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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.

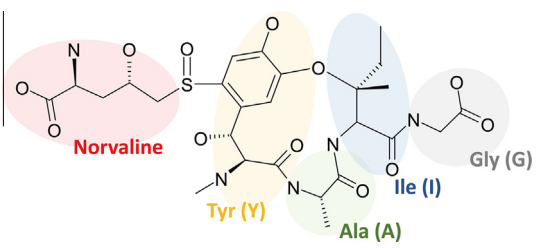

Fig. 1. Structure of ustiloxin B. Amino acid components are indicated by circles.

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://

www.ncbi.nlm.nih.gov/nuccore; gene ID: 7917922), our sequence analysis indicated that a transcription factor for the ustiloxin B cluster is encoded by a gene in the combined form of AFLA_095080 and AFLA_095090 (named *ustR*). Therefore, we constructed an overexpression (OE) strain of this putative gene encoding a transcription factor, $ustR^{OE}$, by replacing 1069 bp of the upstream region of AFLA_095080 with the strong constitutive promoter region of *tef1* (Kitamoto et al., 1998) in the genome of *A. flavus* CA14 Δ*ku70* Δ*pyrG* Δ*niaD* (Fig. S1B). The procedures for the DNA cassette construction and its transformation were the same as those for the disruption mutants. Primer sets are listed in Table S2. The amplicon size obtained using the primer set of cF/cR for $ustR^{OE}$ showed that the overexpression strain was successfully produced (Fig. S4). Homologous integration of DNA fragments without additional ectopic integration in the transformant was confirmed by Southern blot analysis (Fig. S3).

The expression levels of *ustR* in the $ustR^{OE}$ strains were examined by quantitative reverse transcription polymerase chain reaction (qPCR) experiments. Conidia (10^6/mL) of $ustR^{OE}$ and the control strains were inoculated and grown in 250-mL flasks containing 100 mL V8-juice medium (20% (v/v) V8 juice (Campbell's, Camden, NJ, USA) with 0.3% $CaCO_3$) at 30 °C on a rotary shaker at 170 rpm for four days. Each fungal mycelium was harvested, immediately frozen in liquid nitrogen, and ground, and total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), followed by DNase I digestion (New England Biolabs, Ipswich, MA, USA) and quantification using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Using 2 μg of total RNA as a template, cDNA was synthesized using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Santa Clara, CA, USA). One microliter of the resulting cDNA solution was analyzed by qPCR with Power SYBR Green PCR master mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Three primer sets amplifying ~100-bp regions of AFLA_095080, AFLA_095090, and the region bridging the two genes were used to quantify the expression level of *ustR* (Table S2). Samples were tested in three replicates. Expression levels were normalized to the level of tubulin β-subunit (AFLA_051840) expression in each sample. Standard lines for each primer set were created with ten-fold serial dilutions (10 to 0.00001 pM) of DNA standards amplified using genomic DNA of *A. flavus* CA14 Δ*ku70* Δ*pyrG* Δ*niaD* as a template.

2.4. Fungal cultivation and analysis of ustiloxin B

After precultivating 10^6 conidia in 10 mL potato dextrose broth (Difco) at 170 rpm and 30 °C for 24 h, three replicates for each deletion mutant (two for ΔAFLA_095010) and the control strain were cultivated at 28 °C for seven days in 50-mL glass vials containing an autoclaved medium composed of 2.5 g cracked maize and 1.2 mL sterile water. This condition is the first culture condition under which the compound was found in *A. flavus* culture. The fungal mycelia were then homogenized and extracted with 10 mL 70% aqueous acetone for 2 h at room temperature. After vaporizing the acetone, 300 μL of the aqueous concentrate was mixed with an equal volume of ethyl acetate at room temperature for 1 h, and the water layers were filtered using filter units with a 0.22 μm pore size (Nacalai Tesque, Kyoto, Japan). A 2-μL aliquot of each water extract was separated on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) using a 2.0 × 250 mm Develosil XG-C18 M-5 reversed-phase column (Nomura Chemical, Seto-shi, Aichi, Japan) and eluted with a gradient of water–acetonitrile (100:0 to 0:100 for 30 min) at a flow rate of 0.2 mL/min. A micrOTOF II KIK2 MS (Bruker Daltonics, Billerica, MA, USA) was used for detection. The productivity of ustiloxin B ($C_{26}H_{39}N_5O_{12}S$) was analyzed from extracted ion chromatograms (EICs) of m/z 644.2 ± 0.2 $[M-H]^-$ at a retention time of 9.0 min, which was previously identified as ustiloxin B by comparison with an authentic sample (Umemura et al., 2013), using DataAnalysis (version 4.0, Bruker Daltonics).

To measure fungal growth and ustiloxin B production, three replicates of the deletion mutant (two for ΔAFLA_095010), the control, and the $ustR^{OE}$ strains were cultured in V8-juice liquid medium. After five days of cultivation in 10 mL (30 mL for $ustR^{OE}$) liquid medium in a 50-mL TPP TubeSpin bioreactor tube (Sigma) (100-mL flask for $ustR^{OE}$) at 30 °C and 170 rpm, 23 mL (70 mL for $ustR^{OE}$) acetone was added and incubated at room temperature for 1 h. Mycelia and extracts were separated by filtration through Miracloth (Merck Millipore, Darmstadt, Land Hessen, Germany), and the mycelia were freeze-dried for measurement of their dried cell weight. The extracts were treated and analyzed by LC–MS in the same way as that of maize culture. The production of ustiloxin B was quantified by the peak area of EICs of m/z 644.2 ± 0.1 $[M-H]^-$ at the retention time of 9.0 min using DataAnalysis (version 4.0, Bruker Daltonics). Note that the retention time of the compound is different from that in the case of maize culture, most likely because the contents of the water extract differ between cracked-maize and V8-juice cultures.

2.5. Sequence analysis

The amino acid sequences of AFLA_094930 through AFLA_095150 were screened using BLASTP (Altschul et al., 1990) against the UniProtKB database (Punta et al., 2012) and EzCatDB (Nagano, 2005). The amino acid sequences of AFLA_094940 through AFLA_095110 were subjected to BLASTP searches against the amino acid sequences of all *A. flavus* genes to identify homologous genes in the *A. flavus* genome. The nucleic acid sequences of AFLA_094940 through AFLA_095110, which were predicted by MIDDAS-M to be the ustiloxin B gene cluster, were aligned with the *Aspergillus oryzae* RIB40 genome sequence (GenBank AP007150–AP007177 (Machida et al., 2005)) using MAFFT (Katoh et al., 2002) to examine the *A. flavus* gene modeling in the GenBank database. DNA-binding domains of AFLA_095090 and *A. oryzae* AO090113000035 were searched against the Pfam protein motif database (Punta et al., 2012; Sonnhammer et al., 1997). A putative signal peptide was detected in the precursor peptide of the ustiloxin B biosynthetic gene cluster using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

3. Results and discussion

3.1. Characterization of the ustiloxin B biosynthetic gene cluster by gene disruption and LC–MS analysis

In our previous study, we identified two genes in the cluster predicted by MIDDAS-M, AFLA_094960 and AFLA_095040, as being responsible for ustiloxin B biosynthesis based on gene disruption and LC–MS measurement of ustiloxin B production using cracked-maize cultures (Umemura et al., 2013). In this work, we disrupted additional 21 genes inside and adjacent to the putative ustiloxin B gene cluster to refine the cluster identification and to identify genes indispensable for ustiloxin B biosynthesis. Analysis of water extracts of 23 gene deletion mutants, AFLA_094930 through AFLA_095150, from six-day-old cracked-maize culture and V8-juice culture by LC–MS indicated that the gene cluster, at least the genes from AFLA_094940 to AFLA_095100 is responsible for ustiloxin B production (Fig. 2). The dry cell weight measurement showed that the fungal growth of the deletion mutants and the control strain was nearly equal in the V8-juice liquid medium (Table S3). The independent deletion of AFLA_094940 through AFLA_095100 resulted in a complete or substantial loss of ustiloxin

B productivity in both maize and V8-juice media, except for that of AFLA_095030 (Fig. 2, Table 1). The deletion mutant of AFLA_095030 produced the compound in a comparable manner to the deletion mutants outside the cluster (Fig. 2). This similarity might have arisen because the *A. flavus* genome (GenBank ID: AAIH00000000.2) possesses a gene, AFLA_074100, whose amino acid sequence is identical to that of AFLA_095030 (Table 1).

MIDDAS-M predicted the genes AFLA_094940 to AFLA_095110 to be a cluster based on the co-expression of the genes, demonstrating a functional linkage of the 18 genes to ustiloxin B biosynthesis (Umemura et al., 2013). Contrary to the prediction, the deletion mutant of AFLA_095110 showed an ustiloxin B productivity comparable to that of the control strain in both cracked-maize and V8-juice cultures based on the LC–MS analysis (Fig. 2). Our functional annotation suggested that AFLA_095110 is involved in the transfer of *S*-norvaline to an activated aromatic ring of the ustiloxin tyrosine residue (de Vries and Janssen, 2003; Habig et al., 1974; Snyder et al., 1993), and that no other genes in the cluster can compensate for the loss of the function. The existence of a homologue outside the cluster, AFLA_087240, might have compensated for the function of AFLA_095110 (Table 1). Accordingly, we tentatively assigned AFLA_095110 as a member of the ustiloxin B biosynthetic gene cluster. The deletion of the genes outside the cluster from AFLA_094940 to AFLA_095110 did not affect ustiloxin B productivity. Based on the above results, we concluded that the genes AFLA_094940 through AFLA_095110 formed a cluster for ustiloxin B biosynthesis and were designated as *ustO* to *ustS*, according to their predicted functions annotated from functional domain search against the UniProtKB database (Consortium, 2011; Punta et al., 2012) (Table 1, Fig. 3).

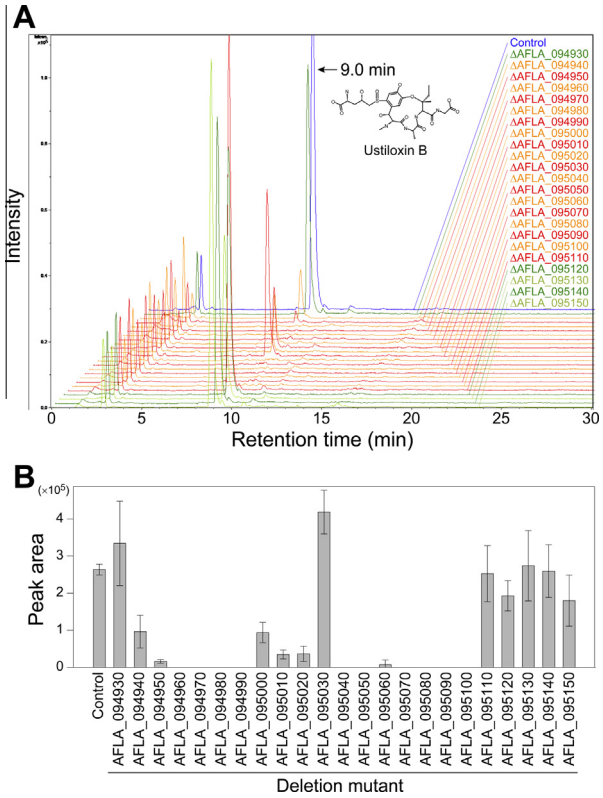

Fig. 2. LC–MS profiles of the deletion mutants for ustiloxin B. (A) EICs of *m*/*z* 644.2 $[M–H]^-$. The retention time of ustiloxin B in water extracts of cracked-maize cultures was 9.0 min. A representative chromatogram is drawn from three replicates for each deletion mutant. The chromatograms for the deletion mutants of the genes predicted by MIDDAS-M to constitute an SMB gene cluster are in yellow and red, and those for the control strain and the genes outside the predicted gene cluster are in blue and green, respectively. (B) The MS peak areas corresponding to ustiloxin B in the control strain and deletion mutants from V8-juice liquid culture water extracts. Averages and standard deviations were calculated from three replicates for each mutant (two replicates instead of three were used for AFLA_095010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The deletion of AFLA_094940 (pyridoxamine 5′-phosphate oxidase family; *ustO*), AFLA_094950 (flavin-containing monooxygenase; *ustF1*), or AFLA_095010 (peptidase S41 family protein; *ustP2*) resulted in the production of a small amount of ustiloxin B. This production most likely occurred because these enzymes are common in microorganisms and have homologs with strong similarity (e-value <1e−80 by BLASTP (Altschul et al., 1990)) in the *A. flavus* genome (Table 1). However, in spite of the absence of their homologs in the genome, the deletion mutants of AFLA_095000 (*ustP1*) and AFLA_095020 (*ustYb*) still produced a small amount of ustiloxin B. Notably, neither gene was identified in the corresponding region of the *A. oryzae* RIB40 genome (GenBank ID: AP007150–AP007177 (Machida et al., 2005)), a close relative of *A. flavus*. Our functional annotation using a BLASTP (Altschul et al., 1990) search against UniProt KB (Consortium, 2011; Punta et al., 2012) indicated that AFLA_095000 (*ustP1*) and AFLA_095010 (*ustP2*) are the N-terminal region and the region containing the catalytic domain, respectively, of a peptidase S41 family protein. These results strongly suggest that the small CDS *ustP1* constitutes a N-terminal moiety of *ustP2*. The function of another small CDS, AFLA_095020 (*ustYb*), is not clear (Table 1) and requires further analysis.

3.2. Increasing the production of ustiloxin B by ustR overexpression

The identified ustiloxin B gene cluster possesses the gene AFLA_095090, which is annotated as a fungal C6-type transcription factor in the GenBank database. However, this gene possesses only an incomplete fungal-specific transcription factor domain (136–383 of 383 in the pfam domain) and lacks any DNA-binding domains. In contrast, *A. oryzae* AO090113000035, which is closely aligned with both AFLA_095080 and AFLA_095090, possesses almost complete domains of a fungal-specific transcription factor (2–383 of 383 in the pfam domain) and a fungal Zn_2Cys_6 binuclear cluster DNA-binding domain (1–37 of 40 in the pfam domain, Fig. S5A). The N- and C-terminal regions of AO090113000035 are identical to those of AFLA_095080 and AFLA_095090, respectively (Fig. S5A). These results strongly suggest that the transcriptional regulator in the ustiloxin B gene cluster, designated as *ustR*, is continuously transcribed from both AFLA_095080 and AFLA_095090 as a single gene (Fig. S5A).

To confirm this possibility, we prepared a *ustR* overexpression strain ($ustR^{OE}$) by replacing the promoter region of AFLA_095080 with that of the *A. flavus TEF1* gene (Fig. S1B). A qPCR analysis revealed that both AFLA_095080 and AFLA_095090 were overexpressed by ca. 15-fold in the $ustR^{OE}$ strain compared with the control strain. Additionally, the "intergenic" region between AFLA_095080 and ALFA_095090 was detected by the qPCR analysis and was overexpressed by ca. 15-fold in the $ustR^{OE}$ strain (Fig. S5B). Consequently, *ustR* was confirmed to be a merged version of the two genes described above.

A number of successful enhancements have been reported for the production of a secondary metabolite by the overexpression of a C6-type transcription factor in the SMB gene cluster (Ahuja et al., 2012; Bromann et al., 2012; Nakazawa et al., 2012). Similarly, the ustiloxin B productivity was enhanced by 4.8-fold by *ustR* overexpression, scored from the areas of the ustiloxin B MS peak (Fig. 4), although the fungal growth measured by dried cell weights was almost equal for both $ustR^{OE}$ and the control strains (Table 2). Thus, it was confirmed that *ustR* is the transcriptional regulator of the gene cluster responsible for ustiloxin B production.

It should be noted that a long intergenic region of >2 kb in size was found between *ustT* and *ustR* (Fig. 3). Interestingly, ~1.6 kb of

Table 1
Genes examined by disruption and LC–MS/sequence analyses.

Gene ID in NCBI	Predicted function[a]	MIDDAS-M detection	Ustiloxin B synthesis[b]	Homologous gene(s) in the genome (e-value)[c]	Gene name[d]	Length (bp)
AFLA_094930	Uncharacterized protein		+++	–	–	
AFLA_094940	Pyridoxamine 5′-phosphate oxidase family	✓	++	AFLA_108810 (5e−87)	*ustO*	814
AFLA_094950	Flavin-containing monooxygenase	✓	+	AFLA_095050 (2e−82) AFLA_053820 (3e−74) AFLA_085530 (6e−28) AFLA_050170 (6e−24)	*ustF1*	1812
AFLA_094960	Cytochrome P450	✓	–	AFLA_122250 (7e−27) AFLA_072360 (6e−26) AFLA_115900 (4e−23) AFLA_101360 (9e−23)	*ustC*	1869
AFLA_094970	Rieske-like protein	✓	–	–	*ustU*	520
AFLA_094980	Allatostatin neuropeptide-like protein	✓	–	–	*ustA*	770
AFLA_094990	Uncharacterized protein	✓	–	–	*ustYa*	910
AFLA_095000	Peptidase S41 family protein	✓	+	–	*ustP1*	337
AFLA_095010	Peptidase S41 family protein	✓	++	AFLA_117010 (6e−83) AFLA_122180 (7e−47) AFLA_060490 (1e−43) AFLA_108490 (5e−34)	*ustP2*	1490
AFLA_095020	Uncharacterized protein	✓	+	–	*ustYb*	545
AFLA_095030	Gamma-glutamyltranspeptidase	✓	+++	AFLA_074100 (0.0) AFLA_109530 (1e−160)	*ustH*	2065
AFLA_095040	Cysteine desulfurase	✓	–	AFLA_109660 (2e−103) AFLA_090820 (3e−74)	*ustD*	1453
AFLA_095050	Flavin-containing monooxygenase	✓	–	AFLA_053820 (7e−132) AFLA_094950 (3e−82) AFLA_085530 (3e−36) AFLA_006150 (1e−32) AFLA_050170 (3e−27)	*ustF2*	1650
AFLA_095060	Tyrosinase	✓	–	AFLA_063220 (2e−39) AFLA_018450 (4e−28) AFLA_067860 (9e−28)	*ustQ*	1350
AFLA_095070	MFS multidrug transporter	✓	–	AFLA_088330 (2e−53) AFLA_041380 (9e−41) AFLA_109610 (3e−40) AFLA_060090 (5e−22)	*ustT*	1791
AFLA_095080	C6 transcription factor	✓	–	–	*ustR*	1827
AFLA_095090	C6 transcription factor	✓	–	–		
AFLA_095100	SAM-dependent methyltransferases	✓	–	–	*ustM*	1233
AFLA_095110	Glutathione S-transferase	✓	+++	AFLA_087240 (8e−68)	*ustS*	732
AFLA_095120	MFS transporter		+++	AFLA_091020 (3e−128) AFLA_121540 (1e−115) AFLA_128470 (8e−114) AFLA_040620 (1e−105) Other 42 hits (>1e−20)	–	
AFLA_095130	Uncharacterized protein		+++	–	–	
AFLA_095140	Pectinesterase		+++	AFLA_023340 (4e−29) AFLA_001410 (5e−27)	–	
AFLA_095150	Xylosidase (C-terminal), Pectinesterase (N-terminal)		+++	AFLA_100100 (1e−92) AFLA_009550 (2e−55) AFLA_004380 (8e−45) AFLA_136480 (3e−39) AFLA_077090 (3e−27) AFLA_119790 (7e−26)	–	

[a] Annotated by BLASTP search against UniProt KB.
[b] According to the peak areas of EICs (*m*/*z* 644.2, negative ion mode) at RT 9.0 min from V8-juice culture water extracts (Fig. 2B).
[c] Amino acid sequences of each gene were screened against the complete gene set in the *A. flavus* genome using BLASTP. Genes with e-values <1e−20, except the query gene, are listed.
[d] Designated in this study.

this region was not found in the *A. oryzae* nucleotide sequence (Fig. 3, dashed line). Because *A. oryzae* RIB40 did not produce ustiloxin B (data not shown), the intergenic region may be involved in the transcriptional regulation of the downstream gene, *ustR*, whose translated product further regulates ustiloxin B productivity.

3.3. Discovery of the precursor protein for ribosomal peptide synthesis in Ascomycetes from gene sequence analysis

Despite its chemical structure, a short cyclic peptide with chemical modifications and a non-protein coding amino acid, norvaline, ustiloxin B is not thought to be synthesized by NRPSs because none of the NRPS-specific catalytic domains (A, C, PCP, or TE) were found in any genes either in the cluster (AFLA_094940 through AFLA_095110) or within the ten adjacent genes outside the cluster by a BLASTP search against the UniProtKB database (Umemura et al., 2013).

In this study, our precise sequence analysis revealed that gene(s) encoding a peptidase (*ustP1* and *ustP2* as a putative single gene) were contained in the cluster, indicating the existence of a peptide sequence(s) serving as a substrate of the enzyme. Interestingly, a detailed sequence analysis of the gene cluster revealed the existence of a highly characteristic protein sequence in the translated product of *ustA* (AFLA_094980), UstA. This protein sequence

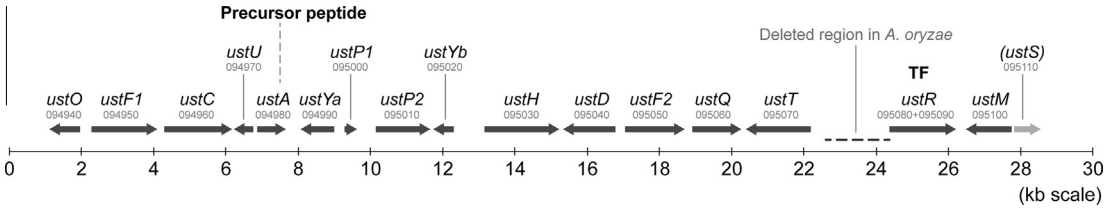

Fig. 3. The ustiloxin B biosynthetic gene cluster of *A. flavus* identified in this study. The genes AFLA_094940 through AFLA_095110 in GenBank were designated as *ustO* through *ustS* based on their functions. The numbers below the gene names in gray are the last six digits of the NCBI gene ID (e.g., "094940" for "AFLA_094940"). AFLA_095110 (*ustS*), the deletion of which did not affect ustiloxin B productivity, is indicated in gray. The region deleted in the corresponding gene cluster of *A. oryzae* is indicated by the dashed line. The total size of the gene cluster is approximately 27 kb including *ustS*.

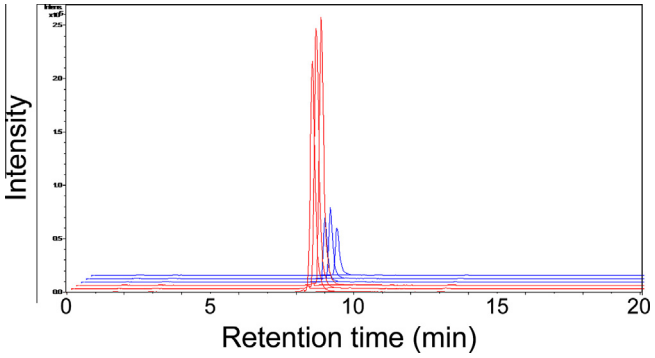

Fig. 4. EICs of *m*/*z* 644.2 in a negative ion mode for *ustR*^{OE} strains (red) and the control strain (blue). The peak areas at RT 8.6 min corresponding to ustiloxin B in water extracts of V8-juice cultures are listed in Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

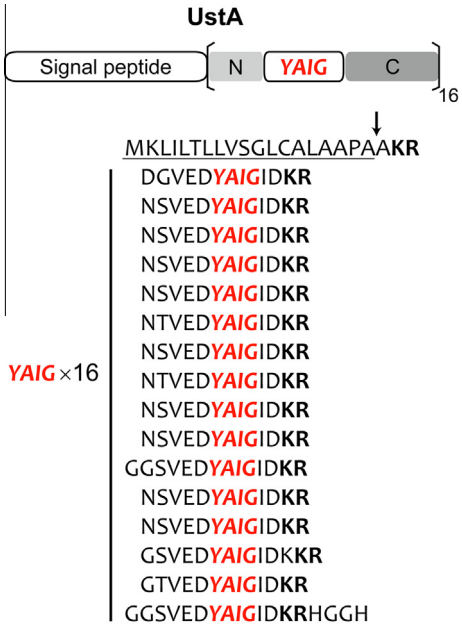

Fig. 5. Protein sequence structure of the ustiloxin B precursor peptide. UstA, the translated product of *ustA* (AFLA_094980), contains a 16-fold repeated sequence that includes YAIG, the exact tetrapeptide of the ustiloxin B cyclic moiety (Fig. 1). The underlined amino acids and the arrow indicate the sequence with a signal peptide characteristic and a cleavage site, respectively. Both of these factors were predicted by the SignalP 4.1 server.

contains a 16-fold repeat of short peptides, including "YAIG," the exact sequence of the ustiloxin B cyclic moiety (Fig. 5). Each repeated sequence unit includes flanking sequences at the N- and C-terminal sides of "YAIG," which might serve as the recognition site for the peptidase(s), UstP1 and/or UstP2. Moreover, the sequence analysis of UstA revealed a signal peptide-like sequence in its N-terminal region (Fig. 5). After transfer to the Golgi apparatus through the endoplasmic reticulum (ER), the peptide is thought to be processed by the endoproteinase Kex2 at the C-terminal side of "KR" (Nielsen et al., 1997; Petersen et al., 2011). These facts strongly suggest that ustiloxin B is biosynthesized through a RiPS pathway and that UstA is the precursor protein of the product.

The RiPS compounds (ribosomal peptides, RiPs) are valuable natural compounds because of their characteristic bioactivity. Bottromycins, for example, are RiPs of *Streptomyces bottropensis* that possess antibacterial ability against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) (Huo et al., 2012). Although the biological activities and natural roles of RiPs are as highly diverse as their biosynthetic pathways, it is a common feature that they are synthesized first as precursor peptides, modified by enzymes, and processed by peptidases (Arnison et al., 2013; Schwarzer et al., 2003). Precursor peptides usually contain an N-terminal leader peptide, a core peptide, and a C-terminal recognition sequence. The leader peptide is important for recognition by post-translational modification enzymes and for export (Oman and van der Donk, 2010; Yang and van der Donk, 2013). The characteristics of the putative precursor peptide of ustiloxin B, UstA, are in complete agreement with other RiPS precursor peptides. Therefore, we conclude that ustiloxin B is synthesized through a RiPS pathway, which is the first characterization of this process in *Ascomycetes*. The genes encoding precursor peptides for α-amanitin and phallacidin, the RiPs of *Amanita* mushrooms, have been reported (Hallen et al., 2007; Luo et al., 2012), but there have

Table 2
Enhanced production of ustiloxin B in the *ustR*^{OE} strain.

Strain	Dry cell weight (g)		Peak area[a]		
	Average[b]	SD[c]	Average[b]	SD[c]	Ratio
Control (*pyrG* revertant)	0.24	0.01	5.9×10^5	9.0×10^4	1
ustR^{OE}	0.24	0.00	2.8×10^6	1.7×10^5	4.8

[a] Evaluated for the MS peaks of *m*/*z* 644.2 at RT 8.6 min.
[b] Averages of three replications.
[c] Standard deviations of three replications.

been no reports of complete RiPS gene clusters in fungi. Therefore, the present work is the first characterization of a complete RiPS gene cluster in fungi.

The precursor peptide UstA is highly unique in its characteristic that its core peptide repeats 16 times; known RiPS precursor peptides contain a single core peptide of the product (Arnison et al., 2013; Condie et al., 2011). There exists a single putative example without evidence of a fivefold repeated core peptide for cyclolinopeptides in *Linum usitatissimum* (Arnison et al., 2013). The *ustA* gene might have acquired its highly repetitive sequence for the core peptide to increase the production of ustiloxin B. Ustiloxin was first found as ustiloxin A in the water extract from false smut balls infected by *Ustilaginoidea virens*; in ustiloxin A, the cyclic amino acid is composed of "YVIG" instead of "YAIG" in ustiloxin B. The absence of an LC–MS peak corresponding to ustiloxin A (m/z = 672.7 $[M-H]^-$) in the water extract from the *A. flavus* culture agrees with the fact that UstA of *A. flavus* does not contain "YVIG" peptides in any of its repeats. Conversely, the precursor peptide for ustiloxins in *U. virens* may include "YVIG" peptide(s) in its repeated core peptide because ustiloxin A was observed in the metabolites of the fungus (Koiso et al., 1992, 1994).

Although there are a few genes whose functions remain unpredicted, we constructed a tentative model for the biosynthetic mechanism of ustiloxin B based on the annotated gene functions (Fig. 6). The contributions of enzymes encoded by the ustiloxin B cluster genes to the moiety of ustiloxin B are indicated in Fig. 6A. Either UstF1 or UstF2 (monooxygenase) must be involved in the hydroxylation of the tetrapeptide, YAIG, and norvaline. UstM (methyltransferase) is thought to be related to the *N*-methylation of the tyrosine residue. UstQ (tyrosinase) must contribute to the oxygen atom crosslinking the aromatic ring of tyrosine and the sidechain of isoleucine, while the oxygen atom can be derived from the monooxygenase, UstF1 or UstF2. The sulfur atom, which links the cyclic peptide intermediate (ustiloxin F) and norvaline, could be added by UstD (cysteine desulfurase) and UstS (glutathione S-transferase). The inclusion of the oxygen atom bound to the sulfur atom might result partially from UstC (cytochrome P450).

Based on the annotation of enzyme functions, we provided a preliminary prediction of the biosynthetic pathway (Fig. 6B). First, UstA must be processed by the subtilisin-like endoprotease Kex2 that is outside the ustiloxin B gene cluster, at the C-terminal side of "KR", after transfer to Golgi apparatus through the endoplasmic reticulum (ER) (Julius et al., 1984; Nielsen et al., 1997). To process the precursor peptide further, at least two peptidases are necessary to cleave the N-terminal and C-terminal sides of "YAIG" core peptide. One of the two peptidases must be UstP (serine peptidase). The other peptidase can be UstH, which is involved in hydrolysis of a tri-peptide, glutathione, as γ-glutamyl transpeptidase in the basic metabolism. γ-Glutamyl transpeptidase is a dual-functional enzyme that can hydrolyze glutathione to glutamate and cysteinyl-glycine and transfer the γ-glutamyl moiety of glutathione to other amino acids (Tate and Meister, 1981; Okada et al., 2006). In ribosomal peptide synthesis, cyclization of the precursor peptide is considered to occur before the N-terminal side of the core peptide, the leader peptide, is cleaved (Oman and van der Donk, 2010). The modifications and cyclization must be carried out by enzymes, including UstM, UstD, UstS, and UstC, to produce ustiloxin B through the cyclic peptide intermediate, ustiloxin F (Koiso et al., 1998) (Fig. 6).

The discovery of many RiPS pathways in bacteria has largely depended on the development of high-throughput genome sequencing technologies during the first decade of the 21st century. For example, after the inclusion of cyanobactins among RiPs was first revealed with the identification of the patellamide biosynthetic gene cluster in *Prochloron didemni*, several other related compounds were identified in *Prochloron* and other species using

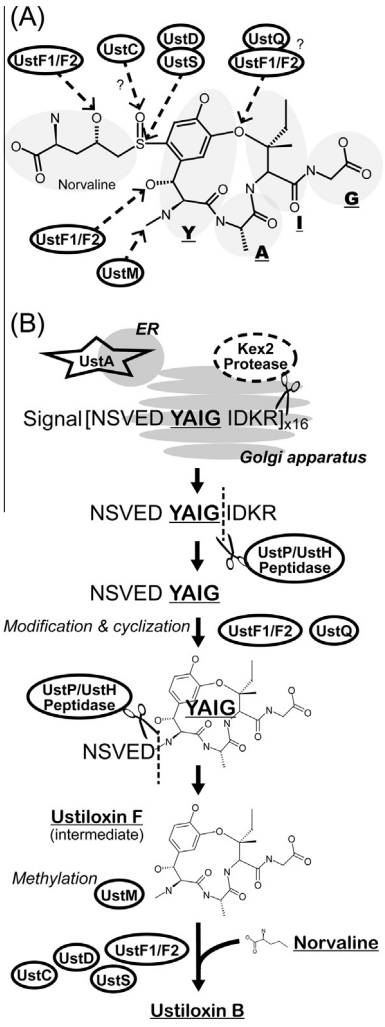

Fig. 6. Putative biosynthetic mechanism of ustiloxin B based on the sequence analyses. UstA is indicated by the star-shaped marker, whereas enzymes involved in the biosynthesis are indicated by ovals. The chemical structures of ustiloxin intermediates were drawn using the ISIS software. (A) Schematic view of contributions to ustiloxin B biosynthesis by enzymes encoded by the genes. (B) Putative biosynthetic pathway of ustiloxin B.

the gene information encoding the precursor peptide (Velasquez and van der Donk, 2011). However, although ~400 genomes of *Ascomycetes*, including filamentous fungi, have been sequenced (http://www.ncbi.nlm.nih.gov/genome/), no RiPS pathways have been reported so far in this division. One possible reason for this is that RiPS genes in *Ascomycetes* may have different characteristics from those in other species. In fact, all genes in the identified ustiloxin B cluster contain many introns (in the NCBI gene modeling), which may suggest that their genetic origin is not bacterial. Another factor is the length of ribosomal peptide sequences. The RiPs of *Amanita* mushrooms, α-amanitin and phallacidin, are composed of eight and seven amino acids, respectively (Hallen et al., 2007), whereas ustiloxin B contains only four amino acids (YAIG) in the ribosomal cyclic moiety. Among more than 50 "YAIG" sequences in the complete peptide sequences of *A. flavus*, it is quite difficult to identify the RiPS precursor gene encoding "YAIG" if it does not repeat. However, if a RiPS precursor peptide contains a highly repeated sequence, we can detect it from genome sequences using an algorithm to detect highly repeated peptide sequences. In fact, we found other examples of genes encoding a repeated peptide with a signal peptide sequence at its N-terminal in *Aspergillus* and *Fusarium* (*e.g.* NCBI FVEG_13634 in *Fusarium verticillioides*).

Our finding that fungal RiPS precursor peptides can contain a highly repeated core of peptides will contribute greatly to the discovery of unidentified RiPS pathways and compounds in *Ascomycetes*, which will be facilitated by advances in genome sequencing technologies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.04.011.

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