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Developing a genetic system for functional manipulations of *FUM1*, a polyketide synthase gene for the biosynthesis of fumonisins in *Fusarium verticillioides*

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Abstract: Fumonisins are mycotoxins produced by *Fusarium verticillioides*, a filamentous fungus that is a widespread pathogen of corn. The biosynthesis of fumonisins is catalyzed by an iterative modular polyketide synthase (PKS). The study of the biosynthetic mechanism for these reduced fungal polyketides has been challenging due to the difficulties in detecting the intermediates with a linear carbon chain and manipulating the 7-domain PKS gene from the filamentous fungus. Here, we described the development of a genetic system for functionally manipulating the methyltransferase domain of *FUM1* that is responsible for the assembly of a dimethylated 18-carbon chain. Using a two-stage screening strategy, including both positive and negative screenings, we were able to generate mutant strains with a specifically changed active-site in *FUM1*. LC-MS analyses indicated that biosynthetic intermediates were detectable in the early stage of culture. The results represent the first functional manipulation of the PKS involved in the biosynthesis of fumonisins.

Keywords: Fumonisin, Polyketide synthase, Mycotoxin, Fusarium verticillioides

1 Introduction

Filamentous fungi are rich sources for polyketide metabolites, some of which have important biological activities [1]. Among them, fumonisins are a group of mycotoxins produced by *Fusarium verticillioides*, which cause several fatal animal diseases, including leukoencephalomalacia in horses [2, 3], pulmonary edema in swine [4], and cancer in rats [5]. In addition, fumonisins are associated with human esophageal cancer [6, 7].

Polyketides are biosynthesized through sequential decarboxylative condensations of short carboxylic acids, which is catalyzed by polyketide synthases (PKSs) [8]. Recently, several genomes of filamentous fungi have been sequenced. The sequencing data revealed a large number of putative PKS genes existing in filamentous fungi [9]. These fungal PKS genes outnumber those found in the genomes of *Streptomyces avermitilis*[10] and *Streptomyces coelicolor*[11], actinomycete species that are traditionally regarded as the most abundant source for polyketide metabolites. Fungal PKSs are clas-

sified into three groups: (i) PKSs for single aromatic ring; (ii) PKSs for polycyclic aromatic ring; and (iii) PKSs for reduced complex-polyketides [12, 13]. They are defined as iterative type I PKSs because they usually contain only a single set of domains, in contrast to the non-iterative modular type I PKSs and monofunctional type II PKSs found in bacteria [1]. Typically, a module consists of a β-ketoacyl synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP). Many PKSs, especially those for reduced polyketides, also contain additional domains, such as β-ketoacyl reductase (KR), dehydratase (DH), enoylreductase (ER), and methyltransferase (MT) (Fig. 1). A single-modular PKS can catalyze multiple cycles of chain elongation [1, 14].

Although information for the biosynthetic mechanism for aromatic fungal polyketides has begun to emerge in the recent years [15–22], the mechanism for the reduced polyketides remains unknown. Two major difficulties are associated with the studies of the reduced fungal polyketides. One is due to the structural feature of the metabolites, which often have a linear carbon backbone resembling fatty acids. This feature not only makes their detection difficult but also often leads to no intermediates accumulated in the gene-disruption mutants because the putative precursors could be utilized by other pathways. Another difficulty is related to the genetic manipulation of the multi-domain genes in filamentous fungi. The PKS genes responsible for the biosynthesis of reduced fungal polyketides are more complex than those for aromatic polyketides. For example, *FUM1* responsible for assembling the 18-carbon chain of fumonisins in *F. verticillioides* was predicted to code seven domains, KS–AT–DH–MT–ER–KR–ACP (Fig. 1) [23]. The gene-disruption approach, most commonly through inserting a selection marker, such as hygromycin [23, 24] or a phleomycin [25] resistance gene into the target gene, has been performed to study this group of PKSs. However, this type of manipulations could only provide evidence for the relatedness of the PKS gene to the biosynthesis of the metabolite. In this study, our goal was to develop a genetic system for functionally manipulating the individual domains of the fungal PKS for reduced polyketides. Reported here are results obtained from an active-site point mutation of the methyltransferase domain of *FUM1*.

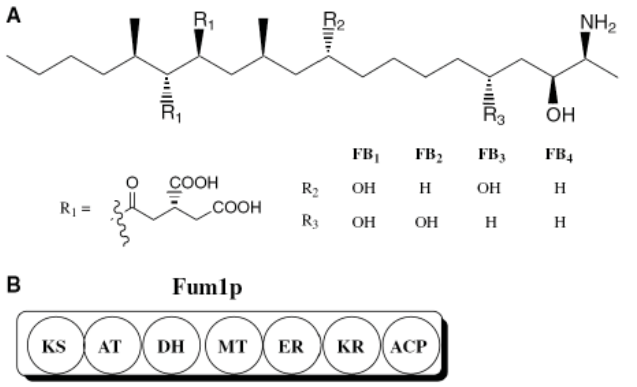

Fig. 1. Chemical structure of B-series fumonisins (A) and domain organization of fumonisin Fum1p, the polyketide synthase responsible for the assembly of the carbon backbone of fumonisins (B). *Abbreviations*: β-ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), methyltransferase (MT), enoylreductase (ER), β-ketoacyl reductase (KR), and acyl carrier protein (ACP).

2 Materials and methods

2.1 Strains and culture media

F. verticillioides wild-type strain A0149 (FGSC number 7600) was provided by Dr. David Gilchrist (University of California, Davis). YPD medium and V-8 juice agar were used to produce mycelia and conidia [23], and cracked maize kernels (CMK) for fumonisin production [23, 26].

2.2 DNA manipulation and construction of plasmids

Escherichia coli DH5-α strain was used as the host for general DNA preparation, and the pGEM-zf series from Promega (Madison, WI) for cloning and DNA sequencing. Plasmid preparation and DNA extraction were carried out with Qiagen kits (Valencia, CA), and all other manipulations were carried out according to standard methods [27]. To introduce site directed mutagenesis at the *S*-adenosylmethionine (SAM) binding site of the *FUM1* MT domain, pUCHΔmt was constructed by means of SOE (splicing by overlap extension) [28], involving three PCR amplifications. The first PCR was

Table 1
The sequence of primers used in this study

Primer	Sequence
MT1	5′-CTGGACTCGGCTTGGTGGGAATTCTCCATC-3′
MT2	5′-TCCGCCGCCAAGCTTAGCACCAATCTCAAGAAC-3′
MT3	5′-GGTGCTAAGCTTGGCGGCGGAGCTCAAGTTATC-3′
MT4	5′-AGATACGGGGTGGTACCTAGCAACCTTGAC-3′
LMT1	5′-GAAAAGTGTTGTCGTCCAGTC-3′
LMT2	5′-TCAAAGGAACCTGACTCAAAG-3′
RMT1	5′-ACCAACACATCCCTAATTTCC-3′
RMT2	5′-GATTGTGACCTCTCCGTAGC-3′

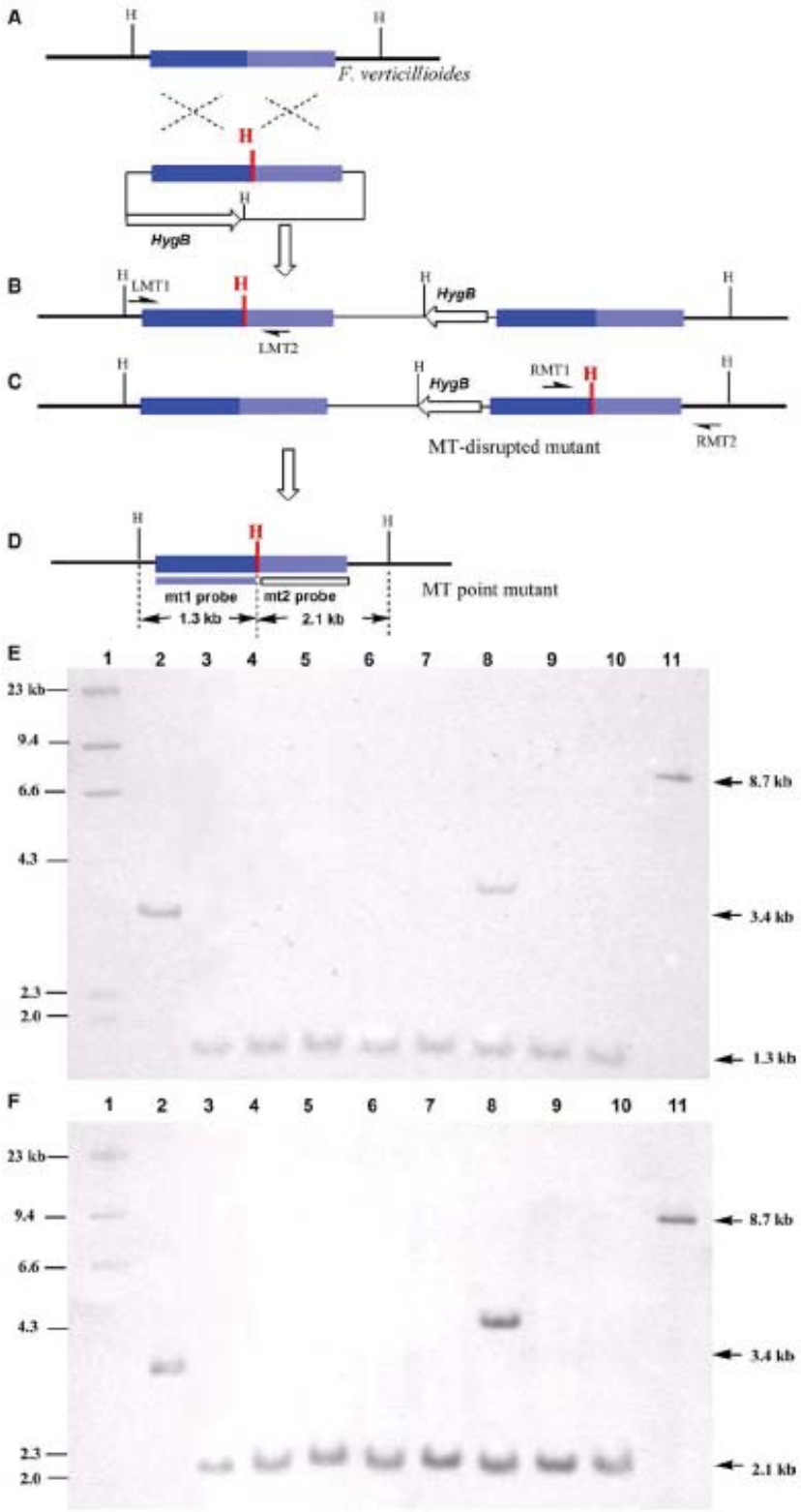

Fig. 2. Screening and confirmation of *F. verticillioides* mutants. (A) Homologous recombination between *FUM1* of *F. verticillioides* and plasmid pUCHΔmt. *Abbreviations*: H: *Hin*dIII; *HygB*: hygromycin B resistant gene. (B) and (C) Gene disruption mutants resulted from a single crossover at the upstream homologous region (B) or at the downstream homologous region (C). The positions of the PCR primers used to screen the homologous recombination are indicated with half-arrows. (D) MT domain mutants resulted from a second crossover at the homologous regions. (E) and (F) Confirmation of the mutants by Southern hybridization with probe mt1 (E) and mt2 (F). The positions of the probes are indicated by bars in (D). The genomic DNA was digested with *Hin*dIII and hybridized with a DIG-labeled probe. The size of hybridizing signals is also indicated in (D). Lane 1, size markers; lane 2, the wild type; lanes 3–10, mutants; lane 11, pUCHΔmt (Digested by *Kpn*I). Note that there are two integrations, one homologous and the other random, in lane 8.

carried out with Cos6B as template [23], using primers MT1 and MT2 (Table 1). The 1187-bp product (mt1) contained a point mutation introduced by the MT2 primer, in which the original sequence, 5'-GGCACC-3', in the SAM binding motif of the MT domain was changed to 5'-AAGCTT-3' (Gly^{1470}–Thr^{1471} of Fum1p changed to Lys^{1470}–Leu^{1471}). This change also created a *Hin* dIII site to facilitate the mutant identification in the later stage. The second PCR was similarly carried out using primers MT3 and MT4 to produce a 1185-bp product (mt2). The third PCR used the mixture of mt1 and mt2 as templates and MT1 and MT4 as primers to get the final 2352-bp product. The fragment was digested with *Eco* RI/*Kpn* I and cloned into the same sites of pT7Blue–3 (Novagen) for sequencing and further into *Sal* I/*Kpn* I sites of pUCH2-8 [23] to yield pUCHΔmt. This construct contains an *E. coli* hygromycin B resistance gene (*Hyg* B) fused to the Promoter 1 from *C. heterostrophus*[29] (Fig. 2).

2.3 Transformation of F. verticillioides

The protocol used to isolate and transform protoplasts was similar to that described by Salch and Beremand [30] with the following modifications. Conidia were germinated in flask containing 30 ml liquid YPD medium on a 120 rpm rotary shaker at 30°C for approximately 7–8 h. The conidia were collected and digested at 30°C for 30–60 min in 20 ml of 0.7 M NaCl containing 5 mg/ml Novozyme (Sigma), 25 mg/ml driselase (Sigma), and 0.5 mg/ml chitinase (Sigma). For transformation, 5 μg of plasmid DNA was diluted with an equal volume of 2× STC buffer to 100 μl and mixed with 100 μl protoplasts, and transformation was mediated with PEG 8000 buffer (30% PEG 8000; 10 mM Tris–HCl, pH 8.0; 50 mM $CaCl_2$). The protoplast–DNA mixture was added to 4.5 ml molten regeneration medium and dispensed onto the surface of 20 ml solidified regeneration medium. After an overnight incubation, this regeneration medium was overlaid with 10 ml of 1% water agar supplemented with 525 μg/ml hygromycin B (Calbiochem, La Jolla, CA), making the final concentration of hygromycin B to 150 μg/ml. Hygromycin-resistant colonies appeared on the plates in approximately 7 days of incubation at 28°C.

2.4 Screening of F. verticillioides mutants

The screening of mutants involves two stages. The first stage is to select for gene disruption mutants resulted from a single crossover of the homologous regions. The hygromycin-resistant colonies on YPD plates were transferred to a 2 ml YPD liquid medium supplemented with 300 μg/ml of hygromycin B for further selection. Single conidia were isolated from the surviving colonies, and the culture grown from the conidia were subjected to PCR and Southern analysis to identify the gene disruption mutants. In the second stage, the mutants were selected for the loss of hygromycin B resistance. Single conidia-derived cultures were allowed to grow in 250-ml Erlenmeyer flasks containing 50 ml liquid YPD medium without hygromycin B. After five-day growth, a 50-μl of the culture was transferred to a 50-ml fresh liquid YPD medium. The transfer process was repeated every five days. After 4–5 serial transfers, the culture was serial-diluted and spread on YPD plate to produce single colonies. The individual single colonies were then replica-plated on two YPD plates, one with hygromycin B and one without. After two-day growth, colonies that did not grow on the plate with hygromycin B but grew on the plate without hygromycin B were selected as putative gene replacement mutants.

2.5 Southern hybridization

Genomic DNA of *F. verticillioides* was prepared as described previously [31]. Immobilon^{TM}–NY^{+} Transfer Membrane (Millipore, Bedford, MA) was used for blot analysis. Southern hybridization was performed using DIG DNA Labeling and Detection Kit (Roche, Indianapolis, IN) according to the manufacturer's manual. Ten micrograms of genomic DNA was used in the hybridizations. Two 1.2 kb probes (mt1 and mt2, Fig. 2) were amplified by PCR using primers MT1-MT2 and MT3-MT4 (Table 1), respectively, with Cos6B as template.

2.6 Co-culture of mutants and analysis of metabolites

The procedure for co-culturing the MT mutant with *FUM6* mutant is identical to that described previously [32]. *FUM6* is a *F. verticillioides* mutant generated by replacing part (1.5 kb) of the coding region of *FUM6* gene with the hygromycin resistance gene (*Hyg* B) [33]. For metabolite production and analysis, the single-conidia derived strains were inoculated in 25 g CMK medium and allowed to grow at 25°C in dark for 3–4 weeks as described previously [32]. The CMK cultures were extracted with water/acetonitrile (1:1, v:v). The extracts were filtered and subjected to HPLC-ELSD (high performance liquid chromatography-evaporative light scattering detection) or LC-ESMS (liquid chromatography-electrospray mass spectrometry) analysis using the same procedures described previously [32].

3 Results and discussion

3.1 Development of the screening procedure for mutants

Our goal was to specifically change the active site of a PKS domain so that a fungal strain with a non-func-

tional PKS domain would be generated. In order to reach this goal, the first thing was to develop a relatively efficient and reliable screening procedure suitable for this filamentous fungus. Both positive (the first stage) and negative (the second stage) screenings are required in order to identify the mutant. Therefore, the one-stage protocol developed previously [23] to screen resistant colonies will not be appropriate for our purpose. In the first stage screening, approximately 3–7 hygromycin-resistant colonies were obtained in a typical transformation using 5 μg of plasmid DNA and 10^7 protoplasts. The colonies resulted from plasmid DNA integration into the fungal genome. PCR was used to identify those resulted from a homologous recombination. The primers LMT1–LMT2 and RMT1–RMT2 (Table 1) were used to detect the mutants with a crossover at the upstream (Fig. 2B) and the downstream (Fig. 2C), respectively, homologous regions. The engineered *Hin* dIII site in the mutated MT domain was used to distinguish the mutants from the wild-type. Using this selection approach, we obtained four mutants, mt-7, mt-21, mt-42, and mt-52, from 70 hygromycin-resistant colonies. Mutant mt-42 gave a 1, 473-bp PCR product when the first pair of primers was used (data not shown). The product was digested by *Hin* dIII to give two fragments of 204 and 1269-bp, indicating a homologous recombinant resulting from a crossover at the upstream homologous region (Fig. 2B). In contrast, the PCR products from the wild type or those resulted from random insertions were not digestible by the enzyme although the products were the same size (data not shown). Mutant mt-7, mt-21, and mt-52 gave a 1, 689-bp PCR product when the second pair of primers was used. A digestion of the product by *Hin* dIII gave two DNA fragments with the expected size of 376 and 1, 313-bp (data not shown), indicating a crossover at the downstream homologous region. After the single crossover mutants were confirmed, they were grown on YPD medium without hygromycin B to select for a second homologous recombination, which will generate either the MT point mutant or reversed mutant (the wild type). Among 1, 880 single colonies that were derived from four gene-disrupted mutants, 32 lost hygromycin B resistance. Among them, 14 were identified as MT point mutants by PCR and Southern analysis, and the rest were reversed mutants. Two representative Southern hybridization results are shown in Fig. 2E and F. When the genomic DNA of the mutants was digested with *Hin* dIII and hybridized with mt1 and mt2 probe, the MT point mutants exhibited 1.3 and 2.1 kb signals, respectively, while the wild type gave a 3.4 kb signal when hybridized with either of the probes (Fig. 2). Thus, the mutants contain a point mutation at the active site of the MT domain of *FUM1* in *F. verticillioides*.

3.2 Metabolite analysis in MT mutants

The fourteen MT point mutants and the 4 gene disruption mutants (mt-7, mt-21, mt-42, and mt-52) were inoculated in CMK medium for 3 weeks for the metabolite analysis. The wild-type strain produced a characteristic pattern of fumonisins. FB_1 was the predominant metabolite with a $[M + H]^+$ of *m/z* 722.8 (Fig. 3A and B), whereas FB_3 and FB_2 were two minor peaks with the same $[M + H]^+$ of *m/z*

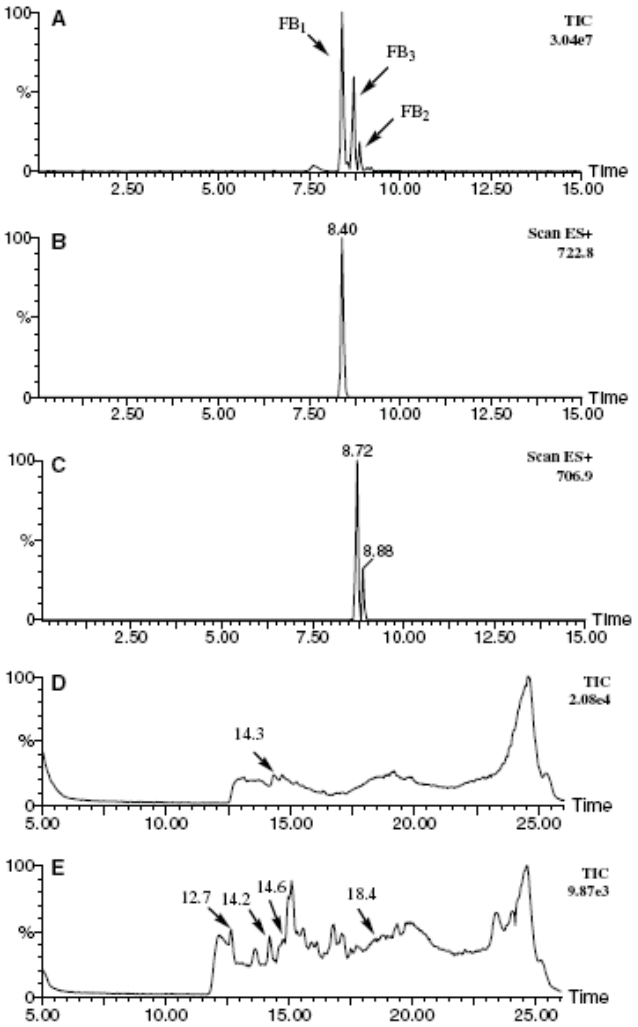

Fig. 3. LC-MS analysis of metabolites isolated from the wild type strain (A-C) and the co-cultured mutants. (A) Total ion current (TIC) of metabolites from a 3-week old culture of the wild type strain. The B-series fumonisins, FB_1, FB_3, and FB_2, are indicated with arrows. (B) Mass scan of the TIC for FB_1 in (A). (C) Mass scan of the TIC for FB_3 and FB_2 in (A). (D) Total ion current (TIC) of metabolites from a 3-day old *FUM6*-deleted mutant. (E) TIC of metabolites from a 5-day old co-culture of the MT domain mutant and *FUM6*-deleted mutant. The four peaks related to biosynthetic intermediates are indicated with arrows. (F) Mass scan of the TIC for a demethylated 3-keto intermediate in (E). (G) Mass scan of the TIC for a normal 3-keto intermediate in (E). (H) Mass scan of the TIC for a normal tetrahydroxy-intermediate in (E). (I) Mass scan of the TIC for a demethylated dihydroxy-intermediate in (E).

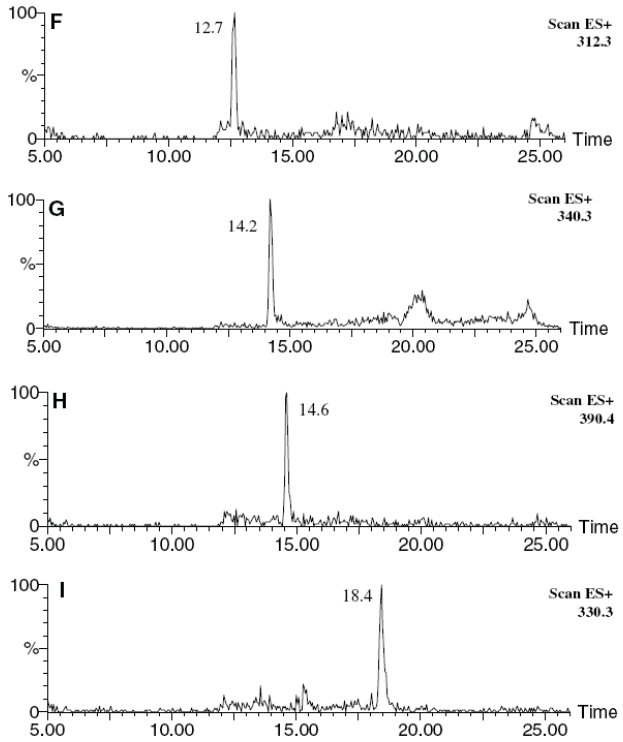

Fig. 3 (*continued*)

706.9 (Fig. 3A and C). As expected, the 4 gene disruption mutants did not produce any fumonisins (data not shown). In the point mutants, no fumonisin was detected, whereas approximately 4 unknown peaks appeared on LC-MS. One of them gave a $[M + H]^+$ of *m/z* 679.8, which was most close to that of demethylated FB_3 or FB_2. However, extensive MS–MS analyses showed that none of the compounds had the linear carbon skeleton of fumonisins. Therefore, no detectable amount of fumonisins or the demethylated analogs was produced in the point mutants. Two possibilities exist for the result. One is that the point mutation at the MT active site resulted in an inactive Fum1p. Another possibility is that the putative demethylated intermediate (stearyl) that was assembled by the point-mutated Fum1p was not recognized by downstream enzymes, such as Fum8p and Fum6p, in the pathway (Fig. 4). Alternatively, the C_{18}-intermediate could be a substrate for fatty acid metabolic pathways, such as the β-oxidation. Intermediates derailed into other pathways are not uncommon in fungal polyketide biosynthesis [34]. This is possible especially when the mutants were allowed to grow a prolonged period of time. In order to test if the point-mutated Fum1p was active, we adopted the co-culture approach. This approach enables two different *FUM* mutant strains to functionally complement each other, so that the final metabolites, which are much more stable and easier for detection, could be produced [32]. When the MT point mutant of *FUM1* was co-cultured with the *FUM6*-deleted mutant [33], FB_1 was clearly produced in the culture (data not shown). The fact that the fumonisin production was restored in the co-cultures indicated that downstream enzymes were functional in the MT point mutant because it was able to convert the intermediates produced by the *FUM6* mutant to the final product. As reported previously [32, 33], the culture of the *FUM6* mutant alone did not accumulate any detectable intermediates when it was allowed to grow for 2–4 weeks. Several small peaks were observed on LC-MS from the culture of the *FUM6* mutant that was 3–7 days old (Fig. 3D). These peaks eventually disappeared if the culture was allowed to grow longer, as observed previously [32].

To gain more information, we analyzed the metabolites in the co-cultures 3–7 days after inoculation. Our previous experience with these mutants showed that intermediates were accumulated only at the early stage of the cultures [32]. Similar to those observed when the deletion mutants of *FUM1* and *FUM6* were co-cultured [32], the 3 to 7-day old co-cultures of MT mutant and *FUM6* mutant produced the normal (methylated) intermediates. For example, the peak with a $[M + H]^+$ of *m/z* 340.3 was predicted for the 3-keto intermediate (Figs. 3E and G and 4), whereas the peak with a $[M + H]^+$ of *m/z* 390.4 for a tetrahydroxylated intermediate (Fig. 3E and H) [32]. In addition to these previously observed peaks, the co-cultures also contained several peaks with mass corresponding to demethylated intermediates. For example, the peak with a $[M + H]^+$ of *m/z* 312.3

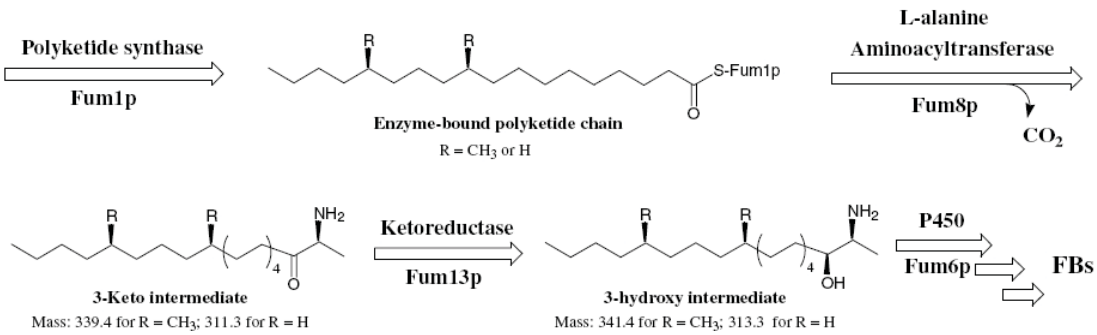

Fig. 4. The early steps of the fumonisin biosynthetic pathway. The mass of relevant intermediates discussed in the text is included in the figure.

is corresponding to the demethylated 3-keto intermediate (Figs. 3E and F and 4), and the peak with a $[M + H]^+$ of *m/z* 330.3 to the demethylated dihydroxy intermediate (Fig. 3E and I). The yield of these intermediates in the mutants was extremely low and eventually disappeared in the older cultures. Nevertheless, the results suggest that *FUM1* with a MT point mutation was functional. The results also suggest that the putative demethylated C_{18} intermediate produced in the MT mutant might be further metabolized by other pathways. Interestingly, the demethylated intermediates were not observed when the MT mutant was cultured alone. The reason for this result is not clear, but might be related to the inability of the culture to produce the more stable, normal (methylated) intermediates of fumonisin, as expected when co-cultured with *FUM6* mutant [32].

In bacteria, the genetic manipulation of PKS domains has proven a powerful way to elucidating the domain structure and function of PKSs [1, 8]. Recently, this approach also has been successfully used in the studies of aromatic fungal polyketides [15, 21]. However, this type of genetic manipulations has not been conducted in reduced fungal polyketides due to inherent difficulties in detecting the metabolites and the lack of an efficient genetic system for the filamentous fungi. In this work, we have developed a genetic system through point mutating the active site of the MT domain of *FUM1* in *F. verticillioides*. This system should be useful in studies of the biosynthetic mechanism of fumonisins as well as other similar fungal polyketides, implied by the large number of PKSs discovered in genome sequencing projects of filamentous fungi.

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