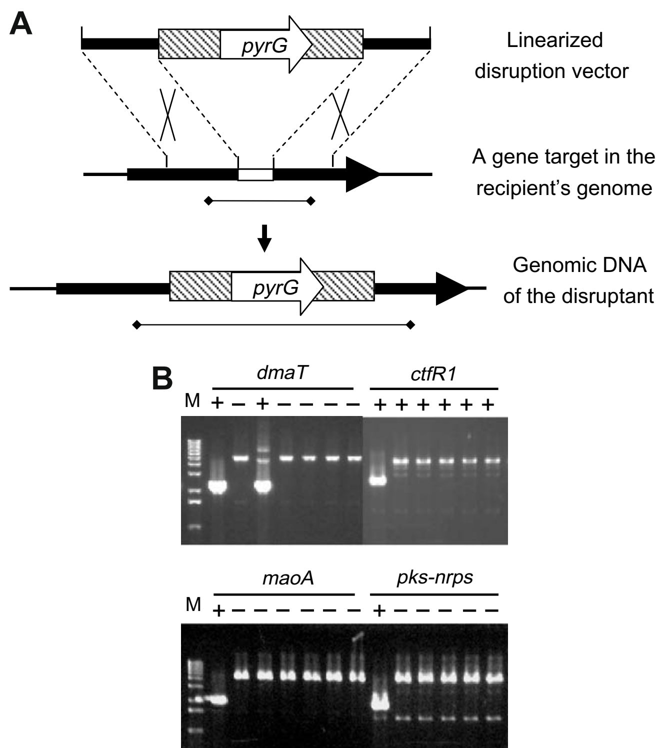

Fig. 2. Disruption of *dmaT*, *ctfR1*, *maoA* and *pks–nrps* genes. (A) Schematic representation of the gene disruption strategy. The *A. flavus* ΔkuΔ70ΔpyrG recipient is defective in the non-homologous end-joining pathway. The linearized vector is the *pyrG*-containing fragment plus the two flanking regions of a gene to be targeted. The white segment in the gene target is the region replaced by the *pyrG* selectable marker after double-crossover recombination. The tagged lines under the genomic DNA are the PCR products amplified by primers derived from specific genomic locations. (B) PCR confirmation of gene disruption. The first lane in each panel is the recipient strain, and others are transformants. The PCR products for intact wild-type genes are *dmaT*: 2.4 kb, *ctfR1*: 2.8 kb, *maoA*: 2.1 kb, and *pks–nrps*: 2.0 kb. The large PCR products are the evidence that the *pyrG* marker (2.7 kb) correctly inserted into respective targeted genes; the sizes are *dmaT*: 5.0 kb, *ctfR1*: 5.0 kb, *maoA*: 4.7 kb, and *pks–nrps* 4.7 kb. The label above each lane is for CPA production, +: positive; −: negative. M: 1 kb DNA ladder.

produce abundant conidia but only a few sclerotia that are larger than 400 μm in diameter whereas S-strain isolates sporulate sparsely and produce numerous sclerotia that are smaller than 400 μm (Cotty, 1989; Horn and Dorner, 1999). The S-strain isolates are very stable in aflatoxin production and typically produce higher amounts than the L-strain isolates, which vary greatly in aflatoxin production (Bayman and Cotty, 1993). The genetic relationship between L and S strains is still not understood. Type I deletion in *norB-cypA* is associated with S strain and also with the L strain evolved from S strain after the loss of aflatoxin production, such as MS5-6 and NM1-6 (Chang et al., 2006). The divergence of S strain from L strain occurred between 1 and 3 Ma (Ehrlich et al., 2005). The genetic differences between the two morphotypes are further manifested by the variations found in the subtelomeric regions (Table 1). In the United States, almost all S-strain isolates produce aflatoxins and CPA in contrast to about three quarters of L-strain isolates (Horn and Dorner, 1998). Each *A. flavus* morphotype may be under different selective forces. The two L-strain isolates MS5-6 and NM1-6 evolved from S strain still retained an intact subtelomeric region (Table 1), which suggests that lack of PCR products in certain locations of the S-strain isolates may be caused by deletion after the divergency.

Taking into account the intrinsic inadequacy of the PCR technique, relative criteria were adopted in interpreting possible regions associated with CPA production. TX5-1s is quite different from other *A. flavus* isolates and its *O*-methyltransferase gene (*omtA*; GenBank Accession No. DQ17679) sequence only has 93% identity to others, forming a unique single nucleotide polymorphism (SNP) pattern different from other well-defined SNP patterns (unpublished results). The lack of PCR products from locations D and E of TX5-1s likely is due to low nucleotide identity in the primers. The isolates MS1-1, NC3-6, SC3-5 and TX21-9, which belong to the previously categorized pattern B group, did not yield PCR products from location C. These isolates have been shown to contain variations in sequences beyond the *norB* gene (Chang et al., 2005). *A. parasiticus* strains differ from CPA-producing *A. flavus* isolates in region A to E. The sequence of the PCR fragment (0.9 kb) only amplified from location C of *A. parasiticus* SU-1 has only 88% DNA sequence identity plus 1% gap to that of *A. flavus* NRRL 3357 instead of the 98–99% identity known for other genes (Ehrlich et al., 2005). Chemical synthesis of CPA has been achieved (Haskins and Knight, 2005), but the biosynthetic steps have not yet been fully elucidated. The enzyme that catalyzes the conversion of cycloacetoacetyl-L-tryptophanyl (cAATrp) and dimethylallyl pyrophosphate to β-cyclopiazonic acid in *P. cyclopium* has been purified (McGrath et al., 1977). The *dmaT* gene likely encodes the corresponding enzyme in *A. flavus*. The *pks–nrps* gene product likely uses two molecules of acetate (acetyl-CoA) along with tryptophan to generate cAATrp. The role of *maoA* is not known. The final step in CPA biosynthesis is the oxidative cyclization of β-CPA by an β-cyclopiazonate oxidocyclase (Holzapfel and Kruger, 1992). The β-cyclopiazonate oxidocyclase of *P. cyclopium* consists of five isoenzymes. Each one contains one covalently linked flavin molecule, giving the isoenzymes a yellow color (Schabort and Potgeiter, 1971). The *ord2* gene predicted to encode a FAD-linked oxidoreductase (Fig. 1) is a likely candidate for the oxidocyclase gene. CPA is produced by other *Aspergillus* species, such as *A. oryzae*, *A. tamarii*, *A. fumigatus*, *A. versicolor*, *A. phoenicis* and *A. clavatus* (Vinokurova et al., 2003). A search of available genome databases of *A. oryzae*, *A. fumigatus*, and *A. clavatus* indicates that dimethylallyl tryptophan synthase genes are present, but no genes homologous to *maoA* and *pks–nrps* are next to the dimethylallyl tryptophan synthase genes in *A. fumigatus* and *A. clavatus*. For *A. oryzae*, which is closely related to *A. flavus*, deletion and rearrangement in the corresponding subtelomeric region resulted in truncation of the *pks–nrps* gene in strain RIB40 and a loss of the three genes in strain B62.

Functional studies have demonstrated that the majority of genes for aflatoxins, sterigmatocystin, trichothecene, and fumonisin biosynthesis are organized in clusters (Brown et al., 1996; Desjardins and Proctor, 2007; Yu et al., 2004). However, not all genes within the boundaries of a gene cluster are required for biosynthesis of secondary metabolites. Only eleven genes in the 22-gene cluster are required for fumonisin biosynthesis (Brown et al., 2007; Proctor et al., 2003). For trichothecenes, three other pathway genes either occur alone (*Tri101*) or form a 2-gene mini cluster (*Tri1* and *Tri16*) (Kimura et al., 2007). Three mini-gene clusters located on a 1.3-Mb chromosome are associated with dothistromin biosynthesis, and non-dothistromin genes are interspersed within each mini-cluster (Zhang et al., 2007). Studies on genes involved in patulin (White et al., 2006) and ochratoxin A (Karolewiez and Geisen, 2005; O'Callaghan et al., 2006) biosynthesis suggest a more fragmented organization of genes involved. In this study, disruption of *ctfR1* did not abolish CPA production and a 3-kb region before *maoA* appears not to contain any genes, which suggests that *maoA*, *dmaT*, *pks-nrps* may be a mini-cluster. Several other genes associated with metabolic and catalytic functions, such as *moxA*, *oxyA*, *ord1* and *ord3* also are clustered along with another transcription factor gene, *ctfR2* (Fig. 1). Despite inconclusive clues from

the PCR profiling of this region, these genes warrant further functional characterization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2008.11.002.

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