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Identification of csypyrone B1 as the novel product of *Aspergillus oryzae* type III polyketide synthase CsyB

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ABSTRACT

As a novel superfamily of type III polyketide synthases (PKSs) in microbes, four genes, *csyA*, *csyB*, *csyC*, and *csyD*, were found in the genome of *Aspergillus oryzae*, an industrially important filamentous fungus. Although orthologs of *csyA*, *csyC*, and *csyD* genes are present in a closely related species, *Aspergillus flavus*, *csyB* gene is unique to *A. oryzae*. To identify its function, we carried out overexpression of *csyB* gene under the control of α-amylase promoter in *A. oryzae*. 3-(3-Acetyl-4-hydroxy-2-oxo-2*H*-pyran-6-yl)propanoic acid, named csypyrone B1, was identified as a CsyB product. Feeding experiments of ^{13}C-labeled acetate indicated that five acetate units were incorporated into csypyrone B1. Two possible mechanisms are proposed for the biosynthesis of cycpyrone B1: (1) condensation of succinyl-CoA with three acetyl/malonyl-CoAs, and the following pyrone ring cyclization; (2) condensation of butyryl-CoA with three acetyl/malonyl-CoAs, and the following pyrone ring cyclization and side-chain oxidation.

1. Introduction

Filamentous fungi have long been considered as producers of versatile secondary metabolites and industrially important enzymes including therapeutic proteins. In particular, *Aspergillus oryzae* is well recognized as an important microbe in Japanese fermentation industry. Although it belongs to the section Flavi same as *Aspergillus flavus*, it does not produce aflatoxin and thus is generally recognized as safe organism.[1,2] Its genome was sequenced by Japanese consortium[2] and compared with those of *Aspergillus nidulans*[3] and *Aspergillus fumigatus*.[4] The genome size of *A. oryzae* is approximately 29% and 34% larger than those of *A. nidulans* and *A. fumigatus*, respectively. This size differences are considered to be largely due to sequence acquisition in *A. oryzae*. Most of the significant gene expansion was found to have occurred in secondary metabolism. In addition to terpenoid biosynthesis genes, a number of polyketide synthase (PKS) genes have been found in *A. oryzae*.[1] In its thirty iterative type I PKS genes, we recently identified a novel PKS-NRPS (non-ribosomal peptide synthase) hybrid gene *cpaA* involved in cyclopiazonic acid biosynthesis.[5,6] However, functions of most type I PKSs in *A. oryzae* have not been confirmed yet. Previously, we reported the presence of 4 type III PKS genes, *csyA*, *csyB*, *csyC*, and *csyD*, in the *A. oryzae* strain RIB40.[7] Type III PKS has a single domain architecture of ketosynthase with an active site for multi-step reactions (substrate priming, decarboxylation, condensation, cyclization, and aromatization).[8] Although bacterial and plant type III PKS superfamily members share an absolutely conserved Cys-His-Asn catalytic triad, they exhibit lower sequence similarities resulting in their variable catalytic functions.[9] In the plant type III PKSs, divergent functions have been reported such as stilbene synthase, acridone synthase, benzalacetone synthase, 2-pyrone synthase, and aloesone synthase.[10] Although similar divergent functions could be expected in microbial type III PKSs, functional analysis on fungal type III PKSs has been very limited. Only recently, type III PKS for pentaketide alkylresorcylic acid in *Neurospora crassa* was identified.[11]

Among 4 type III PKS genes identified in *A. oryzae*, the expression of *csyA* and *csyD* occurred during 1–7 days of culturing in DPY medium while the *csyB* was expressed during 1–3 days.[7] In oligotrophic condition, the expression of *csyB* gene was enhanced though the expression of *csyA* remained unaltered. CsyD lacks the conserved catalytic triad required for PKS activity, indicating that it may be functionally inactive.

Following our study on CsyA product,[12] we carried out the overexpression of CsyB and identified its product as novel polyketide 3-(3-acetyl-4-hydroxy-2-oxo-2*H*-pyran-6-yl)propanoic acid, named csypyrone B1.

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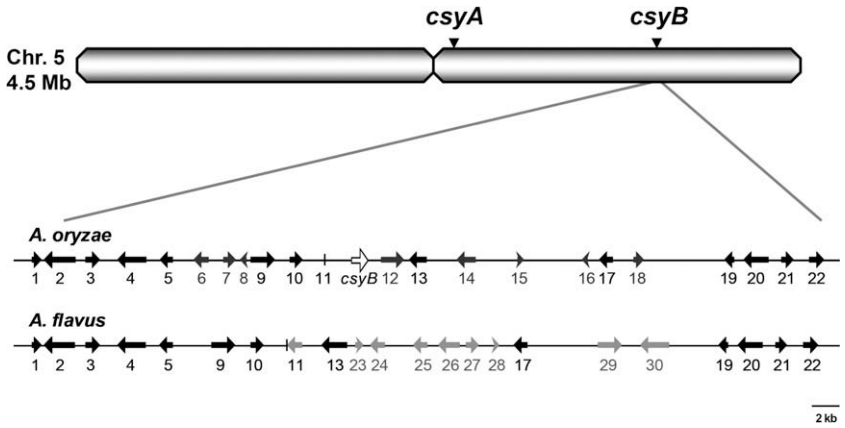

Figure 1. Genomic organization of the *csyB* surrounding region in *Aspergillus oryzae* and the related region in *Aspergillus falvus*. Genes inferred are indicated by arrows. Arrows in black represent the common genes in *A. oryzae* and *A. flavus* genes, and arrows in grey are species-specific genes. The *csyB* gene is shown as white arrow.

2. Results and discussion

2.1. Comparative genomic organization of the type III PKS regions in *A. oryzae* and *A. flavus*

The sequencing of whole genomes of *A. oryzae* and *A. flavus* revealed that the two fungi are closely related each other.[13] A comparative search of the type III PKS genomic regions in the two species revealed that no *csyB* ortholog is present in *A. flavus* although *A. flavus* possesses *csyA*, *csyC*, and *csyD* orthologs. The genomic region surrounding these *csyA*, *csyC*, and *csyD* genes is quite similar, respectively, in both species. The region surrounding the *csyB* gene in *A. oryzae* was compared with the corresponding region of *A. flavus* (Fig. 1) ORFs 1–5 and ORFs 19–20 were more than 98% identical with each other. On the other hand, in the corresponding region between ORF 5 and ORF 19 in *A. flavus*, missing are ORFs 6–8, *csyB*, 12, 14, 16, and 18. In addition ORF 9–11, 13, and 17 show less similarity (72–97%) than ORFs 1–5 and ORFs 19–22. These data suggest that horizontal transfer of region between ORF 6–18 may have occurred in *A. oryzae* or missing genes may have been lost in *A. flavus*. *CsyB* gene is unique to *A. oryzae* showing 47% identity with *A. flavus csyA* ortholog. Some of the genes surrounding *cysB* in *A. oryzae* might be involved in tailoring a product of CsyB. (Table 1)

2.2. Overexpression of *csyB*

In order to investigate the function of *csyB* in *A. oryzae*, expression system driven by *amyB* promoter was adapted as previously utilized for expression of fungal PKSs.[9,14–20] The expression plasmid pTA-csyB was transformed into *A. oryzae* M-2-3 by protoplast-PEG method.[21,22] The *A. oryzae* M-2-3 transformant with pTAex3 served as a control. Although it has not been confirmed whether *A. oryzae* M-2-3 possesses the intact *csyB* gene, type III PKS-related metabolites have not been detected in our inductive PKS expression experiments using *A. oryzae* M-2-3 as a host fungus.

Table 1
Comparison of the *csyB* surrounding regions in *Aspergillus oryzae* and *Aspergillus flavus*

ID	*A. oryzae*	*A. flavus*	Predicted function	Identity(%) (Ao/Af)
1	AO090701000556	AFLA_060280	Hydrolase	99.6 (248/248)
2	[b]	AFLA_060290	Hydrolase	99.4 (828/825)
3	AO090701000557	AFLA_060300	Ketol-acid reductoisomerase	100 (400/400)
4	AO090701000558	AFLA_060310	α-Glucosidase	98.8 (767/770)
5	AO090701000560[a]	AFLA_060320	Unknown	98.8 (331/349)
6	AO090701000561		Oxidoreductase	(405/)
7	AO090701000562[a]		Dioxygenase	(285/)
8	AO090701000563		Unknown	(178/)
9	AO090701000564	AFLA_060330[a]	Acyl esterase	96.9 (593/552)
10	AO090701000565	AFLA_060340	Unknown	92.9 (344/339)
11	[b]	AFLA_060350	Unknown	93.9 (33/342)
csyB				
	AO090701000566		Type III polyketide synthase	(397/)
12	AO090701000567[a]		Transporter	(572/)
13	AO090701000568	AFLA_060360[a]	TolB protein	72.1 (437/606)
14	[b]		Unknown	(439/)
15	AO090701000569[a]		Unknown	(150/)
16	[b]		Unknown	(162/)
17	AO090701000572	AFLA_060420	Glycosyl transferase	85.4 (371/371)
18	AO090701000573[a]		Unknown	(258/)
19	AO090701000575[a]	AFLA_060450	*o*-Methyltransferase	98.3 (243/242)
20	AO090701000576	AFLA_060460	Unknown	99.4 (663/663)
21	AO090701000577	AFLA_060470[a]	Epimerase	99.4 (317/317)
22	AO090701000578	AFLA_060480[a]	NADH dehydrogenase	96.5 (404/402)

(Ao/Af): Predicted protein length, respectively.
[a] The revised locus number by hand annotation.
[b] Unnumbered gene.

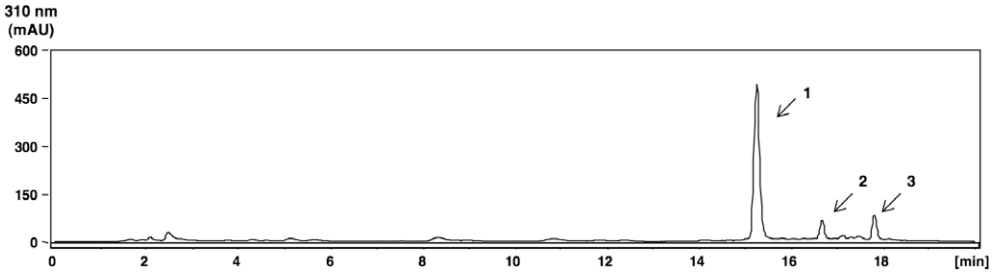

Figure 2. HPLC analysis of *Aspergillus oryzae* transformant with pTA-csyB products. HPLC condition: Inertsil ODS-SP column (2.1 × 100 mm) with a solvent system of acetonitrile containing 1% acetic acid (solvent B) and H_2O containing 1% acetic acid (solvent A) at flow rate 0.2 ml/min at 40 °C. Separation was performed solvent B with 10% in 5 min, 10–60% for 10 min, then 60% for 5 min. Elution was monitored at 310 nm. **1**, csypyrone B1; **2**, compound B2; **3**, compound B3.

Table 2
NMR data of csypyrone B1

Position	^{13}C δ ppm	^{1}H (multi., J in Hz) δ ppm	HMBC $^{1}H \rightarrow ^{13}C$
2	161.0		
3	100.4		
4	181.1		
5	101.6	6.00 (1H, s)	3, 4, 6, 9
6	169.7		
7	205.4		
8	30.2	2.67 (3H, s)	3, 7
9	29.2	2.83 (2H, t, J = 7.3)	5, 6, 10, 11
10	29.9	2.78 (2H, t, J = 7.3)	6, 9, 11
11	175.2		
(4-OH)		16.72	

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data were measured in $CDCl_3$ by JEOL ECA-500 spectrometer.

Since we earlier noted the transcription of *csyB* gene during 1–3 day culturing of *A. oryzae* RIB40 in both the Czapek-Dox and DPY media,[7] the *A. oryzae* M-2-3 transformant with pTA-csyB was grown under normal condition for 2 days, and then cultured in induction media containing maltose for further 3 days. As shown in Fig. 2, HPLC analysis of the ethyl acetate extracts of the pTA-csyB transformants revealed a major peak (B1) at retention time of 15.2 min, and two minor peaks, B2 and B3, at retention times 16.7 and 17.8 min, respectively. None of these peaks were detected in the control transformant (Supplementary Fig. S1). By reverse phase silica gel column chromatography and preparative HPLC, 8.0 mg of compound B1 was obtained from 1 l induction culture medium.

2.3. Structural analysis of the compound B1 and its biosynthesis

Compound B1 was subjected to physicochemical analysis including LC–MS and NMR. Molecular formula of the compound was determined to be $C_{10}H_{10}O_6$ by LC–ESI-TOFMS analysis (*m/z* 227.0543 $[M+H]^+$, calcd for $C_{10}H_{11}O_6$, 227.0550). Its ^{13}C and ^{1}H NMR data are summarized in Table 2. Correlations observed by a hetero-nuclear multiple-bond correlation (HMBC) spectrum are shown in Table 2. With these spectral data, its chemical structure was determined to be 3-(3-acetyl-4-hydroxy-2-oxo-2*H*-pyran-6-yl)propanoic acid, which we designated as csypyrone B1. Csypyrone B1 is similar to germicidin (6-(2-butyl)-3-ethyl-4-hydroxy-2-pyrone), which is produced by *Streptomyces viridochromogenes* and shows an inhibitory effect on the germination of its own arthrospores[23] (Fig. 3).

In the *Streptomyces coelicolor* A3(2) genome sequence, three ORFs for type III PKS were found. One of them, *sco7221* was reported to code for Gcs PKS involved in germicidin biosynthesis.[24] Gcs is a unique type III PKS that could utilize a variable starter CoA ester and catalyze the first condensation with malonyl-CoA,

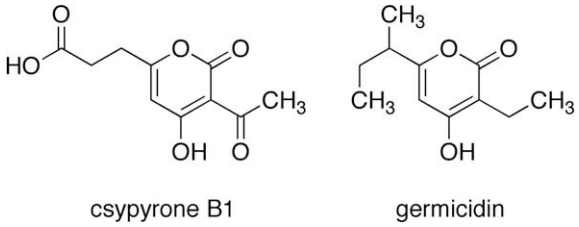

Figure 3. Structures of csypyrone B1 and germicidin.

second condensation with either ethylmalonyl-CoA or methylmalonyl-CoA, and then cyclize to form a pyrone ring.

To confirm the polyketide origin of csypyrone B1, feeding experiment with [1,2-$^{13}C_2$]acetate was carried out using the *csyB* overexpressing strain of *A. oryzae*. Incorporation of acetate units at C6–C9 ($^{1}J_{c-c}$ = 51 Hz) and C10–C11 ($^{1}J_{c-c}$ = 56 Hz) was observed clearly. Thus, the moiety including propanoic acid side-chain (C6 and C9–C11) is considered to be derived from two acetate units, that could probably be succinyl-CoA from TCA cycle or butyryl-CoA. In addition, comparatively weak ^{13}C–^{13}C couplings were observed at C4–C5 ($^{1}J_{c-c}$ = 61 Hz), C7–C8 ($^{1}J_{c-c}$ = 44 Hz) and C2–C3 ($^{1}J_{c-c}$ = 82 Hz) (Fig. 4). This result indicates that C2–C5 and acetyl side-chain (C7 and C8) are from three acetate units.

Possible biosynthetic mechanism for csypyrone B1 is proposed as follows. CsyB catalyzes formation of acetoacetyl-CoA from acetyl-CoA with malonyl-CoA, and 3-oxoadipyl-CoA from succinyl-CoA with malonyl-CoA. These two ketides are coupled to form the branched intermediate, of which pyrone ring cyclization gives csypyrone B1. If butyryl-CoA serves as a substrate in place of succinyl-CoA, host fungus oxidizes the alkyl side-chain to propanoic acid moiety (Fig. 5).

Although it is necessary to carry out in vitro CsyB reaction analysis to identify its actual substrates, expression of CsyB in *Escherichia coli* has not been successful so far. Thus, other expression systems will be utilized for further in vitro experiments to determine direct substrates of CsyB catalyzing condensation. Also,

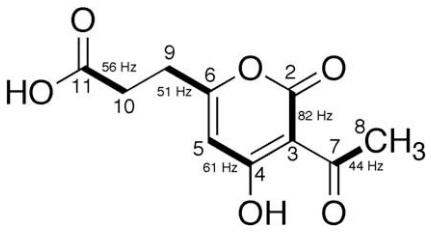

Figure 4. ^{13}C–^{13}C Labeling pattern of csypyrone B1 from [1,2-$^{13}C_2$]acetate. Bold lines indicate the ^{13}C–^{13}C coupled carbon pairs incorporated from [1,2-$^{13}C_2$]acetate. Coupling constants $^{1}J_{C-C}$ are shown in Hz.

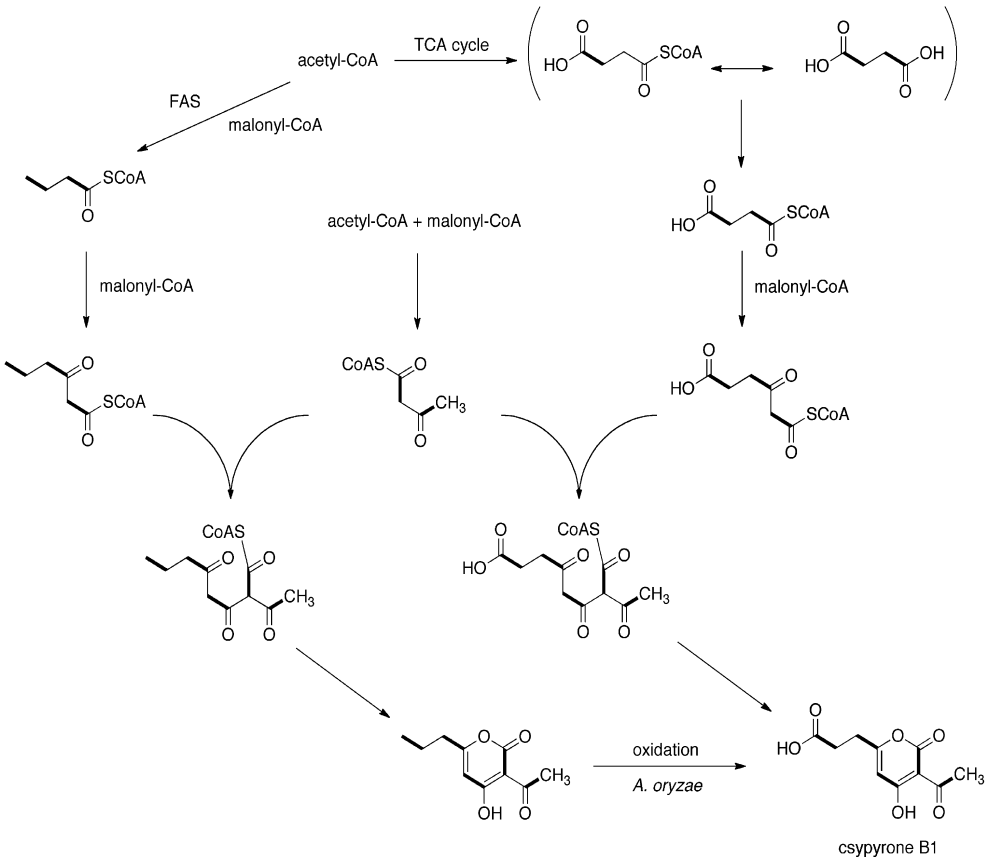

Figure 5. Proposed biosynthesis of csypyrone B1.

isolation and structure analysis of compounds B2 and B3 are now underway in our laboratory.

3. Conclusion

CsyB gene coding for type III PKS is unique to *A. oryzae* with no ortholog present even in the close relative *A. flavus*. By inductive expression in *A. oryzae*, csypyrone B1, 3-(3-acetyl-4-hydroxy-2-oxo-2*H*-pyran-6-yl)propanoic acid, was identified to be a product of CsyB. Feeding experiments indicated that csypyrone B1 could be biosynthesized by condensation of succinyl-CoA and three acetyl/malonyl-CoAs, and the following pyrone ring cyclization.

4. Experimental

4.1. Organism and culture media

DPY medium[22] and potato dextrose agar plates were used for culturing *A. oryzae* RIB40 and *A. oryzae* M-2-3 strains. For induction culture, Czapek-Dox-maltose medium containing 1% polypeptone (Wako, Japan) was used.

4.2. Construction of the *csyB* overexpression strain

The *csyB* gene was amplified from the cDNA of *A. oryzae* RIB40 strain with the specific forward primer (CACGTGATGATTGAGC CACTACC) and reverse primer (CACGTGTCACGCATGAAGGTAG). The PCR amplification was carried out using Phusion DNA polymerase (New England Biolabs). The amplified product was cloned into the *Sma* I digested expression vector pTAex3 under the control of *amyB* promoter and *argB* selection marker to yield the plasmid pTA-csyB. After confirmation of the cloned sequence, the plasmid was subsequently transformed into *A. oryzae* M-2-3 (*argB*$^-$) by protoplast-PEG method.[21,22] The *A. oryzae* M-2-3 transformant with pTAex3 served as a control.

4.3. HPLC and LC–MS analysis of the CsyB product

The pTA-csyB transformant and the control transformant with pTAex3 were grown in 10 ml of induction medium at 30 °C for 3 days. After removal of mycelia by filtration, culture media were extracted with ethyl acetate under acidic condition. Ethyl acetate extracts were evaporated and resulting residues were dissolved with acetonitrile for HPLC and LC–MS analysis. Analytical HPLC was carried out on an Agilent 1100 HPLC system using an Inertsil ODS-SP reverse-phase column (2.1 × 100 mm; GL science, Japan) with a solvent system of acetonitrile containing 1% acetic acid (solvent B) and H_2O containing 1% acetic acid (solvent A) at flow rate 0.2 ml/min at 40 °C. Separation was performed solvent B with 10% in 5 min, 10–60% for 10 min, then 60% for 5 min. Elution was monitored at 310 nm. ESI mass spectra data were obtained on an Agilent 1100 HPLC-microTOF mass spectrometer (Bruker Daltonics).

4.4. Isolation of csypyrone B1

Transformants were first cultured in DPY medium at for 2 days with shaking (160 rpm) at 30 °C, and then transferred to the media containing maltose (5 × 200 ml) and cultured for 3 days with shaking (160 rpm) at 30 °C to induce the expression of *csyB*. After removal of mycelia by filtration, combined induction culture media were subjected to reverse phase silica gel column chromatography (50 g; Cosmosil 75C_{18}-OPN, Nacalai Tesque, Japan). Elution was

carried out with 25% aq. methanol containing 0.1% formic acid. Fractions containing csypyrone B1 were concentrated by evaporation and then dissolved in 50% acetonitrile, which was applied onto an Inertsil ODS-3 JET column (10 × 50 mm; GL science, Japan). Isocratic elution at a flow rate of 1.0 ml/min was carried out with 50% aq acetonitrile containing 0.1% formic acid. Csypyrone B1 was collected and dried up in vacuo. ^{1}H (500 MHz in $CDCl_3$) and ^{13}C (125 MHz in $CDCl_3$) NMR data were measured by ECA-500 spectrometer (JEOL, Japan).

4.5. Physicochemical properties of csypyrone B1

Colorless needles ($CHCl_3$); mp: 135.6–136 °C; UV λ_{max} (MeOH) nm (log ε): 309 (4.45), 224 (4.43); IR (ATR) cm^{-1}: 3624 (–OH), 2923 (–COOH), 1704 (–CO–); HR-ESI-MS $[M+H]^+$ *m/z*: 227.0543 (calcd for $C_{10}H_{11}O_6$: 227.0550). NMR data are summarized in Table 2. ^{1}H NMR spectra are shown in Supplementary Figures S2–S5.

4.6. Feeding experiment using sodium [1,2-$^{13}C_2$]acetate

Fifty mg of sodium [1,2-$^{13}C_2$]acetate (99 atm % enriched, Isotec) was mixed with 100 mg of non-labeled sodium acetate. After dissolved in 2 ml of sterile water, labeled sodium acetate was added to the culture of *A. oryzae* transformant with pTA-csyB (1000 ml) during the maltose induction for 4 days. Csypyrone B1 was isolated as described above and subjected to ^{13}C NMR analysis. ^{13}C NMR spectra fed with sodium [1,2-$^{13}C_2$]acetate are shown in Supplementary Figures S6–S12.

4.7. Sequence analysis

The *csyB* gene and surrounding region sequences were obtained from databases, *A. oryzae*: http://www.bio.nite.go.jp/dogan/Top, *A. flavus*: http://www.aspergillusflavus.org/genomics/. These sequences were hand annotated by using FGENESH program (Softberry, Inc.) and/or GENEID program.[25]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.058.

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