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ARTICLE

Development and Media Regulate Alternative Splicing of a Methyltransferase Pre-mRNA in *Monascus pilosus*

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Two alternatively spliced mRNAs (*d*- and *l*-*MpLaeA*) of a methyltransferase gene (*MpLaeA*) were identified from *Monascus pilosus* IFO4520 and its mutant MK-1. Alternative splicing of the *MpLaeA* pre-mRNA occurred in the 5′-untranslated region (5′-UTR). The alternative splicing patterns of *MpLaeA* were regulated by the fungal growth stage and the principal nutrients: that is, the short *l*-*MpLaeA* mRNA was a constitutive transcript at all growth stages and different carbon or nitrogen sources, but the glutamate and $NaNO_3$ as main nitrogen source could up-regulate the long *d*-*MpLaeA* mRNA form. The long spliced 5′-UTR of *d*-*MpLaeA* blocked GFP expression in *Escherichia coli*, suggesting that *d*-*MpLaeA* mRNA was an ineffective spliced mRNA. Down-regulation of *MpLaeA* by transgenic antisense *d*-*MpLaeA* cDNA resulted in decreasing synthesis of monacolin K in *M. pilosus*. This suggested that the alternative splicing of *MpLaeA* mRNA might regulate the synthesis of monacolin K.

KEYWORDS: Monacolin K; alternative splicing; methyltransferase; *Monascus pilosus*.

INTRODUCTION

Monacolin K (also known as lovastatin) plays an important role in human health. It blocks the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate during cholesterol synthesis by inhibiting the HMG-CoA reductase (*1*, *2*). Monacolin K has been used to treat hypercholesterolemia. Many filamentous fungi can produce monacolin K (*3*), including members of the *Monascus* genus (*4*), which have been used for a long time in Asia to brew various oriental beverages. The genes and enzymes involved in the biosynthesis of monacolin K have been identified and characterized in *Aspergillus terreus* (*2*, *5*); *Monascus* was suspected of possessing a similar monacolin K biosynthetic pathway, and these genes have been cloned recently from *Monascus pilosus* (*6*). Regulation of monacolin K biosynthesis depends on culture conditions, including the composition of the medium, and incubation parameters such as the wavelength of light in *Aspergillus* (*7*, *8*) and *Monascus* (*9*−*11*). However, little is known about the molecular mechanism of the regulation of monacolin K biosynthesis in *Monascus*.

We identified an up-regulated EST in the *M. pilosus* mutant MK-1 by suppressing subtractive hybridization. This EST is homologous to a fungal positive secondary metabolism regulator (*LaeA*), which is a putative methyltransferase (*12*). Methyltransferase catalyzes methyl transfer from *S*-adenosyl-L-methionine (S-AdoMet) to nitrogen, oxygen, or carbon atoms, thereby modifying the structure of proteins, DNA, RNA, and small molecules in a wide spectrum of organisms including bacteria, plants, and mammals (*13*). It therefore plays a role in gene expression regulation (*14*) and can be shown to be involved in fungal secondary metabolism, including monacolin K (*12*, *15*). We report here on the regulation of the alternative splicing *MpLaeA* pre-mRNA and its relationship with the monacolin K synthesis in *M. pilosus*.

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MATERIALS AND METHODS

Culture Conditions and *Monascus pilosus* Strains. Stock cultures of *M. pilosus* IFO4520 and the mutant (MK-1) were maintained on potato dextrose agar (PDA) (BD Difco, USA). Spores of 10−14 days were collected in a sterile solution (0.9% NaCl, 0.2% Tween 80) and filtered with sterile glass wool for further incubation. The media used in this study were potato dextrose broth (PD) and Czapek−Dox broth (CD) (BD Difco, USA) and glucose−glycerol−peptone (GGP) medium (7% glycerol, 3% glucose, 3.8% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$). Liquid cultures were incubated at 25 °C with agitation at 120 rpm.

RNA Analyses. Total RNA was isolated from *M. pilosus* mycelia with RNeasy Plant Mini Kit (Qiagen, Germany) and treated with DNase I (RNase free) (TaKaRa, Japan) for RT-PCR and Northern blot. mRNA was purified from the total RNA with an Oligotex-dT30 (Super) mRNA Purification Kit (TaKaRa, Japan) for suppression of subtractive hybridization (SSH), 5′- or 3′-RACE (rapid amplification cDNA ends), and RT-PCR. SSH was performed with a Clontech PCR-Select cDNA Subtraction Kit (Clontech, TaKaRa, Japan), and mRNA of *M. pilosus* IFO4520 and MK-1 used in SSH experiment was isolated from the fungi incubated in GGP media for 14 days. 5′- and 3′-RACE were obtained with a SMART RACE cDNA Amplification Kit (Clontech) with the primer LF8 for 3′-RACE and the primter LR15 for 5′-RACE. RT-PCR was carried out with an AccessQuick RT-PCR System (Promega, Madison, WI) to monitor the two alternative mRNAs of *MpLaeA* with primers LF22 and LR733 using the total RNA or the purified mRNA. All primers are listed in **Table 1**.

RNA for Northern blots was transferred onto the charged nylon membrane (Roche, Germany) after electrophoresis in a 1.5% agarose−formaldehyde gel. DIG-labeled full-length sense or antisense RNA probes

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Table 1. PCR Primers Used in This Study

primer	sequence (5′−3′)
LF8	GTGGTAGCGTTGTGAGCTGGCCCAG
LR15	CCTCTTCGGTGGCTTGCTTCAGACACTGG
LF22	GGATCCCGCAGTTGTACATAA
LR1522	GATTCAATTGGAAATTGGCTTC
LF296	AGGGACTTCTTCTAGCACTGC
LR733	AGGCGATCCTGCTCCAGGTCG
LDF	ACCTCGTACATAACCCTGTATGTATT
IVF	CCTGATGTTTGGACAACAA
IVR	CAGGCCAACGCACCTGCCTGAG
IVR1	TGCATCGTCAGTTCATCGCGCTG
IVF6	GCTTTCCCAGACTCTCTGCAACG
UTRF	ATCCGCTGGATCCCGCAGTTGTACA
EgFR	GCTGGATCCTGTGTGAAATTGTT
HygF	ATGAAAAAGCCTGAACTCACCG
HygR	CGGTCGGCATCTACTCTATTTCTTT

of *d-cDNA* of *MpLaeA* were generated by in vitro transcription from the cloned *d-cDNA* of *MpLaeA* in pGEM-T easy vector (Promega) by T7 or SP6 RNA polymerase, by following the protocols of the DIG Northern Starter Kit (Roche, Germany). Prehybridization, hybridization, washing, and chemiluminescent detection were also carried out as described in this manual. Stringent washes were given as 2× SSC/0.1% SDS at room temperature for 10 min twice and 0.1× SSC/0.1% SDS at 68 °C for 20 min twice. Images of the chemiluminescence were recorded using the "live acquire" function in the ChemiDoc XRS System (Bio-Rad, Hercules, CA).

DNA Analyses. Genomic DNA was isolated by CTAB protocol (*16*). After a 0.7% agarose gel separation, the digested genomic DNA was capillary blotted onto the charged nylon membrane (Roche, Germany) before being probed with the DIG-labeled *d-cDNA* of *MpLaeA* by PCR with a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche). RCR was carried out with high-fidelity Pyrobest Taq DNA polymerase (TaKaRa). Inverse PCR was done according to the protocol in the *Molecular Cloning Manual* (*16*) to amplify the promoter region of *MpLaeA*. Primers (IVF/IVR) were used for first-round inverse PCR, and the genomic DNA was digested with *Eco*RV; the primers (IVR1/IVF6) for second-round inverse PCR and the genomic DNA were digested with *Xba*I.

GFP Analyses. Standard methods for plasmid isolation and manipulation were performed as described by Sambrook and Russell (*16*). 5′-UTR of *d-* and *l-MpLaeA* cDNA was amplified by primers LF22 and IVR and blunt inserted into the *Sma*I site of pEGFP (Clontech, USA). The inserted direction was checked by restriction enzyme digestion. After insertion, the fused *lacZ* part in pEGFP was removed by inverse PCR with primers (EgFR and UTRF) and recycled by *BamH*I sites in the primers. Therefore, the 5′-UTR was placed between the *lac* promoter and the *EGFP* gene and was located behind the transcription start point and ribosome binding site. These constructs were transformed into *E. coli* JM109. Four independent constructed experiments were to insert *d-* and *l-*5′-UTR before *EGFP*. Quantitative fluorometric assay of EGFP was performed following the *BD Living Colors User's Manual* (Clontech, TaKaRa, USA). Results of mean EGFP activity were from 16 samples of 4 independent constructs for every insert.

Transformation of *M. pilosus*. A plasmid, pMGH, was constructed to introduce a foreign gene into *M. pilousus* IFO4520. pBARMET1(*17*) was used as the starter plasmid and was inserted with the pgpdA-hyg at the *Bgl*II and *Not*I. The *pgdA* promoter was from pBARGPE1 (*17*), and a hygromycin B (hygB) resistance gene was amplified from pMDC45 (*18*) with primers (HygF and HygR). The antisense *d-MpLaeA* was inserted after the fungal *gpdA* promoter of pMGH by *Eco*RI, and the antisense *d-MpLaeA* insert was selected from the transformed clones by restriction enzyme digestion.

Yatalase (TaKaRa, Japan) was used to generate the protoplasts of *M. pilosus* IFO4520, which were grown from spores in PD broth at 25 °C for 16 h. Fungal transformation by electroporation was done with a Gene Pluser II apparatus (BioRad) following the guide in the BioRad Bulletin (*19*), with the modification of keeping the transformed protoplasts after the electroporation in PD broth plus 1.2 M sorbitol overnight at 20 °C before they were spread on PDA solid medium plus 80 μg/mL hygromycin B.

Determination of Monacolin K and Red Pigments. After incubation, the media were filtered by 0.2 μm filters, and the filtered media were directly used to measure monacolin K and red pigments. The monacolin K and red pigments in the fungi were extracted by 10 volumes of 60% ethanol for 2 days and then were measured. Monacolin K was analyzed by high-pressure liquid chromatography (HPLC) with an Agilent 1100 system (Agilent, Palo Alto, CA), following the protocol of Miyake et al. (*11*, *20*). Red pigments of *Monascus* are mixtures of serial similar compounds, and their maximum absorption range is from 490 to 500 nm. The absorbance at 490 nm was used to indicate the production of red pigments.

Nucleotide Sequence Accession Number. The nucleotide sequence of the methyltransferase gene (*MpLaeA*) from *M. pilosus* was submitted to GenBank under accession no. DQ178028.

RESULTS

Isolation of Alternative Spliced cDNAs and Gene of *MpLaeA*. *M. pilosus* IFO4520 and its mutant MK-1 were used in this study from Dr. Miyake (*20*). An up-regulated EST of MK-1 was identified as a homologue of a fungal secondary metabolism regulation gene, *LaeA* (*12*), with suppressive subtractive hybridization. Rapid amplification of cDNA ends (RACE) was used to identify the 5′- and 3′-ends of the full-length cDNA of this EST. LF8 primer was used to amplify the 3′-end of this EST in 3′-RACE, and LR15 primer was used to amplify the 5′-end of this EST in 5′-RACE. Two bands, with a 278 bp difference in length, were generated in the 5′-RACE reaction. According to the sequences of 5′- and 3′- RACE products, LF22 and LR1522 primers were used to amplify the full-length gene from genomic DNA by PCR (**Figure 1A**) and cDNA from mRNA by RT-PCR (**Figure 1B**), respectively. Only one band was amplified from the genomic DNA of *M. pilosus* by PCR, but two bands were amplified from mRNA of *M. pilosus* by RT-PCR with LF22 and LR1522 primers. This suggested that the two bands of RT-PCR were alternative products of this gene, which was called *MpLaeA* (*Monascus pilosus LaeA* homologue). The *MpLaeA* gene is 1900 bp length.

After the two RT-PCR bands had been sequenced, the transcripts of *MpLaeA* seemed to have two alternative spliced mRNAs. The long spliced mRNA was named *d-MpLaeA* (1810 bp), and the short one, *l-MpLaeA* (1532 bp) (**Figure 1A**). There are two introns in the *MpLaeA* gene. The first intron (I_1) contains 278 bp and is alternatively spliced (**Figure 1A**), whereas the second intron (I_2) is 90 bp. Southern blot was used to check the copy number of *MpLaeA* in the *M. pilosus* genome. Only one band of Southern blot of their genomic DNA digested with various restriction enzymes (**Figure 1C**) indicated that one copy of *MpLaeA* exits in *M. pilosus* genome. This is consistent with the notion that the two mRNAs are splice variants of *MpLaeA* pre-mRNA.

The expression levels of *MpLaeA* in *M. pilosus* IFO4520 and MK-1 are different according to the suppressive subtractive hybridization experiments (data not show), but no difference in the genomic sequence and the two alternatively spliced cDNA (*d-MpLaeA* and *l-MpLaeA*) sequences of *MpLaeA* was found between *M. pilosus* IFO4520 and MK-1. To compare their promoters, inverse PCR was used to isolate the promoter. Their 2305 bp promoters were identified with IVF/IVR primers (first-round inverse PCR, genomic DNA digested with *Eco*RV) and IVR1/IVF6 primers (second-round inverse PCR, genomic DNA digested with *Xba*I) (**Figure 1A**). There is also no difference in the sequences of *MpLaeA* promoters between *M. pilosus* IFO4520 and MK-1. Therefore, the differences of expression level between

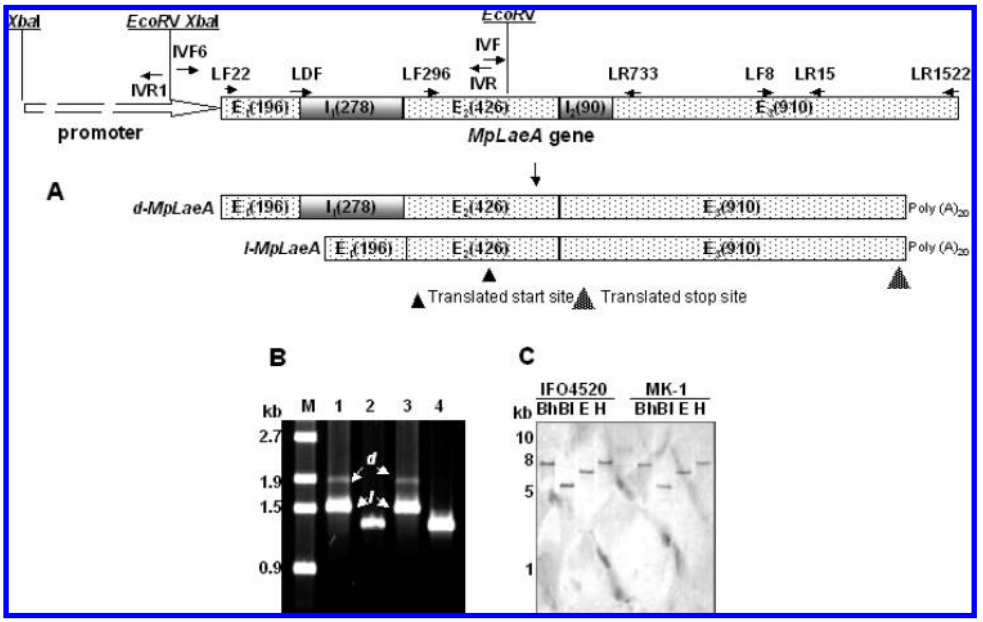

Figure 1. Schematic diagram of *MpLaeA* and its promoter. (**A**) Structure of *MpLaeA*, its promoter, and the two alternatively spliced mRNAs (*d-MpLaeA* and *l-MpLaeA*). The horizontal arrows indicate the locations of the primers used in PCR. Sizes of the introns (I) and exons (E) are indicated in parentheses. (**B**) RT-PCR for generating the two full-length alternative spliced *MpLaeA* cDNAs. Lanes 1 and 3 were samples amplified from *M. pilosus* IFO4520 and MK-1 by primers (LF22 and LR1522), respectively, to get the 1.8 kbp *d-MpLaeA* (d) and the 1.5 kbp *l-MpLaeA* (l); lanes 2 and 4 by primers (LF296 and LR1522), respectively. (**C**) Southern blot of *MpLaeA*. Fifteen micrograms of genomic DNA was digested with *Bam*HI (Bh), *Bgl*I (Bl), *Eco*RI (E), and *Hin*dIII (H) and probed with DIG-labeled *d-MpLaeA* cDNA.

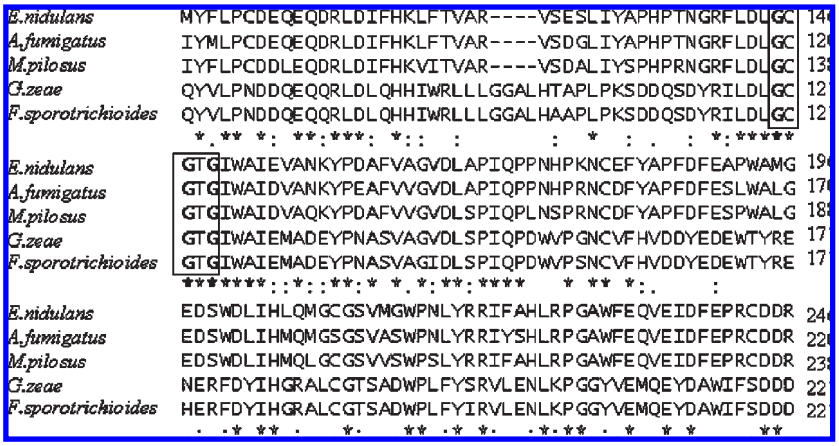

Figure 2. Amino acid alignment of fungal methyltransferases. The GXGXG (in boxes) *S*-AdoMet binding domain suggests putative methyltransferase activity of *MpLaeA*. The GenBank gi are 37622142 for *Emericella nidulans*, 37703327 for *Aspergillus fumigatus*, 42548965 for *Gibberella zeae*, and 28202126 for *Fusarium sporotrichioides*.

M. pilosus IFO4520 and MK-1 did not arise from the genomic sequence of *MpLaeA*.

The two spliced mRNAs were predicted to have the same longest open reading frame (ORF), which encodes a homologue of the methyltransferase (**Figure 2**). The predicted protein has a high sequence similarity with the fungal secondary metabolism positive regulator *LaeA* (*12*). *MpLaeA* and *LaeA* of *E. nidulans* (gi 37622142) show 70% (250/354) sequence identity and 84% positive homology at the amino acid level. The GXGXG *S*-AdoMet binding domain in the predicted amino acid sequence encoded by *MpLaeA* suggests putative methyltransferase activity (**Figure 2**). This pattern of amino acids has been observed in a large number of methyltransferases (*13*), but *MpLaeA* also lacks other domains (*21*) of methyltransferases such as *LaeA* (*12*). In addition, phylogenetic analysis also suggested that *MpLaeA* is a homologue of the methyltransferase gene at both genomic DNA and cDNA levels (data not show).

Developmental Regulation of the Alternative Splicing of *MpLaeA*. To investigate the alternative splicing of *MpLaeA* during development of *M. pilosus*, Northern blot of 20 μg total RNA was used to monitor *d*- and *l-MpLaeA* mRNA. In potato dextrose broth (PD) medium (**Figure 3**, left column), two alternatively spliced *d*- and *l-MpLaeA* mRNAs were transcribed at different growth stages in both *M. pilosus* IFO4520 and MK-1. The level of *l-MpLaeA* mRNA is significantly higher than that of *d-MpLaeA* in PD and GGP media (**Figure 3**). In GGP medium (**Figure 3**, right column), the *l-MpLaeA* mRNA could be detected at all growth stages of *M. pilosus* IFO4520 and the mutant MK-1, whereas the *d-MpLaeA* mRNA could be detected only at the late growth stages (14 days) in *M. pilosus* IFO4520 and at early growth stages (1−10 days) in the mutant MK-1. The transcription level of *d-MpLaeA* mRNA of MK-1 at late growth stage in GGP is lower than that of *M. pilosus* IFO4520. This evidence suggests that the alternative splicing of *MpLaeA*

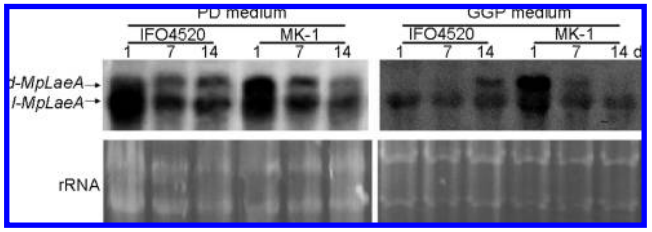

Figure 3. Developmental regulation of the alternative splicing of *MpLaeA* pre-mRNA. Twenty micrograms of total RNA was probed with DIG-labeled full-length antisense RNA probes of *d-MpLaeA*. SYBR-stained rRNA is indicated as a loading control, and RNA marker had been used to verify their size. Washes were at high stringent conditions (2× SSC/0.1% SDS at room temperature for 10 min twice, and 0.1× SSC/0.1% SDS at 68 °C for 20 min twice). 10^7 spores of *M. pilosus* IFO4520 and the mutant MK-1 were grown in 30 mL of potato dextrose broth (PD) medium or GGP broth medium (contains 7% glycerol, 3% glucose, 3.8% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$) at 25 °C with 120 rpm shaking.

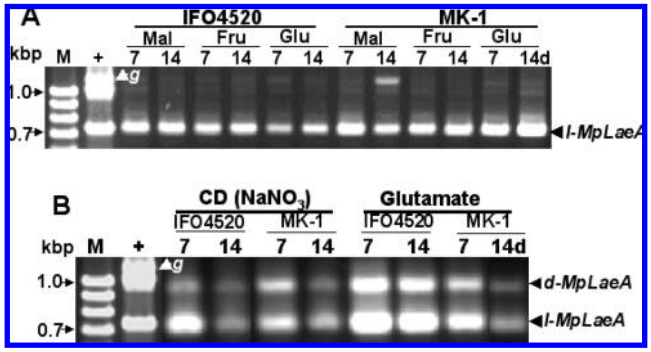

Figure 4. Medium regulation of the alternative splicing of *MpLaeA* pre-mRNA. RT-PCR was performed with a one-tube RT-PCR system by primer set (LF22+LR733) after 27 PCR cycles. The three bands of lane + were the PCR product mixture from the cloned *MpLaeA* gene (band *g*), *d*-cDNA (band *d*), and *l*-cDNA (band *l*) by primer set (LF22+LR733) for size control. (**A**) RT-PCR of *MpLaeA* in various carbon source media. 10^7 spores of *M. pilosus* IFO4520 and its mutant MK-1 were grown in 30 mL of media (3.8% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.2% $NaNO_3$) plus 3% maltose (Mal), 3% fructose (Fru), or 3% glucose (Glu) at 25 °C with 120 rpm shaking. (**B**) RT-PCR of *MpLaeA* in different nitrogen source media. 10^{14} spores of *M. pilosus* IFO4520 and the mutant MK-1 were grown in 100 mL of the media containing 0.1% Czapek−Dox broth of Difco, plus 2.9% glucose, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.2% $NaNO_3$ or 3% glutamate at 25 °C with 120 rpm shaking.

pre-mRNA is developmentally regulated in GGP medium, and alternative splicing patterns of *MpLaeA* are different between *M. pilosus* IFO4520 and its mutant MK-1.

Medium Regulation of Alternative Splicing of *MpLaeA*. As shown above, the transcription and alternative splicing of *MpLaeA* are different in PD and GGP media. Both carbon and nitrogen sources are thought to influence the gene transcription and monacolin K biosynthesis in *A. terreus* and *M. pilosus* (*7*, *20*). Several types of nitrogen and carbon sources were tested for their influence on the synthesis of monacolin K and the alternative splicing of *MpLaeA*. RT-PCR from 5 ng of mRNA was used to monitor the alternative splicing of *MpLaeA* pre-mRNA with the primers LF22 and LR733. The different sizes of amplified bands with the primer set of LF22 and LR733 could discriminate their origins as the + lane shown in **Figure 4**. The 1099bp band was amplified from the *MpLaeA* genomic DNA (*g* band in **Figure 4**), the 1009 bp band was from the *d-MpLaeA* cDNA (*d* band in **Figure 4**), and the 731 bp band was from *l-MpLaeA* cDNA (*l* band in **Figure 4**). *l-MpLaeA* mRNA could be detected in various carbon or nitrogen culture conditions (**Figure 4**), but the *d-MpLaeA* mRNA could only be amplified at all developmental stages in *M. pilosus* IFO4520 and mutant MK-1 (band *d* in **Figure 4B**), which were grown in the medium with glutamate or $NaNO_3$ as the main nitrogen source. The *d-MpLaeA* mRNA level was relatively higher in these two nitrogen-poor source media (**Figure 4B**) compared to in the nitrogen-rich source media (**Figure 4A**). In all of the different carbon source media (**Figure 4A**), alternative spliced *l-MpLaeA* mRNA was the main form, the alternative spliced *d-MpLaeA* mRNA being relatively low in this rich organic nitrogen media. The different carbon and nitrogen sources could affect the alternative splicing of *d-MpLaeA* pre-mRNA.

Blocking of GFP by 5′-UTR of *MpLaeA* in Bacteria. *d*- and *l-MpLaeA* mRNAs possess a differential and long 5′-untranslated region (5′-UTR), 714 bp 5′-UTR for *d-MpLaeA* and 436 bp 5′-UTR for *l-MpLaeA*. Theoretical analysis of these UTRs is shown in **Table 2**. In 5′-UTR, *d-MpLaeA* mRNA has more uORF and a higher free energy than *l-MpLaeA* mRNA. To test the influence of their 5′-UTRs on mRNA stability or translation efficiency, the 5′-UTRs of *d*- and *l-MpLaeA* cDNA were amplified from the cloned cDNA with a primer set of LF22 and IVR. The amplified 5′-UTRs were inserted between the *EGFP* gene and the *lac* promoter in the pEGFP vector (**Figure 5B**). These constructed plasmids were transformed into *E. coli* JM109. The EGFP assay showed that 5′-UTR of *d-MpLaeA* mRNA completely blocked EGFP activities in bacteria (**Figure 5A**) and that

Table 2. Theoretical Analysis of 5′-Untranslated Regions (UTRs) of *MpLaeA* mRNA

5′-UTR of	length (bp)	uATGs [a]	uORFs [b]	size of uORDs (no. of codons)	free energy [c] (kcal/mol)
d-MpLaeA	714	4	3	39, 62, 21	−148.3 to −156.1
l-MpLaeA	436	2	1	73	−86.9 to 91.5

[a] Number of ATG codes present upstream of the authentic translation start site. [b] Number of ORFs found upstream of the authentic translation start site. [c] The mean free energy values are indicated for each UTR for which the lowest and highest values are given. These were calculated using MFOLD (*22*).

5′-UTR of *l-MpLaeA* mRNA decreased EGFP activities by ~40%. The four above independent constructs have been tested; the effects of 5′-UTR on EGFP activities are similar. This indicated that *d-MpLaeA* mRNA might not be translated in fungi and is an ineffectively transcribed mRNA.

Influence of Alternative Splicing of *MpLaeA* on the Secondary Metabolism. To confirm the relationship between *MpLaeA* and fungal secondary metabolism, we tried to decrease the transcription level of *MpLaeA* by transformation. Two published transgenic protocols for the *Monascus* genus (*23*, *24*) were tested without satisfactory results. The only protocol that worked (see Materials and Methods) could produce a few transgenic *M. pilosus* IFO4520, but the transformation was unsuitable for the mutant MK-1.

To decrease the transcription level of *MpLaeA*, the antisense *d-MpLaeA* was transformed into *M. pilosus* IFO4520; three clones of *M. pilosus* IFO4520 with antisense *d-MpLaeA* were obtained. Southern blot showed that these three clones had an extra about 8 kb band except from the 5 kb genomic *MpLaeA*. The extra 8 kb band confirmed that the antisense *d-MpLaeA* had recombined in the genome of *M. pilosus* IFO4520 (**Figure 6A**). These transgenic clones tolerated 80 μg/mL hygromycin B on PDA or GGP solid media. A transgenic clone (clone 2) showed decreased transcription of the two alternative spliced mRNAs, using 2 μg of mRNA for Northern blot (**Figure 6B**). This clone was used to measure the monacolin K production in GGP

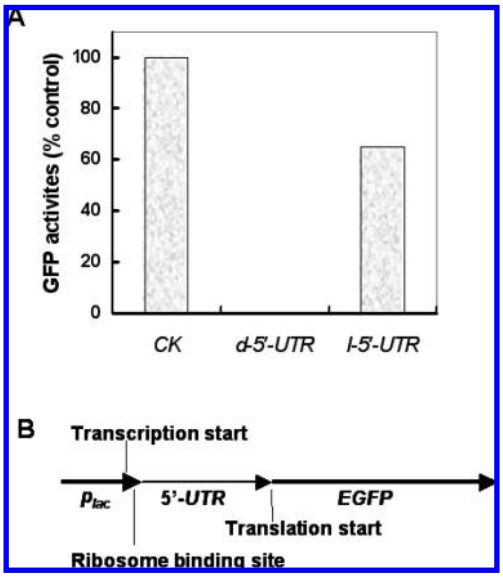

Figure 5. 5′-untranslated regions (5′-UTR) of *MpLaeA* decrease *EGFP* expression. (**A**) Influence of 5′-UTR on EGFP activities. *E. coli* JM109 with *pEGFP* (CK), *pd-UTR-EGFP* (*d*-5′-UTR), and *pl-UTR-EGFP* (*l*-5′-UTR) were checked in Luria−Bertani (LB) media plus 100 μg/mL ampicillin at 37 °C overnight. EGFP activity in the bacteria with *pEGFP* (CK) was taken as 100% of activity in the control. Results are the mean of 16 samples from 4 independent experiments with four constructs. (**B**) Schematic construction of *5′-UTR-EGFP*. The 5′-UTRs of *d*- and *l-MpLaeA* were inserted between the translation start codon (ATG) of the *EGFP* gene and *lac* promoter in the *pEGFP* vector and transformed into *E. coli* JM109.

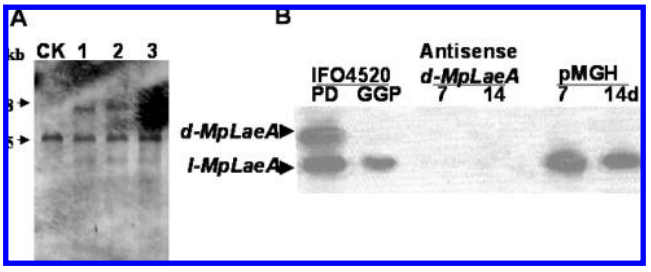

Figure 6. Down-regulation of *MpLaeA* decreases monacolin K production. *M. pilosus* IFO4520 transformed with antisense *d-MpLaeA* cDNA was controlled by *mtr* promoter. (**A**) Southern blot of the transgenic *M. pilosus* IFO4520 clones. Fifteen micrograms of genomic DNA was digested with *Bgl*II and probed with DIG-labeled *d-MpLaeA* cDNA. Except from the 5 kb band, the extra 8 kb band in lanes (1−3) indicated that the antisense *d-MpLaeA* has been recombined into the genome of *M. pilosus* IFO4520. Untransgenic *M. pilosus* IFO4520 served as control (CK). (**B**) Northern blot of the transgenic *M. pilosus* IFO4520. Two micrograms of mRNA was loaded and probed with DIG-labeled antisense *d-MpLaeA* RNA. mRNA of *M. pilosus* IFO4520 grown in PD and GGP media was used as size control.

liquid media. GGP medium was used for growing the transgenic clone to measure monacolin K without hygromycin B, because 20 μg/mL hygromycin B could have strongly induced the production of red pigments (data not show). No monacolin K could be detected in this transgenic clone when it was grown in GGP medium at 25 °C for 14 or 21 days (**Table 3**). This evidence indicated that the down-regulation of *MpLaeA* by antisense *d-MpLaeA* also resulted in less production of monacolin K in *M. pilosus* and that *MpLaeA* also could regulate monacolin K biosynthesis of *M. pilosus* as did *LaeA* in *A. nidulans* (*12*).

Table 3. Down-Regulation of *MpLaeA* Decreases Synthesis of Monacolin K[a]

	monacolin K (μg/g of FW)	fungal fresh wt (g)
pMGH	47 ± 18	1.2 ± 0.2
antisense *d-MpLaeA*		1.2 ± 0.2

[a] About 10^7 spores of *M. pilosus* IFO4520 with the empty vectors *pMGH* and *pMGH-antisense d-MpLaeA* were grown in 100 mL of GGP medium for 14 days at 25 °C.

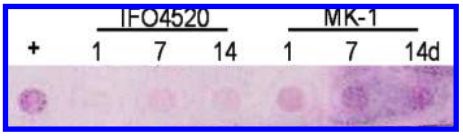

Figure 7. Northern dot blot of *MpLaeA*. Two micrograms of mRNA of *M. pilosus* IFO4520 and MK-1 grown in GGP media was dotted on membrane and probed with DIG-labeled *d-MpLaeA* cDNA; 1 ng of cloned *l-MpLaeA* cDNA was used as positive control (+).

DISCUSSION

MpLaeA is highly homologous to *LaeA*, which is a global positive regulator of fungal secondary metabolism including sterigmatocystin, penicillin, and lovastatin in *A. nidulans* (*12*). This suggests that *MpLaeA* might also be a regulator of the secondary metabolism in *M. pilosus*. Down-regulation of *MpLaeA* (**Figure 6B**) decreased the synthesis of moncolin K in *M. pilosus* (**Table 3**), and total transcripts of *MpLaeA* in *M. pilosus* mutant MK-1 was also higher than in its parent *M. pilosus* IFO4520 (**Figure 7**). The mutant MK-1 showed about 5-fold higher production of monacolin K than its parent *M. pilosus* IFO4520 (*20*). This evidence supports the correlation between the biosynthesis of monacolin K and *MpLaeA* transcripts in *M. pilosus*. *MpLaeA* was predicted to be a methyltransferase with a *S*-AdoMet binding domain. Methyltransferases are known to be specific for a wide range of substrates including proteins, nucleic acids, and small molecules. *MpLaeA* belongs to this large enzyme family with the signature *S*-AdoMet binding domain (*13*) and plays a wide range of biological roles including regulation. Monacolin K of *A. terreus* has two methyl groups derived from *S*-AdoMet, and two proteins (LNKS and LovF) have the methyltransferase domains in monacolin K biosynthetic pathway (*2*). In *A. nidulans*, *LaeA* was feedback-regulated by *aflR* (*12*), but *MpLaeA* has no AflR binding sites in either the gene region or its promoter. Although *A. nidulans LaeA* cDNA possesses a long 5′-UTR (642 bp) (*12*), it was not an alternative spliced gene. This might indicate that *MpLaeA* in *M. pilosus* is regulated by other mechanisms, unlike *LaeA* in *A. nidulans*(*12*).

Although the gene sequences of *MpLaeA* and its promoter are similar, expression and alternative splicing patterns of *MpLaeA* showed differences between *M. pilosus* IFO4520 and its mutant MK-1 (**Figures 3**, **4**, and **7**). In rich organic nitrogen media (GGP or media contains peptone), *M. pilosus* IFO4520 and MK-1 have relatively high productivity of monacolin K and red pigments (*20*), and they also have relatively less alternatively spliced *d-MpLaeA* mRNA (right column in **Figure 3** and **Figure 4A**). The 5′-UTR of *d-MpLaeA* mRNA has more uATGs, more uORFs, and a higher free energy than that of *l-MpLaeA* mRNA (**Table 2**). 5′-UTR has a big influence on mRNA stability and translation efficiency and plays a key role in post-transcriptional gene regulation (*25*). The uATG and uORF in 5′-UTR also controls the expression of the main open reading frame (ORF) and controls gene expression at translation level. In most cases, 5′-UTRs that enable efficient translation are short and relatively unstructured, have a low GC content, and contain no uATG

codons (*26*). The 5′-UTR of *d-MpLaeA* mRNA blocked the expression of fused GFP in *E. coli* (**Figure 5**), indicating that *d-MpLaeA* mRNA is an ineffectively spliced mRNA. The *d-MpLaeA* might not be translated into protein because of the possible secondary structure of its long 5′-UTR. Low levels of alternatively spliced *d-MpLaeA* mRNA might increase the secondary metabolism of *M. pilosus*.

The biosynthesis of fungal secondary metabolites is usually associated with their development and culture conditions (*27*). Monacolin K synthesis in *M. pilosus* was influenced by media (*11*, *20*). When cultured in the media with nitrate or glutamate as the main nitrogen source, *M. pilosus* grew very poorly and could not synthesize monacolin K, but in rich organic nitrogen (peptone) media, the two fungal strains grew well and could synthesize monacolin K and red pigments. The synthesis of monacolin K and red pigments of MK-1 was stronger than that of *M. pilosus* IFO4520 (*20*). These culture conditions increased the alternative spliced *d-MpLaeA* transcript (**Figure 4B**). In rich nitrogen media (GGP), at late growth stage, *d-MpLaeA* of MK-1 was repressed, but it was transcribed in *M. pilosus* IFO4520 (**Figure 3**, right column). Furthermore, when the expression of *MpLaeA* was decreaded by anti-*d-MpLaeA* transformation, production of monacolin K also decreased. These results suggested that *MpLaeA* might regulate the synthesis of monacolin K of *M. pilosus* through the alternative splicing of *MpLaeA* pre-mRNA.

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