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Ribosomal biosynthesis of α-amanitin in *Galerina marginata*

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Abstract

Amatoxins, including α-amanitin, are bicyclic octapeptides found in mushrooms (Agaricomycetes, Agaricales) of certain species in the genera *Amanita*, *Galerina*, *Lepiota*, and *Conocybe*. Amatoxins and the chemically similar phallotoxins are synthesized on ribosomes in *Amanita bisporigera*, *Amanita phalloides*, and *Amanita ocreata*. In order to determine if amatoxins are synthesized by a similar mechanism in another, distantly related mushroom, we obtained genome survey sequence data from a monokaryotic isolate of *Galerina marginata*, which produces α-amanitin. The genome of *G. marginata* contains two copies of the α-amanitin gene (*GmAMA1-1* and *GmAMA1-2*). The α-amanitin proprotein sequences of *G. marginata* (35 amino acids) are highly divergent from *AMA1* of *A. bisporigera* except for the toxin region itself (IWGIGCNP in single-letter amino acid code) and the amino acids immediately upstream (N[A/S]TRLP). *G. marginata* does not contain any related toxin-encoding sequences besides *GmAMA1-1* and *GmAMA1-2*. DNA from two other α-amanitin-producing isolates of *Galerina* (*G. badipes* and *G. venenata*) hybridized to *GmAMA1*, whereas DNA from the toxin non-producing species *Galerina hybrida* did not. Expression of the *GmAMA1* genes was induced by growth on low carbon. RNASeq evidence indicates that both copies of *GmAMA1* are expressed approximately equally. A prolyl oligopeptidase (POP) is strongly implicated in processing of the cyclic peptide toxins of *A. bisporigera* and *Conocybe apala*. *G. marginata* has two predicted POP genes; one, like *AbPOPB* of *A. bisporigera*, is present only in the toxin-producing isolates of *Galerina* and the other, like *AbPOPA* of *A. bisporigera*, is present in all species. Our results indicate that *G. marginata* biosynthesizes amatoxins on ribosomes by a pathway similar to *Amanita* species, involving a genetically encoded proprotein of 35 amino acids that is post-translationally processed by a POP. However, due to the high degree of divergence, the evolutionary relationship between *AMA1* in the genera *Amanita* and *Galerina* is unclear.

Keywords

Amatoxin; *Amanita*; Cyclic peptide

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Appendix A. Supplementary material Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.fgb.2011.12.005.

1. Introduction

Ribosomally encoded cyclic peptides are known from a number of taxa including mammals, plants, bacteria, and fungi (Craik, 2006). Some, like the cyanobactins and cyclotides of symbiotic marine bacteria and plants, respectively, are cyclized head-to-tail (Donia and Schmidt, 2011; Oman and van der Donk, 2010; Walsh et al., 2010). Others, such as the conotoxins of marine snails, are cyclized by multiple disulfide bonds (Han et al., 2008). Collectively, ribosomally synthesized peptides are characterized by small size, high chemical stability, and a wide range of potent biological activities (Craik, 2006).

Most fatal mushroom poisonings are due to the amatoxins, such as α-amanitin, which are present at high concentrations in many species of *Amanita* sect. *Phalloideae* (Wieland, 1986). Amatoxins and the related phallotoxins are bicyclic peptides containing a unique Trp–Cys cross bridge (with the trivial name tryptathionine) and several hydroxylated amino acids (May and Perrin, 2007). In addition, the phallotoxins contain one D-amino acid. Amatoxins are defining inhibitors of eukaryotic RNA polymerase II (pol II) (Novello et al., 1970), whereas phallotoxins specifically bind and stabilize F-actin (Wehland et al., 1977).

Unlike other fungal cyclic peptides, the amatoxins and phallotoxins are synthesized on ribosomes (Hallen et al., 2007a). The gene for α-amanitin from *Amanita bisporigera*, *AMA1*, is predicted to encode a proprotein of 35 amino acids, with the amino acid sequence of the mature toxin (i.e., IWGIGCNP in single-letter amino acid code) flanked by conserved sequences. Genes encoding β-amanitin (IWGIGCDP), phallacidin (AWLVDCP), and phalloidin (AWLATCP) were also found in species of *Amanita* sect. *Phalloideae* (Hallen et al., 2007a). *Amanita* species are predicted to biosynthesize many more cyclic peptides than those currently known, because their genomes contain at least 30 sequences related to *AMA1*. Genes in this extended family are characterized by conserved sequences flanking a hypervariable “toxin” region of 7–10 amino acids (Hallen et al., 2007a; Luo et al., 2010).

In the amatoxin gene family from *Amanita* species, the amino acid immediately upstream of the toxin region is an invariant Pro, and Pro is always the last amino acid in the toxin itself. A member of the prolyl oligopeptidase (POP) family of proline-specific proteases is probably responsible for the initial post-translational cleavage of the proprotein at the carboxyl side of the two Pro residues to release the linear peptide corresponding to the mature toxin (Luo et al., 2009). Evidence in support of this conclusion includes purification and identification of a POP from *Conocybe apala* that can process a synthetic phalloidin precursor to release the linear peptide; identification of a POP gene (*AbPOPB*), that is restricted in taxonomic distribution to toxin-producing species of *Amanita*; and immunocytochemical co-localization of AbPOPB with α-amanitin in tissues of *A. bisporigera* (Luo et al., 2010).

Amatoxins and the chemically related phallotoxins are also found in certain mushroom species in the genera *Galerina*, *Lepiota*, and *Conocybe*, which are phylogenetically not closely related to *Amanita* (Bresinsky and Besl, 1990; Hallen et al., 2003; Matheny et al., 2006). *Galerina* is a genus of cosmopolitan wood-rotting mushrooms that can degrade both hardwoods and softwoods (Enjalbert et al., 2004; Gulden et al., 2001, 2005). Species of

Galerina sect. *Naucoriopsis* can contain levels of α-amanitin higher than *Amanita phalloides*, the most common cause of fatal human mushroom poisoning (Enjalbert et al., 2002). Although species of *Galerina* are relatively small and obscure, they occasionally cause human and animal poisonings worldwide (Enjalbert et al., 2004; Kaneko et al., 2001; Muraoka et al., 1999).

The presence of the same complex metabolite in phylogenetically widely divergent species of fungi raises the question of how this situation evolved, i.e., whether it was by descent from a common ancestor, convergent evolution, or horizontal gene transfer. To address these possibilities, we here identify and characterize the genes for α-amanitin in *Galerina marginata*. The results also give support to the utility of *G. marginata* as an experimental system to elucidate and manipulate the full biosynthetic pathway of α-amanitin.

2. Materials and methods

2.1. Biological materials

Four species of *Galerina* were obtained from Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands, including *G. marginata* (CBS 339.88), *Galerina badipes* (CBS 268.50), *Galerina venenata* (CBS 924.72), and *Galerina hybrida* (CBS 335.88). *G. marginata* CBS 339.88 is monokaryotic and was confirmed to make α-amanitin. *G. venenata* is considered synonymous with *G. marginata* (Gulden et al., 2001, 2005). The cultures were maintained on potato dextrose agar. For DNA isolation, the isolates were cultured in liquid medium for 15–30 d with rotary shaking at 120 rpm at 23 °C. The medium was HSV-2C, which contains (per liter) 1 g yeast extract, 2 g glucose, 0.1 g NH_4Cl, 0.1 g $CaSO_4 \cdot 5\ H_2O$, 1 mg thi amine·HCl, and 0.1 mg biotin, pH 5.2 (Muraoka and Shinozawa, 2000). For induction experiments, the media had the same formulation, except that high carbon (HSV-5C) and low carbon (HSV-1C) media contained 5 g glucose and 1 g glucose, respectively (Muraoka and Shinozawa, 2000).

2.2. Nucleic acid isolation and genome sequencing

Lyophilized fungal mycelia were ground in liquid nitrogen with a mortar and pestle. High molecular weight DNA was isolated using genomic-tip 100/G (Qiagen, Germantown, MD; catalog #10,243) and RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA), following the manufacturers' protocols.

Genomic DNA was sequenced by 454 pyrosequencing at the Research Technology Support Facility (RTSF) at Michigan State University. A general library was constructed using standard protocols and sequenced on a 454 GSFLX Titanium Sequencer. Raw reads were assembled with Newbler and assembled into a searchable database.

2.3. Cloning and gene characterization

AMA1 and *PHA1* are the designations for the α-amanitin- and phallacidin-encoding genes of *A. bisporigera*, respectively; the prefix *Ab* is used to designate other genes from *A. bisporigera*. The prefix *Gm* is used to designate all genes from *G. marginata*. Using *AMA1* as the search query, two homologs were identified in the partial genome survey sequence of

G. marginata and designated as *GmAMA1-1* and *GmAMA1-2*. PCR primers for each gene were designed based on the partial sequences and used to amplify full length copies. The amplicons were cloned into *Escherichia coli* DH5α and sequenced. Homologs of the two *A. bisporigera* prolyl oligopeptidases (*AbPOPA* and *AbPOPB*) were found in the *G. marginata* genome sequence (Luo et al., 2010). The *G. marginata* genes with highest identity to *AbPOPA* and *AbPOPB* were designated as *GmPOPA* and *GmPOPB*, respectively.

PCR primers unique to *GmAMA1-1* and *GmAMA1-2* were designed and used for isolation of genomic clones of each gene. For *GmAMA1-1*, the unique primers were 5′-CTCCAATCCCCCAACCACAAA-3′ (forward) and 5′-GTCGAACACGGCAACAACAG-3′ (reverse). For *GmAMA1-2*, the primers were: 5′-GAAAACCGAATCTCCAATCCTC-3′ (forward), and 5′-AGCTCACTCGTTGCCACTAA-3′ (reverse). For cloning full-length genomic clones of *GmPOPA* and *GmPOPB*, PCR primers corresponding to the amino and carboxyl termini of both genes (which were on four different contigs) were designed from the genome survey sequence. The forward primers were 5′-TTTAGGGCAGTGATTTCGTGACA-3′ and 5′-AACAGGGAGGCGATTATTCAAC-3′, and the reverse primers were 5′-GAACAATCGAACCCATGACAAGAA-3′ and 5′-CCCCCATTGATTGTTACCTTGTC-3′. The primer pairs were used in both combinations and successful amplification indicated the correct pairing of 5′ and 3′ primers. The resulting amplicons were cloned into *E. coli* DH5α and sequenced.

The genomic DNA sequences were used for primer design to obtain full-length cDNAs by Rapid Amplification of cDNA Ends (RACE) using the SMART RACE kit (Clontech, Mountain View, CA). A cDNA copy of *GmAMA1-1* was obtained using primers 5′-CCAACGACAGGCGGGACACG-3′ (5′-RACE) and 5′-GACCTTTTTGCTTTAACATCTACA-3′ (3′-RACE), and of *GmAMA1-2* with primers 5′-GTCAACAAGTCCAGGAGACATTCAAC-3′ (5′-RACE) and 5′-ACCGAATCTCCAATCCTCCAACCA-3′ (3′-RACE). The RACE primers for *GmPOPA* were 5′-CGGCGTTCCAAGGCGATGATAATA-3′ (5′-RACE) and 5′-CATCTCCATCGACCCCTTTTTCAGC-3′ (3′-RACE), and for *GmPOPB* 5′-AGTCTGCCGTCCGTGCCTTGG-3′ (5′-RACE) and 5′ CGGTACGACTTCACGGCTCCAGA-3′ (3′-RACE). Sequences generated from the RACE reactions were used to assemble full-length cDNAs of all four genes.

Alignments of genomic and cDNA copies was done with Spidey (http://www.ncbi.nlm.nih.gov/spidey/) and Splign (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi).

2.4. DNA and RNA blotting

DNA for Southern blotting was digested with *Pst*I and electrophoresed in 0.7% agarose. Probe labeling, blotting, and filter hybridization followed standard protocols (Scott-Craig et al., 1990). Hybridizations were performed for 15 h at 65 °C as described (Luo et al., 2010). Roughly 2 μg of DNA were loaded per lane. Probes were made by labeling genomic DNA of *GmAMA1-1*, *GmPOPA*, and *GmPOPB* with [^{32}P]dCTP.

For the *GmAMA1* induction experiment, *G. marginata* was cultured in HSV-5C media for 30 d and then transferred to HSV-5C or HSV-1C and grown for an additional 10 d. The resulting mycelia were lyophilized and stored at −80 °C prior to RNA extraction (Hallen et al., 2007b). Full-length cDNA was prepared using the GeneRacer RACE kit, following the manufacturer's protocols. Hybridization probes were amplified from the *GmAMA1* cDNA using a specific 5′ primer (5′-ATGTTCGACACCAACTCCACT-3′) and GeneRacer 3′ nested primer (5′-CGCTACGTAACGGCATGACAGTG-3′). Probe labeling, RNA gel electrophoresis, and blotting followed standard protocols (Scott-Craig et al., 1990). Each lane was loaded with 15 μg total RNA.

2.5. Nucleotide sequence accession numbers

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: JN827311 for *GmAMA1-1*, JN827312 for *GmAMA1-2*, JN827313 for *GmPOPA*, and JN827314 for *GmPOPB*.

2.6. Amanitin extraction and analysis

G. marginata was cultured in HSV-5C media for 30 d and then transferred to fresh HSV-1C medium for an additional 10 d. After harvest, the mycelium was lyophilized and stored in at −80 °C. A portion of dried mycelium (0.2 g) was ground in liquid nitrogen and mixed with 2 ml methanol:water:0.01 M HCl (5:4:1) (Enjalbert et al., 1992; Hallen et al., 2003). The suspension was incubated at 22 °C for 30 min and then centrifuged at 10,200 × *g* for 10 min at 4 °C. The supernatant was collected and filtered through a 0.22 μm filter. Chromatographic separation was done on a C18 column (Vydac 218TP54) attached to an Agilent Model 1100 HPLC with detection at 230, 290, and 305 nm. Elution solution A was water + 0.1% trifluoroacetic acid, and solution B was acetonitrile + 0.075% trifluoroacetic acid. The flow rate was 1 ml/min with a gradient from 100% A to 100% B in 30 min. An α-amanitin standard (Sigma A2263) was dissolved in water at a concentration of 100 μg/ml. Loadings were 40 μl unknown or 20 μl standard.

3. Results

3.1. Identification of the α-amanitin genes in G. marginata

One plate of 454 pyrosequencing was performed on *G. marginata* genomic DNA and assembled into contigs totaling approximately 73 MB. This is approximately two times the expected size of the genome based on the average of known basidiomycetes. The sequences were put into a database and searched using *AMA1*, *PHA1*, *AbPOPA*, and *AbPOPB*. The contigs defined in these searches were used to design PCR primers for amplification of full length sequences of each gene.

Two homologs of *AMA1* were found in the genome of *G. marginata* and named *GmAMA1-1* and *GmAMA1-2*. *GmAMA1-1* contains three introns and *GmAMA1-2* contains two (Fig. 1 and Supplementary Fig. S1). The three introns of *GmAMA1-1* are 53, 60, and 60 nt in length in the identical locations as the three introns of *AMA1* (Hallen et al., 2007a). The first intron in both *GmAMA1-1* and *GmAMA1-2* interrupts the third codon before the stop codon. *GmAMA1-1* and *GmAMA1-2* differ at eight nucleotides out of 108 nucleotides in the coding

region (i.e., from the ATG through the TGA stop codon), two of which result in amino acid changes and six of which are silent (Fig. 2). There are numerous nucleotide differences between *GmAMA1-1* and *GmAMA1-2* in the 5′ and 3′ untranscribed regions but also large stretches of identity. The major difference between *GmAMA1-1* and *GmAMA1-2* is that the latter gene is 104 bp shorter. The indel spans the second intron of *GmAMA1-1* in the 3′ UTR (Fig. 1) and accounts for the presence of only two introns in *GmAMA1-2* (Fig. 1 and Fig. S1).

The translational start site of a gene is generally assumed to be the first in-frame ATG codon after the transcriptional start site. For *GmAMA1-1*, this indicates a start site that is analogous to *AMA1* of *A. bisporigera*. For *GmAMA1-2*, however, there is an in-frame ATG that is 78 nucleotides upstream of the ATG indicated in Fig. 2, which would result in a proprotein of 61 amino acids instead of 35 as predicted for *AMA1* and *GmAMA1-1*. We cannot exclude that this is the actual translational start of *GmAMA1-2*. However, *AMA1, PHA1*, and *GmAMA1-1* all lack any in-frame ATG codons between their transcriptional starts and the indicated ATG, and therefore it is unlikely that *GmAMA1-2* translation starts at the upstream ATG. Another argument against the first ATG being the actual translational start is that POPs preferentially act on peptides shorter than 40 amino acids (Szeltner and Polgár, 2008).

Assuming that translation of *GmAMA1-2* begins at the second ATG, *GmAMA1-1* and *GmAMA1-2* both encode 35-amino acid proproteins, the same size as the proprotein of *AMA1* in *A. bisporigera* (Hallen et al., 2007a). The toxin-encoding region (IWGIGCNP) is in the same relative position as it is in *AMA1*. There are 31 nucleotide differences between *GmAMA1-1* and *AMA1* in the coding region of 108 nucleotides (from the ATG through the stop codon). This results in a low level of amino acid conservation except for the toxin region and the amino acids immediately upstream of the toxin region (N[A/S]TRLP) (Fig. 2).

The proproteins of almost all of the members of the large family of genes related to *AMA1* and *PHA1* in *A. bisporigera*, *A. phalloides*, and *Amanita ocreata* start with a conserved five amino acid motif, MSDIN (Hallen et al., 2007a; Luo et al., 2009). The first five amino acids of the two *G. marginata* α-amanitin genes are MFDTN. To test if the *G. marginata* toxin-encoding genes were part of a family as was found in *A. bisporigera*, the *G. marginata* database was searched with the upstream and downstream amino acid sequences of *GmAMA1-1* and *GmAMA1-2* and no additional related sequences were identified. In addition, searching the *A. bisporigera* database with the conserved regions of *GmAMA1-1* and *GmAMA1-2* did not reveal any related sequences in the *A. bisporigera* genome beyond the known MSDIN family members previously described (Hallen et al., 2007a; Luo et al., 2009).

3.2. Distribution of α-amanitin genes in the genus Galerina

Within the genus *Amanita*, *AMA1* and *PHA1* are present only in section *Phalloideae*, which contains all of the known amatoxinand phallotoxin-producing species in this genus (Hallen et al., 2007a). To explore the distribution of the α-amanitin genes in relation to toxin production in *Galerina*, we compared four species of *Galerina* by DNA and RNA blotting. Recent taxonomic revision of this genus indicates that *G. marginata* and *G. venenata* are

synonyms, whereas *G. hybrida* and *G. badipes* are still considered to be separate species (Enjalbert et al., 2004; Gulden et al., 2001, 2005). *GmAMA1-1* hybridized to all three α-amanitin producers (*G. marginata, G. badipes,* and *G. venenata*) but not to the toxin nonproducer, *G. hybrida* (Fig. 3). In contrast to *Amanita* species, which exhibit multiple hybridizing bands when probed with *AMA1* or *PHA1*, the pattern in *Galerina* was less complex. Only two bands were present, indicating that *GmAMA1* is not part of an extended gene family in *G. marginata*. Genomic DNA digested with additional restriction enzymes also produced a similar pattern of only two hybridizing bands (data not shown). This pattern of hybridization is consistent with the genome survey sequence, which indicates that *G. marginata* has two and only two sequences related to *GmAMA1-1*. The genome survey sequence and cDNA analysis indicate that both genes encode α-amanitin (Fig. 2), and that our isolate of *G. marginata* does not make other compounds related to α-amanitin. In particular, the genome survey sequence did not contain a DNA sequence that could encode β-amanitin, which differs from α-amanitin by one amino acid (Asp in place of Asn). HPLC analysis of *G. marginata* CBS 339.88 indicated that it does not produce β-amanitin in detectable quantities (Fig. 4). The *G. marginata* sample contained ~0.4 mg α-amanitin/g dry weight.

3.3. Regulation of GmAMA1 by low carbon

Successful amplification of *GmAMA1-1* and *GmAMA1-2* by reverse transcriptase PCR with gene-specific primers indicated that both genes are transcribed in culture. This conclusion was supported by RNASeq performed at the DOE Joint Genome Institute (unpublished results). More than 50 million expression sequences were obtained from a culture of *G. marginata* grown on low carbon. Approximately equal numbers of gene-specific sequence reads were obtained from *GmAMA1-1* and *GmAMA1-2*.

Expression was also studied by RNA blotting. Muraoka and Shinozawa (2000) showed that α-amanitin production in *Galerina fasciculata* is up-regulated on low glucose medium (carbon starvation). Expression of *GmAMA1-1* and/or *GmAMA1-2* were also up-regulated by carbon starvation in *G. marginata* and *G. badipes* (Fig. 5). Due to their high nucleotide similarity, this experiment could not distinguish between expression of *GmAMA1-1* and *GmAMA1-2*. As expected from the DNA blot results, RNA from the toxin nonproducer, *G. hybrida*, gave no signal when the fungus was grown in either high or low carbon (Fig. 5).

3.4. Prolyl oligopeptidase genes in G. marginata

Several lines of evidence implicate a prolyl oligopeptidase (POP) in the processing of the proproteins of amatoxins and phallotoxins (Luo et al., 2009, 2010). The *G. marginata* partial genome survey contained two homologs of the *POP* genes of *A. bisporigera*. Genomic PCR, reverse transcriptase PCR, and RACE were used to obtain full length copies of the two genes and determine their intron/exon structures (Fig. 6). *GmPOPA* has 18 introns, which is the same number as *AbPOPA*, and *GmPOPB* has 17 introns, one fewer than *AbPOPB* (Luo et al., 2010). The amino acid sequences of the predicted translational products of *GmPOPA* (738 amino acids) and *GmPOPB* (730 amino acids) are 57% identical to each other. The *GmPOPA* protein is 65% identical to *AbPOPA* and 58% identical to *AbPOPB*, and *GmPOPB* is 57% identical to *AbPOPA* and 75% identical to *AbPOPB*.

Sequences hybridizing to *AbPOPA* are present in amatoxin and phallotoxin-producing and non-producing species of *Amanita*, whereas *AbPOPB* is present only in the amatoxin-producing species (Luo et al., 2010). Similarly, by DNA blotting *GmPOPA* was found to be present in all four specimens of *Galerina*, but *GmPOPB* was not present in the amanitin non-producing species *G. hybrida* (Fig. 3). The high similarity of the hybridization patterns when genomic DNA of *G. venenata* and *G. marginata* were probed with *GmAMA1*, *GmPOPA*, and *GmPOPB* is consistent with them being the same species (Gulden et al., 2001). The association of *POPB* with amanitin production in both *A. bisporigera* and *G. marginata*, and the higher amino acid identity of *GmPOPA* to *AbPOPA* and of *GmPOPB* to *AmPOPB* are consistent with a role for *POPB* in amanitin biosynthesis in the two species. All other basidiomycetes in GenBank and at the DOE Joint Genome Institute (JGI) have single POP genes, which are probably functional homologs of *POPA*.

4. Discussion

Amatoxins and phallotoxins are the first ribosomally encoded cyclic peptides described in the kingdom Mycota, a taxonomic group that contains an abundance of nonribosomal peptide synthetases (Walton et al., 2004). Like species of *Amanita*, *G. marginata* also synthesizes α-amanitin on ribosomes. The α-amanitin genes in *G. marginata* show significant resemblance to their homologs in *Amanita* mushrooms, including intron/exon structure, proprotein length, and probable post-translational processing by a dedicated prolyl oligopeptidase (POP). The *G. marginata* genes differ from those of *A. bisporigera* in several ways. First, the proproteins share little amino acid identity except in the toxin region itself (IWGIGCNP) and in the amino acid motif (N[A/S]TRLP) found immediately upstream of the toxin region. One of these amino acids is a completely conserved Pro residue, which would be necessary for recognition by the processing POP. It is possible that the other upstream conserved amino acids (i.e., N[A/S]TRL) are important for recognition of the proproteins by POPB or for cyclization, if this step occurs before proteolytic processing. Second, *G. marginata* contains two nearly identical copies of the α-amanitin gene, whereas *A. bisporigera* has only one. Conversely, *A. bisporigera* has two copies of the gene for phallacidin (*PHA1*), whereas *G. marginata* does not biosynthesize phallotoxins nor have any genes that could encode these cyclic heptapeptides (Hallen et al., 2007a). Third, the genome of *G. marginata* contains only two sequences related to the α-amanitin genes, whereas. *A. bisporigera* has a large family of related sequences (>30 members), which encode predicted, but chemically unknown, cyclic peptides. These predicted peptides contain 7–10 amino acids and most of them lack Trp and Cys and therefore cannot form tryptathionine, which is characteristic of the amatoxins and phallotoxins.

G. marginata and other species of *Galerina* are well-known to make α-amanitin (Enjalbert et al., 2004; Muraoka et al., 1999; Muraoka and Shinozawa, 2000). No species of *Galerina* has been reported to produce phallotoxins, but some have been reported to make β-amanitin, which differs from α-amanitin in having Asp in place of Asn. The difference between these two forms of amanitin is probably genetically encoded and not catalyzed by, e.g., a transamidase, because the genome of *A. phalloides* contains a gene that could encode β-amanitin (Hallen et al., 2007a). Our isolate of *G. marginata* does not synthesize β-amanitin and the genome lacks a gene for β-amanitin. *G. marginata* produces, at most, traces of β-

amanitin in culture (Benedict et al., 1966; Benedict and Brady, 1967). We have not detected β-amanitin in several North American specimens of *Galerina* (unpublished results). Therefore, it appears that some species or isolates of *Galerina* do make β-amanitin and others do not. Other forms of amanitin, such as γ-amanitin and ε-amanitin, differ from α-amanitin and β-amanitin in their pattern of hydroxylation, which is not expected to be genetically encoded directly.

Several lines of evidence implicate a prolyl oligopeptidase (POP) in the initial processing of the proproteins of amatoxins and phallotoxins. First, in the extended MSDIN family of *Amanita*, and as we have now shown for the α-amanitin genes of *G. marginata*, the flanking Pro residues are completely conserved. During posttranslational processing, one Pro remains in the mature toxin and the other is removed with the flanking peptide. Second, an enzyme that proteolytically cleaves a synthetic phalloidin proprotein, isolated from the phalloidin-producing fungus *C. apala*, was identified as a POP (Luo et al., 2009). The same enzyme cleaves at both Pro residues to release the mature linear peptide (AWLATCP in the case of phalloidin). Third, toxin-producing species of *Amanita* have two *POP* genes, whereas all other sequenced basidiomycetes have only one. One of the *Amanita POP* genes, *AbPOPB*, is taxonomically restricted to toxin-producing species, like *AMA1* and *PHA1* themselves (Luo et al., 2010). Fourth, the distribution of AbPOPB and α-amanitin overlap in mushroom tissues (Luo et al., 2010), indicating a cytological connection between α-amanitin biosynthesis and accumulation. The results in the current paper indicate that *G. marginata* also has two *POP* genes, like *A. bisporigera* but unlike other, toxin non-producing species of mushrooms. *GmPOPB* is absent from the toxin nonproducing species *G. hybrida*, and therefore it cannot have an essential housekeeping function. We hypothesize that *AbPOPB* and *GmPOPB* are dedicated to biosynthesis of the amatoxins and/or phallotoxins in their respective species.

Galerina and *Amanita* are in different families of the Agaricales (Strophariaceae and Amanitaceae, respectively) (Kirk et al., 2008). This raises the question of the evolutionary origins of the capacity to biosynthesize the same complex secondary metabolite, which requires multiple coordinated biosynthetic activities and encoding genes. Our results indicate that the fundamental biosynthetic pathway is the same in both taxa, probably including proprotein processing by a POP. However, it remains unclear from our studies if this situation is the result of horizontal gene transfer, descent from a common ancestor, or convergent evolution. The fact that the extended MSDIN family members of *Amanita* are closer in sequence to each other than are the α-amanitin genes of *Amanita* and *Galerina* suggests that, if α-amanitin biosynthesis in the two genera has a common evolutionary origin, expansion of the *Amanita* MSDIN family occurred after the split. Identification of the biosynthetic genes for amatoxins and phallotoxins in other genera, such as *Lepiota* and *Conocybe*, might shed light on these alternative evolutionary scenarios.

G. marginata has several advantages as an experimental system for further work on the elucidation and manipulation of the amatoxin biosynthetic pathway. First, *G. marginata* can be cultured under laboratory conditions, unlike most species of *Amanita* (Benedict et al., 1966; Benedict and Brady, 1967; Muraoka and Shinozawa, 2000; Zhang et al., 2005). Second, *G. marginata* produces α-amanitin in culture and production can be induced by

carbon starvation. Third, genomic sequencing and genetic studies should be facilitated by the availability of a toxin-producing monokaryotic strain of *G. marginata*. Fourth, the panoply of toxin genes is reduced from >30 members to only two in *G. marginata*, simplifying the analysis of novel compounds that might be produced by genetic manipulations. Fifth, *G. marginata* clearly has the enzymes necessary for the post-translational processing of the proprotein of *GmAMA1* into mature α-amanitin, which must include cyclization, formation of the Trp-Cys cross-bridge of tryptathionine, and several hydroxylations. Therefore, it is reasonable to expect that *G. marginata* will correctly catalyze at least some of the required biosynthetic steps with non-natural peptides, such as cyclization and tryptathionine formation, leading to novel cyclic peptides based on the α-amanitin scaffold.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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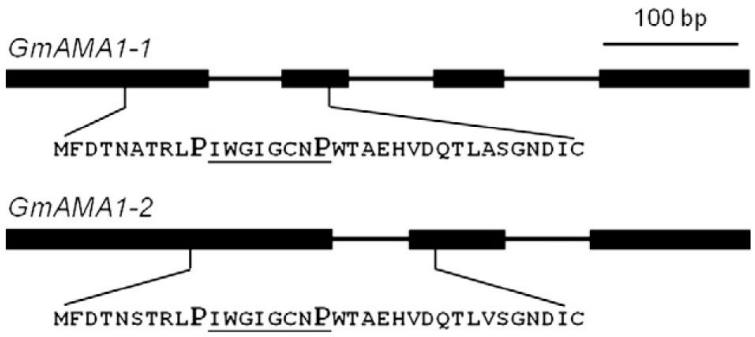

Fig. 1.

Gene structure of the two α-amanitin genes (*GmAMA1-1* and *GmAMA1-2*) in *Galerina marginata*. Exons are indicated by heavy lines and introns by thin lines. The predicted proprotein sequences and their locations are indicated below.

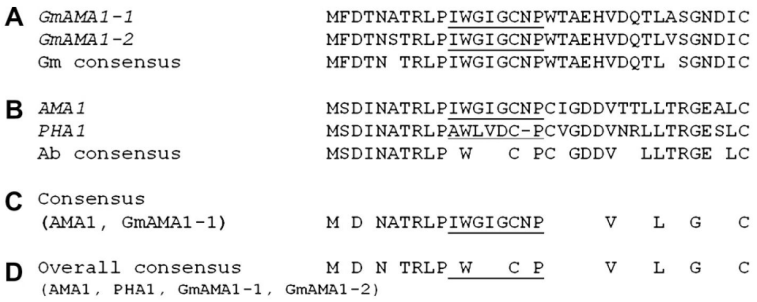

Fig. 2.

Alignments of the predicted amino acid sequences of the proproteins of α-amanitin-encoding genes in *G. marginata* and *A. bisporigera*. (A) Alignment of the two copies of the α-amanitin-encoding genes in *G. marginata* (*GmAMA1-1* and *GmAMA1-2*). (B) Alignment of *AMA1* (encoding α-amanitin) and *PHA1* (encoding phallacidin) from *A. bisporigera* (*Ab*) (from Hallen et al., 2007a). A gap was introduced in the sequence of *PHA1* because phallacidin has one fewer amino acid than α-amanitin. (C) Consensus between *AMA1*, the α-amanitin-encoding gene of *A. bisporigera*, and copy 1 (*GmAMA1-1*) of the α-amanitin-encoding gene of *G. marginata*. (D) Consensus among *AMA1*, *PHA1*, *GmAMA1-1*, and *GmAMA1-2*.

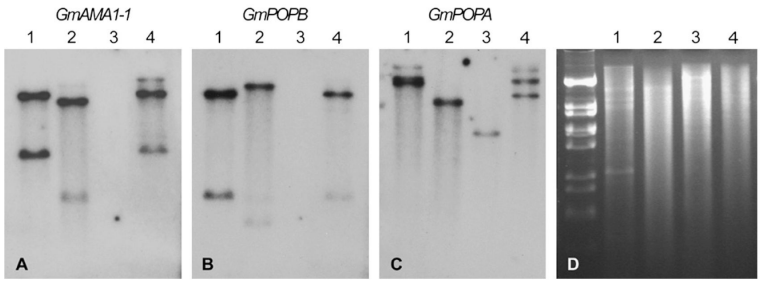

Fig. 3.

DNA blot of *Galerina* species. Lane 1, *G. marginata*; lane 2, *G. badipes*; lane 3, *G. hybrida*; lane 4, *G. venenata*. Panel A Probed with *GmAMA1-1*; panel B probed with *GmPOPB*; panel C, probed with *GmPOPA*; panel D, gel stained with ethidium bromide.

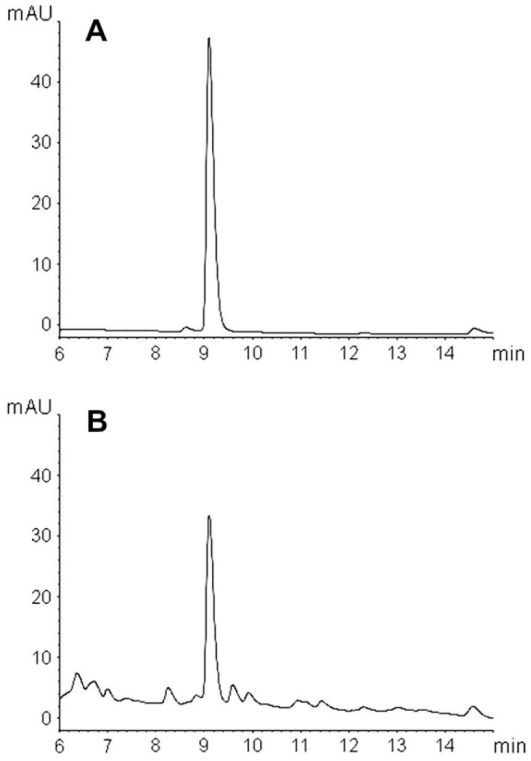

Fig. 4.

Reverse-phase HPLC analysis of amatoxins in *Galerina marginata* strain CBS 339.88 grown on a medium containing low carbon (see Materials and Methods). (A) α-amanitin standard. (B) extract of *G. marginata*. Elution was monitored at 305 nm.

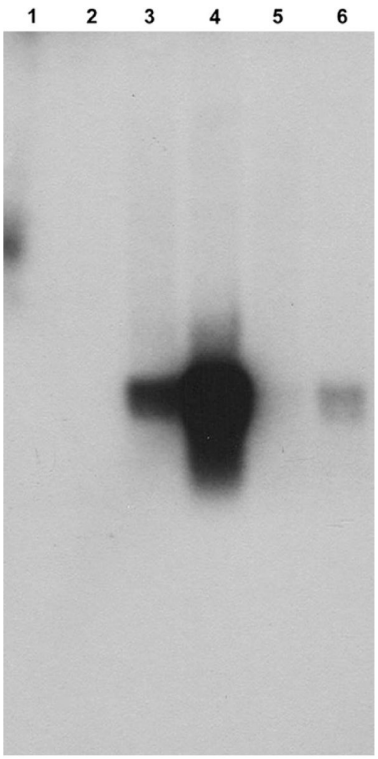

Fig. 5.

RNA blotting of *Galerina* strains under different growth conditions. The probe was *GmAMA1-1*. Lane 1: *G. hybrida* grown on high carbon. Lane 2: *G. hybrida*, low carbon. Lane 3: *G. marginata*, high carbon. Lane 4: *G. marginata*, low carbon. Lane 5: *G. badipes*, high carbon. Lane 6: *G. badipes*, low carbon. Each lane was loaded with 15 μg total RNA. The major band in lanes 3, 4 and 6 is ~300 bp. The higher molecular weight signal in lane 1 is spurious.

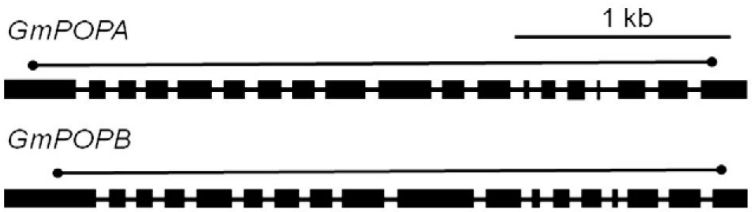

Fig. 6.

Structures of *GmPOPA* and *GmPOPB* encoding putative prolyl oligopeptidases from *G. marginata*. Thick bars indicate exons and thin bars indicate introns. The lines above the gene models indicate the positions of the coding regions.