Functional Complementation of Fumonisin Biosynthesis in *FUM1*-Disrupted *Fusarium verticillioides* by the AAL-Toxin Polyketide Synthase Gene *ALT1* from *Alternaria alternata* f. sp. *Lycopersici*

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Received January 23, 2008

Fumonisins and AAL-toxins are mycotoxins produced by several widespread fungal pathogens of crops. The carbon backbone of the mycotoxins originates from a highly reduced, acyclic polyketide, a C_{18} chain for fumonisins and a C_{16} chain for AAL-toxins. Fungal reduced polyketides are assembled by iterative modular polyketide synthases (PKS), and their biosynthetic mechanism is not very clear. Here, we cloned the PKS gene, *ALT1*, from the tomato pathogen *Alternaria alternata* f. sp. *Lycopersici* and introduced it into *Fusarium verticillioides* 5777, which does not produce fumonisins due to a disrupted fumonisin PKS gene, *FUM1*. An *ALT1* transformant of strain 5777 produced fumonisin B series as well as fumonisin analogues. The results provide experimental evidence for the function of *ALT1*, which encodes a PKS for mycotoxin biosynthesis. The results also show that the C16-synthesizing *ALT1* is able to support the C_{18} fumonisin biosynthesis in *F. verticillioides*, suggesting that the final size of the fungal reduced polyketides is not determined by the PKSs alone. Unlike other PKSs, these PKSs do not have a thioesterase/cyclase domain. The release of polyketide precursors that are covalently attached to the PKSs involves a distinct mechanism, which probably determines the structure of the final products.

Fungal polyketides are biosynthesized by iterative polyketide synthases (PKS).[1,2] The enzymes contain only a single set of domains that are repetitively used during the polyketide chain elongation. Fungal PKSs are classified into three groups based on the reduction level of their products, nonreducing PKS (NR-PKS), partially reducing PKS (PR-PKS), and highly reducing PKS (HR-PKS).[2,3] NR-PKSs synthesize aromatic polycyclic compounds, such as norsolorinic acid (aflatoxin precursor) and tetrahydroxy naphthalene. PR-PKSs synthesize cyclic compounds such as 6-methylsalicylic acid (6-MSA), which has an aromatic ring but results from a partially reduced intermediate. HR-PKSs synthesize nonaromatic compounds such as fumonisins, AAL-toxins, T-toxins, and lovastatin.

Among the three groups of fungal PKSs, the mechanism for HR-PKSs to control product structure is the least understood.[1,2] The domain architecture of HR-PKSs typically consists of KS-AT-DH-MT-ER-KR-ACP (Figure 1). In addition to the essential domains, KS (β-ketoacylsynthase), AT (acyl transferase), and ACP (acyl carrier protein), it contains a complete set of β-keto processing domains, KR (β-ketoacyl reductase), DH (dehydratase), and ER (enoylreductase), as well as a methyltransferase (MT). Interestingly, HR-PKSs do not have an obvious way of terminating and offloading the nascent polyketide chain, as the synthases do not contain a thioesterase (TE) or Claisen-like cyclase (CLC) domain.[4] Furthermore, fungal highly reduced polyketides, such as fumonisins, AAL-toxins, and T-toxins, have an acyclic polyketide chain. Thus, they cannot be released through intramolecular cyclization as seen in the partially reduced polyketides, such as 6-methylsalicylic acid.

In the group of highly reduced polyketides, fumonisins and AAL-toxins are mycotoxins that are economically important and structurally interesting. Fumonisins are produced by the widespread corn pathogen *Fusarium verticillioides*, are known to cause several fatal diseases in livestock, and are associated with human esophageal cancer and neural tube defects.[5] AAL-toxins are produced by the tomato pathogen *Alternaria alternata* f. sp. *Lycopersici* and are pathogenicity factors essential for the fungi to infect plants.[6] Fumonisins and AAL-toxins are known as sphinganine-analogue mycotoxins (SAMT) due to their structural similarity to sphinganine (dihydrosphingosine, DHS),[7] which is the backbone precursor of sphingolipids. They are competitive inhibitors of sphinganine *N*-acetyltransferase (ceramide synthase).[5,8] The inhibition of ceramide and sphingolipid biosynthesis leads to various diseases in animals and plants.

Both fumonisins and AAL-toxins contain a highly reduced, dimethylated, acyclic polyketide chain (Figure 1). The main differences are the length and the postpolyketide modifications of the backbone. For fumonisins, the 18-carbon backbone (carbon 3−20) is derived from acetate, and C-1 and C-2, as well as the C-2 amino, are derived from alanine.[9,10] The two methyl groups at C-12 and C-16 are derived from methionine.[11] The hydroxyls at C-5, C-10, C-14, and C-15 of FB_1 are derived from molecular oxygen.[12] Therefore, the initially synthesized carbon chain is a highly reduced polyketide. The polyketide-originated backbone (carbons 2−17) of AAL-toxins is two carbon atoms shorter than that of fumonisins, and the C-1 and amino group are derived from glycine. The fumonisin PKS (Fum1p) has been the subject of several studies,[13–17] while none of the AAL-toxin biosynthetic genes have been studied for their function. Our earlier studies have shown that both the KS domain and chain-releasing enzyme are important to the production of a distinct group of products.[15,17] In this study, we showed that the AAL-toxin PKS gene (*ALT1*) is able to functionally complement fumonisin biosynthesis in a *F. verticillioides* mutant with a disrupted fumonisin PKS gene (*FUM1*). The results provide new evidence for the mechanism of HR-PKS-catalyzed fungal polyketide biosynthesis.

Results and Discussion

FUM1 is a 8163 bp gene encoding fumonisin PKS (Fum1p) in *F. verticillioides*.[13] Strain 5777 was originally isolated as a naturally occurring fumonisin-nonproducing strain of *F. verticillioides*.[18] It was recently shown that this strain carries 20 nucleotide mutations in *FUM1*, including a G5415A mutation that introduces a premature stop codon and disrupts the translation of Fum1p.[19] In the AAL-toxin-producing *A. alternata*, an 8286 bp open reading frame was predicted to encode a putative PKS gene, *ALT1* (personal communication with Dr. Motoichiro Kodama at Tottori University, Japan). We first amplified a 9130 bp fragment by multistep PCR

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10.1021/np8000514 CCC: $40.75
Published on Web 04/25/2008

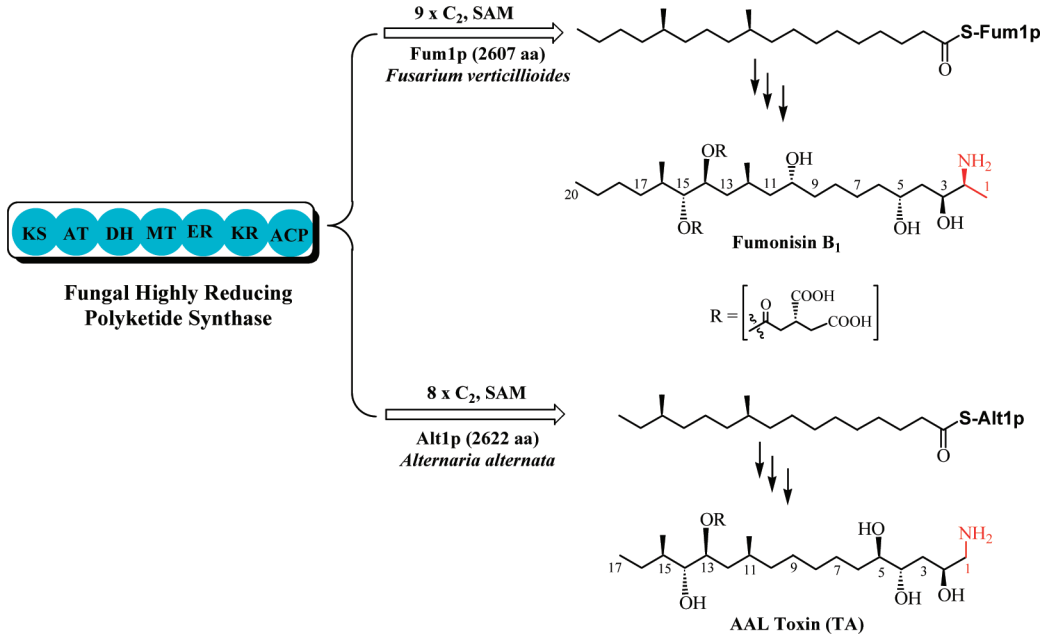

Figure 1. Domain organization of fungal highly reducing polyketide synthases, fumonisin Fum1p from *Fusarium verticillioides* and AAL-toxin Alt1p from *Alternaria alternata*. The structure of fumonisin B_1 and AAL-toxin TA, which are the most common sphinganine-analogue mycotoxins in the fungi, is shown. KS, β-ketoacylsynthase; AT, acyl transferase; DH, dehydratase; MT, methyltransferase; ER, enoylreductase; KR, β-ketoacyl reductase; ACP, acyl carrier protein; SAM, *S*-adenosyl methionine.

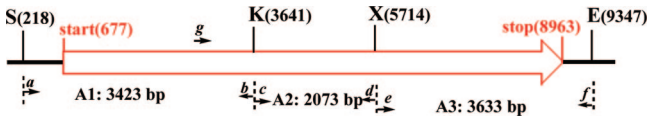

Figure 2. Strategy to amplify the open reading frame of *ALT1* and its 5′ and 3′ flanking regions using PCR. The small arrows indicate the locations of the primers, with *a* through *f* referring to primers ALT-1-F through ALT-6-R, respectively, and *g* referring to primer SEQ-2-F in Table 1. The relevant restriction enzyme sites are indicated, S for *Spe*I, K for *Kpn*I, X for *Xba*I, and E for *Eco*RI. The numbers following the restriction enzyme sites indicate the relative positions of the sites within the DNA fragment sequenced by the Kodama laboratory. The start site and the stop site of *ALT1* are also indicated. Three PCR fragments, A1, A2, and A3, were amplified by the respective primer pairs as indicated in the scheme and eventually assembled into vector pUCH2-8 to produce the construct pUCH-*ALT1* for fungal transformation.

from the genome of *A. alternata*. This fragment contains the entire coding region of *ALT1*, as well as a 459 bp 5′-noncoding region and a 384 bp 3′-noncoding region (the putative promoter and terminator) (Figure 2).

The fragment was cloned into pUCH2-8[13] to form plasmid pUCH-*ALT1*, which was transformed into the protoplasts of *F. verticillioides* wild type and strain 5777. The single colonies that appeared on hygromycin B-containing plates were the putative transformants and subjected to PCR verification. To verify *ALT1* integration into *F. verticillioides* genome, primers SEQ-2-F/ALT-2-R (Table 1 in the Supporting Information), which are located inside the *ALT1* coding region, were used to amplify a specific 873 bp product (Figure 1 in the Supporting Information). Fifty of strain 5777-derived colonies were checked by PCR, and 15 of them were shown to contain *ALT1*. Forty-eight of the wild-type-derived colonies were checked, and 18 of them contained *ALT1*. The results suggest that the integrated plasmid in many of the hygromycin-resistant colonies had probably rearranged, which is common in this filamentous fungus.[13,15,17,19]

The metabolites produced by the transformants as well as the wild type and strain 5777 of *F. verticillioides* were analyzed by HPLC-ELSD and ESIMS. The wild-type strain produced the B series fumonisins (Figure 3A). The introduction of *ALT1* into the wild type did not alter the level of fumonisin production in most transformants, but a few transformants produced a lower level or no fumonisins (data not shown). The reduced fumonisin production in these wild-type transformants suggests that *ALT1* probably inserted into a site important to fumonisin biosynthesis or regulation. In all transformants, no new metabolites were observed.

Strain 5777 did not produce any fumonisins (Figure 3B). The major product of the strain appeared to be a pigment. The pigment was always present in the transformants derived from strain 5777. The transformants containing *ALT1* were screened for new metabolites using HPLC-ELSD and ESIMS. The majority of transformants had a metabolite profile similar to the parent strain 5777 (shown as example, 5777-*ALT1*-5, in Figure 3D). The results show that the integration of this heterologous PKS gene into *F. verticillioides* genome has little effect on the metabolite production. However, one of the transformants, 5777-*ALT1*-4, produced a group of compounds that closely migrated with each other and with fumonisins on HPLC (Figure 3C). The yield of the compounds was approximately 20% of the fumonisins produced by the wild type. ESIMS and HRFABMS analyses of these compounds gave two similar series of $[M + H]^+$ signals. In addition to the typical $[M + H]^+$ signals for fumonisins, 722.4 (FB_1), 706.4 (FB_2/FB_3), 690.4 (FB_4), a new series of $[M + H]^+$ signals, including 720.4, 704.4, and 688.4 *m/z*, was also observed (Figure 2 in the Supporting Information). The latter are two mass units lower than that of the corresponding fumonisins, which are most likely the 3-keto fumonisins that have been reported previously.[20–22] The results show that *ALT1* can *in trans* complement the fumonisin production in the *FUM1*-disrupted mutant. The results also show that the heterologous gene was expressed in strain 5777-*ALT1*-4 with properly spliced introns and that the produced enzyme was able to interact with all the downstream enzymes in the fumonisin biosynthetic pathway. The coproduction of 3-keto analogues of fumonisins suggests that the heterologous PKS probably could not interact fully with the 3-ketoreductase (Fum3p) in the fumonisin pathway.[20,21]

Fumonisin biosynthetic genes, including *FUM1*, have been the subject of extensive investigations in several laboratories.[13–15,17,19–28]

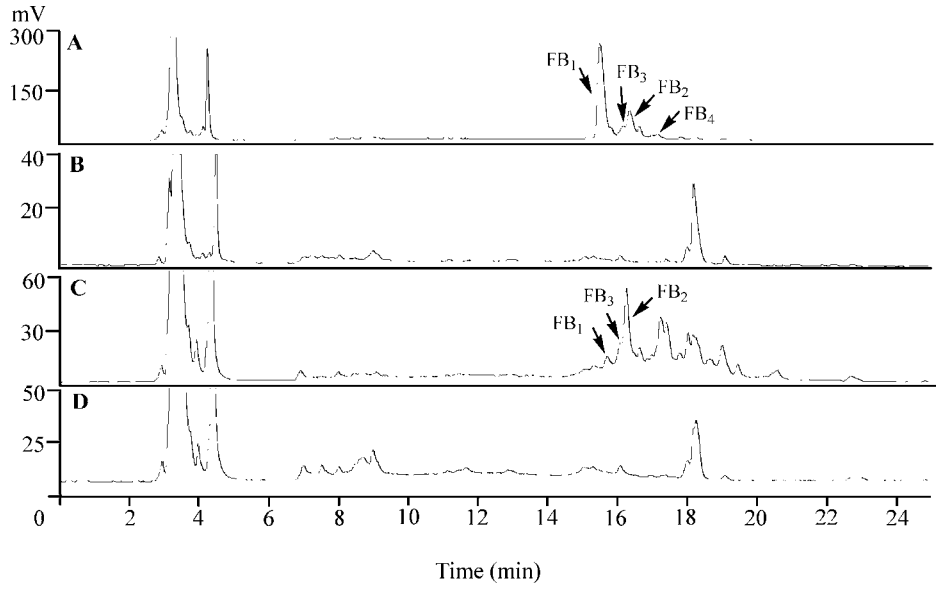

Figure 3. HPLC-ELSD analysis of metabolites produced by the wild-type (A), strain 5777 (B), *ALT1* transformant 5777-*ALT1*-4 (C), and *ALT1* transformant 5777-*ALT1*-5 (D). The peaks for fumonisins are indicated with arrows.

In contrast, although several biosynthetic genes for AAL-toxins have been sequenced (personal communication with M. Kodama), none of the genes have been studied for their function. Our results provide experimental evidence for the function of *ALT1*, which is a mycotoxin-synthesizing PKS gene.

Fum1p synthesizes an 18-carbon chain in *F. verticillioides*, while Alt1p makes a 16-carbon chain in *A. alternata*. The two synthases have identical domain organization, KS-AT-DH-MT-ER-KR-ACP, and share a similarity/identity of 72%/60% for the entire amino acid sequences. Our results showed that Alt1p can support the biosynthesis of the 18-carbon-derived fumonisins in *F. verticillioides*, demonstrating that Alt1p is able to make a carbon chain longer than it normally makes. This is the first experimental evidence for the catalytic flexibility of the fungal PKS. It is probable that the fungal PKS could synthesize a series of polyketide intermediates on the single-module enzyme template, but only certain intermediates with a specific size could be released by the chain-releasing enzyme (Fum8p for fumonisins).[29] Thus, the final size of products would ultimately be determined by the specificity of the chain-releasing enzyme. This is consistent with the domain organization of the PKSs. Distinct from bacterial PKS and fungal NR-PKS, Fum1p and Alt1p do not contain a thioesterase (TE) domain for releasing bacterial reduced polyketides[1] or a Claisen-like cyclase (CLC) domain for releasing fungal nonreduced, aromatic polyketides.[4] Furthermore, since the products are acyclic compounds, the polyketide-releasing through simultaneous cyclization, as seen in fungal PR-PKS,[2] cannot be involved.

In both *FUM* and *ALT* gene clusters, there is a gene (*FUM8* or *ALT4*) encoding an α-oxoamine synthase type of enzyme (personal communication with Kodama).[23] This type of enzyme is known to catalyze the decarboxylative condensation between an amino acid and an acyl chain.[30] In the biosynthesis of fumonisins the linear 18-carbon chain is released by condensing with the α-carbon of L-alanine, whereas in AAL-toxins the linear 16-carbon is released by condensing with the α-carbon of glycine (Figure 1). Thus, the chain-releasing mechanism in SAMT biosynthesis involves new carbon−carbon bond formation and the introduction of a new functional group (amino). This is distinct from any known polyketide releasing mechanism and could provide a new way to generating fungal polyketide analogues by metabolic engineering.

Experimental Section

Strains, Plasmids, Reagents, and Media. *F. verticillioides* A0149 (FGSC number 7600) was used as the wild-type strain. The *FUM1* mutant strain 5777[18] and plasmid pUCH2-8[13] that contains hygromycin B resistance gene were obtained from Dr. Robert Proctor (U.S. Department of Agriculture, Peoria, IL). *Escherichia coli* strain DH5-α was used as the host for general recombinant DNA preparation. Plasmid pMECA and pGEM-Zf series (Promega, Madison, WI) were used for DNA cloning and sequencing. Plasmid preparation and DNA extraction were carried out with Qiagen kits (Valencia, CA), and other general molecular biology manipulations were executed according to the standard protocols.

All oligonucleotide primers for PCR were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Taq DNA polymerase and T4 DNA ligase were purchased from Promega (Madison, WI); Deep Vent DNA polymerase and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Hygromycin B was purchased from Calbiochem (La Jolla, CA). Other chemicals were purchased from either Fisher Scientific (Springfield, NJ) or Sigma-Aldrich (St. Louis, MO).

Luria−Bertani (LB) medium was used for general *E. coli* cell propagation. For *F. verticillioides*, V8 medium was used for conidiospore production and YPD medium was used for mycelial growth.[13] GYAM medium and CMK medium were used for fungal metabolite production.[13]

Construction of Plasmid pUCH-*ALT1*. The DNA sequence information for *ALT1* from *A. alternata* was kindly provided by M. Kodama at Tottori University, Japan. On the basis of this information, we designed a series of primers to amplify a 9130 bp fragment from the *A. alternata* f. sp. *Lycopersici* genomic DNA. This fragment includes the *ALT1* coding region (starting at no. 677 and ending at no. 8963) and the flanking 5′- and 3′-noncoding regions. Because of the large size, the complete 9130 bp fragment was assembled from three smaller fragments that were separately amplified by PCR and confirmed by DNA sequencing (see the Supporting Information).

Transformation of *F. verticillioides* and Screening of Mutants. The protocols used to produce and transform protoplasts were essentially identical to those described previously.[14] For protoplast transformation, 5 μg of plasmid DNA was transformed into about 1×10^7 protoplasts and plated on regeneration medium plate containing 150 μg/mL hygromycin B. After approximately 5−7 days of incubation at room temperature, all hygromycin B-resistant colonies that appeared on the plates were transferred into 2 mL of YPD liquid medium supplemented with 300 μg/mL hygromycin B for further selection. The surviving colonies were putative transformants and subjected to verification by PCR. For PCR analysis, the genomic DNA of transformants was prepared following a fungal DNA quick preparation protocol. Briefly, the fungal mycelia were collected from 1 mL of 4-day YPD culture; then a 400 μL extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 100 mg of sterile sea sand (Mallinckrodt, Paris, KY) were mixed with the mycelia. After incuba-

tion at 65 °C for 20 min, the mixture was agitated on a Fisher Vortex (Genie 2) at maximal speed for 10 min to break the cells. The extract was centrifuged at 13 200 rpm for 10 min on an Eppendorf desktop centrifuge, and the supernatant was mixed with the same volume of cold *i*-PrOH to precipitate DNA. The DNA pellet was washed with 1 mL of 70% EtOH twice and dissolved in 50 μL of H_2O. For PCR, 4 μL of the DNA solution was used as template.

Analysis of Metabolites. For metabolite production, various strains of *F. verticillioides* were cultured on CMK medium as described previously.[14,22] Briefly, 1 mL of overnight liquid YPD culture derived from single conidia was inoculated into CMK medium (25 g) and allowed to grow at room temperature in the dark for 4 weeks. The CMK cultures were extracted with 50 mL of H_2O/CH_3CN (1:1, v/v). The extracts were filtered and subjected to analysis using a high-performance liquid chromatography (HPLC) system (Prostar-210, Varian, Walnut Creek, CA) coupled to an evaporative laser scattering detector (ELSD2000, Alltech, Deerfield, IL). The column was Alltima C18LL (5 μm, 250 × 4.6 mm i.d., Alltech, Deerfield, IL), and the experimental conditions and program were the same as described previously.[14,22] The metabolites separated by HPLC were collected and further analyzed by ESIMS and high-resolution fast atom bombardment mass spectrum (HRFABMS, Fisons Autospec Q, Manchester, England) at the Nebraska Center for Mass Spectrometry (UNL, Lincoln, NE).

Acknowledgment. We thank R. Proctor at U.S. Department of Agriculture, Peoria, IL, for providing *FUM1* mutant strain 5777, M. Kodama at Tottori University, Japan, for providing the DNA sequence information for *ALT1*, and R. Cerny and K. Wulser at the Nebraska Center for Mass Spectrometry, University of Nebraska−Lincoln, for assistance in MS analyses. This work was supported in part by NSF (MCB-0614916) and NSF China (No. 30428023). The research was performed in facilities renovated with support from NIH (RR015468-01). C.V. was supported by the UCARE program at University of Nebraska−Lincoln.

Supporting Information Available: The construction of plasmid pUCH-*ALT1*, PCR primer sequences and screening of the fungal transformants, and MS data for fumonisins and analogues produced by the transformants. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP8000514