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Characterization of the biosynthetic gene cluster for the ribosomally synthesized cyclic peptide ustiloxin B in *Aspergillus flavus*

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ABSTRACT

Ustiloxin B is a secondary metabolite known to be produced by *Ustilaginoidea virens*. In our previous paper, we observed the production of this compound by *Aspergillus flavus*, and identified two *A. flavus* genes responsible for ustiloxin B biosynthesis (Umemura et al., 2013). The compound is a cyclic tetrapeptide of Tyr-Ala-Ile-Gly, whose tyrosine is modified with a non-protein coding amino acid, norvaline. Although its chemical structure strongly suggested that ustiloxin B is biosynthesized by a non-ribosomal peptide synthetase, in the present study, we observed its synthesis through a ribosomal peptide synthetic (RiPS) pathway by precise sequence analyses after experimental validation of the cluster. The cluster possessed a gene (AFLA_094980), termed *ustA*, whose translated product, UstA, contains a 16-fold repeated peptide embedding a tetrapeptide, Tyr-Ala-Ile-Gly, that is converted into the cyclic moiety of ustiloxin B. This result strongly suggests that ustiloxin B is biosynthesized through a RiPS pathway and that UstA provides the precursor peptide of the compound. The present work is the first characterization of RiPS in *Ascomycetes* and the entire RiPS gene cluster in fungi. Based on the sequence analyses, we also proposed a biosynthetic mechanism involving the entire gene cluster. Our finding indicates the possibility that a number of unidentified RiPSs exist in *Ascomycetes* as the biosynthetic genes of secondary metabolites, and that the feature of a highly repeated peptide sequence in UstA will greatly contribute to the discovery of additional RiPS.

1. Introduction

Fungi produce various secondary metabolites, many of which are bioactive and useful as medicines (e.g., penicillin, cyclosporine, and lovastatin) (Keller et al., 2005). In the late 20th century, with the advent of gene cloning, it became apparent that fungal secondary metabolites are biosynthesized by clusters of coordinately regulated genes, and most of these clusters possess polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), dimethylallyltryptophan synthases, or terpene cyclases with specific sequence motifs (Keller and Hohn, 1997; Keller et al., 2005). Recently, another type of secondary metabolites, ribosomally synthesized peptides or ribosomal peptides (RiPs) have become known particularly in bacteria (Arnison et al., 2013). In fungi, however, only two RiPs (α-amanitin and phallacidin) are known in *Amanita* mushrooms (Hallen et al., 2007).

We recently identified a gene cluster associated with ustiloxin B production in *Aspergillus flavus* using MIDDAS-M, an algorithm that predicts secondary metabolite biosynthetic (SMB) gene clusters based on the concurrent expression of contiguous genes in the genome rather than relying on the presence of core genes for secondary metabolism production, such as PKS or NRPS (Umemura et al., 2013). Although *A. flavus* is known to produce many secondary metabolites, including aflatoxin (Yabe and Nakajima, 2004; Yu, 2012), aflatrem (Nicholson et al., 2009), and cyclopiazonic acid

Abbreviations: EIC, extracted ion chromatogram; ER, endoplasmic reticulum; LC–MS, liquid chromatography–mass spectrometry; NRPS, non-ribosomal peptide synthetase; OE, overexpression; PKS, polyketide synthase; qPCR, quantitative polymerase chain reaction; RiP, ribosomal peptide; RiPS, ribosomal peptide synthetic; SMB, secondary metabolite biosynthetic; TF, transcription factor.

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(Chang et al., 2009), it has never been known to produce ustiloxins until our recent report. According to the MIDDAS-M prediction, the ustiloxin B gene cluster is composed of 18 genes, AFLA_094940 through AFLA_095110, including genes encoding a fungal C6-type transcription factor, a major facilitator superfamily transporter, and a cytochrome P450. We prepared three types of deletion strain, one that had 13 genes deleted and two that had an internal gene in the cluster deleted independently, and observed the loss of ustiloxin B production by these deletion mutants (Umemura et al., 2013).

Ustiloxin B consists of a tetrapeptide, Tyr-Ala-Ile-Gly (YAIG), which is circularized at the side chains of Tyr and Ile and modified with a methyl group, a hydroxyl group, and a non-protein-coding amino acid, norvaline, at the tyrosine (Fig. 1; the history of ustiloxins is briefly described in the Supplementary Information). The structure of the compound strongly indicates that ustiloxin B is synthesized by an NRPS, particularly because it contains the non-proteinogenic amino acid norvaline. However, our previous functional domain analysis revealed that no gene in or flanking the cluster encodes a protein with the NRPS-specific catalytic domains A, C, PCP, and TE (Strieker et al., 2010; Umemura et al., 2013).

In the present study, we discovered a gene encoding a protein with a repeated YAIG motif that exactly matches the cyclic peptide moiety of ustiloxin. Further, we found a peptidase-encoding gene presumably processing the precursor protein, UstA, adjacent to *ustA*. This discovery and subsequent sequence analyses strongly suggest that ustiloxin B is biosynthesized not by an NRPS but by a ribosomal peptide synthetic (RiPS) pathway. The RiPS pathway was first discovered for nisin and subtilin in 1988 (Banerjee and Hansen, 1988; Buchman et al., 1988; Kaletta and Entian, 1989; Kaletta et al., 1989), and many RiPS pathways have been discovered since the first decade of the 21st century in bacteria, plants, and fungi (*Basidiomycetes*) (Arnison et al., 2013; Huo et al., 2012; Velasquez and van der Donk, 2011; Wang et al., 2013; Yang and van der Donk, 2013). However, the existence of the RiPS pathway has not been reported in any *Ascomycetes*, including *Aspergillus* species, to date. First, we verified the MIDDAS-M prediction by preparing each disruption mutant for the predicted 18 genes plus five adjacent genes outside the predicted cluster, followed by an LC–MS analysis of ustiloxin B production. Second, we observed enhanced ustiloxin B productivity caused by the overexpression of the C6-type transcription factor. Third, we performed an extensive functional characterization of the translated products deduced from all of the genes in the cluster using a bioinformatics approach. Based on the analytical results, we proposed a RiPS pathway model for ustiloxin B biosynthesis.

2. Materials and methods

2.1. Strains and genome sequence

A. flavus strain CA14 Δ*ku70* Δ*pyrG* Δ*niaD* was used for the construction of transformants. The gene-annotated genome sequence of *A. flavus*, GenBank EQ963472–EQ966232 (Yu et al., 2008), was used to design primers and analyze the sequences of the ustiloxin B biosynthetic gene cluster. The procedure for DNA extraction is described in the Supplementary Methods.

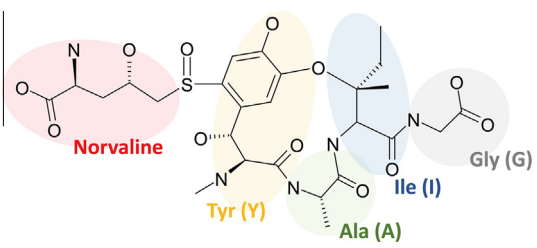

Fig. 1. Structure of ustiloxin B. Amino acid components are indicated by circles.

2.2. Gene disruption and transformation

The disruption of the 16 *A. flavus* genes predicted by MIDDAS-M to be components of the ustiloxin B gene cluster (AFLA_094940 through AFLA_095110 except AFLA_094960 and AFLA_095040, whose deletion mutants were prepared in our previous study (Umemura et al., 2013)) and five adjacent genes (AFLA_094930 and AFLA_095120 through AFLA_095150) was accomplished via protoplast transformation (Min et al., 2007; Szewczyk et al., 2006) with *pyrG* as the selectable marker, as previously described (Umemura et al., 2013). Briefly, deletion cassettes were constructed via fusion PCR (Szewczyk et al., 2006) by first amplifying ~1 kb of the upstream and downstream regions of each target gene coding sequences and a 1.8 kb fragment of *pyrG* originating from *Aspergillus nidulans* using the KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). After gel-extraction of the specifically amplified DNA fragments, the fragments were fused by PCR using KOD-Plus and a primer set of 5F and 3R (Fig. S1A). The primer sets are listed in Table S1. The *A. flavus pyrG* gene fragment was also amplified using primers of Af-*pyrG*-F/R to construct a *pyrG* revertant as a control.

For fungal transformation, conidia (10^6/mL) of *A. flavus* CA14 Δ*ku70* Δ*pyrG* Δ*niaD* were placed into 300-mL flasks containing 100 mL potato dextrose broth (Difco, Franklin Lakes, NJ, USA) supplemented with 1.12 g/L uracil and incubated at 30 °C on a rotary shaker at 170 rpm for two days. The cultures were harvested and washed with 0.8 M NaCl solution. A solution containing 100 mg of lysing enzyme (Sigma, St. Louis, MO, USA), 100 mg of Yatalase (TaKaRa, Otsu-shi, Shiga, Japan), and 50 mg of cellulase (Yakult, Tokyo, Japan) in 30 mL 10 mM NaH_2PO_4 and 0.8 M NaCl was added to the fungal mycelia. This mixture was gently shaken at 100 rpm and 30 °C for 3 h. The cell wall debris was removed with a cell strainer (Corning, Corning, NY, USA), and the filtrate was centrifuged at 3500 rpm in an AR510-04 rotor (TOMY, Tokyo, Japan) at 4 °C for 20 min. After discarding the supernatant, the pellet was washed twice with 1 mL of 1.2 M sorbitol, 50 mM $CaCl_2$, and 10 mM Tris–HCl (pH 7.5) and suspended in 100 μL of the solution. Approximately 1 μg of each final DNA fragment was mixed with a 100-μL aliquot of the protoplasts on ice. After incubation on ice for 20 min, 1 mL of 50% polyethylene glycol (Mr 3350, Sigma), 10 mM Tris–HCl (pH 7.5), and 10 mM $CaCl_2$ solution was added, mixed by tapping, and incubated at room temperature for 20 min. Each transformation solution was plated on the surface of regeneration medium agar (35 g Czapek-Dox broth (Difco), 52.86 g $(NH_4)_2SO_4$, and 10 g agar in a final volume of 1 L). The plates were incubated at 30 °C for 3–5 days.

Three putative transformants for each deletion mutant (except ΔAFLA_095010, for which only two transformants could be obtained) were independently isolated from a single conidium, subjected to DNA isolation, and screened by amplifying loci outside and inside the target genes by PCR with the respective primer sets of cF/cR and incF/incR (Table S1). The amplicon sizes analyzed by electrophoresis showed that all deletion mutants and the revertant as the control strain were successfully obtained (Fig. S2). Further, Southern blot analysis showed a single band without any extra significant signals for all of the transformants (Fig. S3). These results clearly indicate the homologous integration of DNA fragments without ectopic insertion in all of the transformants above.

2.3. Overexpression of ustR

Although only AFLA_095090 was annotated as a gene encoding fungal C6-type transcription factor in the NCBI database (http://