JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 287–293 287
DOI:10.1021/jf903139x

# Identification of the *mokH* Gene Encoding Transcription Factor for the Upregulation of Monacolin K Biosynthesis in *Monascus pilosus*

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Monacolin K is a secondary metabolite synthesized by polyketide synthases (PKS) from *Monascus*. The monacolin K biosynthetic gene cluster, *mokA*−*mokI*, has been characterized in *Monascus pilosus*. The *mokH* gene encoding Zn(II)2Cys6 binuclear DNA binding protein is assumed to be an activator for monacolin K production. In this study, the *mokH* gene was cloned and driven by the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter for overexpression in *M. pilosus*. The transformants containing an extra copy of the *mokH* gene were obtained and verified by PCR and Southern hybridization. The transcripts of *mokH* in the transformants were expressed significantly higher than those of the wild-type strain. The transformants were stably inherited through the next generation, as determined by observation of the enhanced green fluorescent protein (EGFP). The transformant T-mokH1 also showed a 1.7-fold higher production of monacolin K than the wild-type strain in a time course analysis. Analysis of the RT-PCR products demonstrated that the monacolin K biosynthetic genes in the transformant were expressed to a greater extent than those in the wild-type strain. These results indicated that *mokH* upregulated the transcription of monacolin K biosynthetic genes and increased monacolin K production.

**KEYWORDS: Monacolin K; *Monascus pilosus*; transcription factor; glyceraldehyde-3-phosphate dehydrogenase**

## INTRODUCTION

*Monascus* spp. are filamentous fungi that produce secondary metabolites with polyketide structures and are known as as monacolin K, J, L, M, and X, all of which belong to the cholesterol synthesis inhibitor group (*1*−*4*). Dimerumic acid (antioxidant) and γ-aminobutyric acid (GABA) (hypotensive agent) are also found in *Monascus*-fermented product (*5*, *6*). In addition, the red pigments produced by *Monascus* species have been applied to various foods as natural colorants. Therefore, *Monascus* rice products are usually used as healthy foods in Asia. Monacolin K, or lovastatin, differs slightly in structure from compactin. Monacolin K contains a methyl group derived from *S*-adenosyl-L-methionine (SAM) at the C-6 position of the nonaketide-derived backbone that is absent in compactin (*7*). In our previous study, the monacolin K biosynthetic gene cluster in *Monascus pilosus* contains nine putative open reading frames involved in monacolin K biosynthesis (*8*). These nine genes, *mokA*−*mokI*, were highly homologous to the genes of the lovastatin biosynthetic gene cluster of *Aspergillus terreus* and the compactin biosynthetic gene cluster of *Penicillium citrinum* (*7*, *9*). They include two polyketide synthase genes thought to be responsible for the synthesis of nonaketide and diketide. Functional inactivation of the nonaketide synthase gene, *mokA*, demonstrates that it is essential for monacolin K biosynthesis (*8*).

The *mokH* gene encoding the transcription factor shares 54% and 49% similarity with the *lovE* gene of the lovastatin gene cluster and the *mlcR* gene of the compactin gene cluster, respectively (*7*−*9*). These transcription factors all exhibit a Zn(II)2Cys6-type zinc finger nucleotide binding domain, which is thought to be an activator of lovastatin and compactin production (*10*, *11*). Transformation of an extra copy of the *lovE* gene into the wild-type *A. terreus* results in a 7- to 10-fold overproduction of lovastatin (*10*). Likewise, an additional copy of the *mlcR* gene, fused to the promoter and terminator of *Aspergillus nidulans* 3-phosphoglycerate kinase (*pgkA*), can upregulate the expression of the compactin biosynthetic genes in *P. citrinum* (*11*). The transformant, *pgkA*(P)::*mlcR*, produces 10−15% more compactin in comparison to the parent strain. In this study, we characterized the function of the *mokH* gene in the transcription of the *mokA*−*mokI* genes and its productivity in monacolin K biosynthesis. An extra copy of the *mokH* gene driven by the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter of *M. pilosus* was integrated into the chromosome of

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Published on Web 12/07/2009

*M. pilosus*. Polymerase chain reaction (PCR) and Southern hybridization were used to confirm the fusion of *gpd*(P)::*mokH* and the copy numbers of transformants. Monacolin K expression and the productivity of its biosynthesis were also analyzed by reverse-transcription PCR and HPLC, respectively. Our results revealed that the *mokH* gene is a positive regulator of monacolin K biosynthesis.

## MATERIALS AND METHODS

**Strain Used and Growth Conditions.** *M. pilosus* BCRC38072, a monacolin K-producing strain isolated from red rice (anka) collected from a local traditional market, was used in this study. The strain was incubated on YM agar (DIFCO 271120, Detroit, Michigan) for one week, and spore suspensions were obtained by washing cultured YM agar plates with distilled water. Mycelium was harvested after incubation for 15 days at 25 °C with constant agitation in liquid medium (7% glycerol, 3% glucose, 3% monosodium glutamate, 1.2% polypetone, 0.2% $NaNO_3$, and 0.1% $MgSO_4 \cdot 7H_2O$).

**Nucleic Acid Manipulations.** Fungal genomic DNA was isolated by liquid nitrogen treatment according to the method developed by Bingle et al. (*12*). Total RNA from *M. pilosus* was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) for Northern hybridizations and RT-PCR analyses. Southern and Northern hybridizations were performed using the DIG system (DIG wash and buffer set) (Roche Diagnostics, Mannheim, Germany). The manipulations of DNA and RNA transfer, immobilization, and hybridization were carried out as described by Sambrook et al. (*13*). The *mokH* gene probe was DIG-labeled by PCR amplification using the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). The primer set used for the *mokH* gene was pmkHf: ACCTCATCGCTCCAGACCAT and pmkHr: CTGCGAGAGAATGAGAGTGC.

**Construction of the *mokH* Gene Expression Vector.** The glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter from plasmid pAN7-1 (*14*) was replaced with a 1.1 kb *Bgl*II-*Sal*I fragment of the *gpd* promoter using the primer set (gpd1: AGATCTGATCTGATATCAATGAGTACAAC and gpd2: GTCGACTTTTGATAGAGTGATAGATATTAG) from *M. pilosus* BCRC38072 (GenBank accession no. DQ984142) to obtain the plasmid pMS. The PCR-amplified product of the 1.5 kb *mokH* gene was introduced into the plasmid pMS by digestion of the *Sal*I-*Bam*HI site blunted by the End-It DNA End-repair kit (Epicenter, Madison, WI) to obtain the plasmid pMS-H. The PCR-amplified product of a 3.5 kb fragment containing the promoter, the *mokH* gene, and the terminator from pMS-H was introduced into the plasmid pMS-0.5hsp (*15*) by digestion of the *Cla*I site blunted with the End-It DNA End-repair kit to obtain the plasmid pMSmokH.

**Transformation of *M. pilosus* BCRC38072.** The protoplast was prepared according to the method developed by Chen et al. (*15*). The conidia from a one-week culture of *M. pilosus* were incubated in 100 mL of Vogel medium at 30 °C for 16−18 h. The mycelia were harvested on miracloth (Millipore, Bedford, USA) and washed in MA digestion solution (0.1 M maleic acid, pH 5.5, and 1.2 M $(NH_4)_2SO_4$). The mycelia were digested for 4−5 h using 100 mg of Yatalase (Takara, Tokyo, Japan), 100 mg of lysing enzyme (Sigma, St. Louis, USA), and 100 $\mu$L of $\beta$-glucuronidase (Sigma, St. Louis, USA) in 50 mL of MA digestion solution. To remove undigested mycelia, protoplasts were harvested by passing them through miracloth and by centrifugation at 1000 rpm for 10 min (Sorvall, Wilmington, USA). The protoplasts were maintained in 80% STC (1 M sorbitol, 50 mM Tris pH 8.0, 50 mM $CaCl_2$) and 20% PTC (40% PEG 4000, 50 mM Tris pH 8.0, 50 mM $CaCl_2$), and dimethylsulfoxide (DMSO) was added to a final concentration of 1%. For genetic transformation of *M. pilosus*, 100 $\mu$L of protoplasts was mixed with 1 mM aurintricarboxylic acid (ATA) and 5 $\mu$g of DNA. The mixtures were incubated on ice for 30 min. An amount of 1 mL of PTC was added, and the sample was mixed gently. Following incubation at room temperature for 20 min, the protoplast mixtures were added to 15 mL of SYP medium (1 M sorbitol, 0.1% yeast extract, 0.1% peptone, and 2% agar) containing 60 $\mu$g of hygromycin B/mL. Plates were incubated at 28 °C, and transformants were detected by PCR and Southern hybridization.

**Measurement of Red Pigment.** To determine red pigment and monacolin K levels, the strains were incubated in 50 mL of liquid medium for 15 days at 25 °C. The aliquots of *M. pilosus* and the transformant culture were cleared of cells and filtered with a 0.2 mm filter. The supernatants were analyzed at 500 nm with a spectrophotometer for the determination of red pigment levels.

**Measurement of Monacolin K.** Aliquots of the *M. pilosus* culture were cleared of cells and filtered through a 0.2 mm filter. The supernatants were analyzed by high-performance liquid chromatography (HPLC) performed on a Waters system (Waters, Milford, MA) fitted with a reverse-phase $C_{18}$ column (LichroCART 250-4, Rp-18e, 5 $\mu$m). The HPLC parameters were as follows: solvent A, 0.1% phosphorus acid in water; solvent B, acetonitrile; 35% A and 65% B in 30 min; flow rate, 1.5 mL $min^{-1}$; and detection by UV spectroscopy (Waters 600 pump and 996 pgotodiode array detector). A standard monacolin K compound (Sigma, St. Louis, USA) was used to confirm the HPLC analysis.

**Fluorescence Microscopy.** EGFP expression in transformant hyphae were compared by fluorescence microscopy to that of the wild-type hyphae. Fluorescence photomicrography was conducted on the specimens using a fluorescence microscope (Leica RXA, Mannheim, Germany). The red filter (Chroma, VT, USA), with peak excitation at 450−490 nm and peak suppression at 525−575 nm, was used to detect EGFP.

**Reverse-Transcription PCR.** Total RNA from 0.2 g of mycelial powder was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with 2 U of RNase-free DNase for 1 h. The first strand cDNA was synthesized by SuperScript III Reverse Transcription with random hexamers (Invitrogen, Carlsbad, CA) at 50 °C for 1 h with 5 $\mu$g of RNA and used as the template for PCR. The PCR amplification was carried out on a PCR system 2700 thermocycler (Applied Biosystems, Foster City, CA). The 50 $\mu$L reaction mixture contained 1 $\mu$L of cDNA as template, 0.2 mM primers, 2 units of Taq DNA polymerase, and 800 mM dNTPs. The nine primer sets listed in **Table 1** were used to carry out the monacolin K biosynthetic gene amplification performed in this study. The reaction conditions included an initial denaturation for 5 min at 96 °C, followed by 25 or 30 cycles for 1 min at 96 °C, 1 min at 52 or 55 °C, and 1 min at 72 °C with a final extension time of 7 min at 72 °C. The PCR products were confirmed by electrophoretic analysis in a 1.2% agarose gel.

## RESULTS

**Sequence Analysis and Construction of the pMSmokH Expression Vector.** The biosynthesis of polyketide in fungi has many layers of regulation. Some layers are controlled by environmental factors, while others play a role in a genetic regulation of elements of secondary metabolism. The position of the transcription factor within a polyketide gene cluster has implications for its regulation (*16*). The monacolin K biosynthetic gene cluster contains the transcription factor *mokH*, which is similar to *lovE* and *mlcR* in lovastatin and compactin biosynthesis, respectively (**Figure 1A**). Analysis of the cDNA sequence revealed that the *mokH* gene includes one intron, which generally has a splicing junction at GU-AG, in contrast to the intron numbers of *lovE* and *mlcR* (**Figure 1B**). However, they all contain the cysteine-rich Zn(II)2Cys6-type zinc finger nucleotide-binding domain that shares the consensus sequence $CX_2CX_6CX_{11}CX_2CX_6C$.

Glyceraldehyde-3-phosphate dehydrogenase (GPD), which catalyzes the reversible oxidation and phosphorylation of glyceraldehyde-3-phosphate to produce 1,3-diphosphoglycerate, is one of the key enzymes in the Embden−Meyerhof−Parnas pathway, also known as the glycolytic pathway. The GPD protein comprises 5% of all cellular protein in eukaryotes (*17*, *18*). It is a well-known house-keeping gene that is considered to be highly and constitutively expressed. Construction of the expression vector using the *gpd* promoter from *M. pilosus* can increase the expression of the *mokH* gene. The primer set used in this study, based on the Bacterial Artificial Chromosome (BAC) library constructed from *M. pilosus* BCRC38072 (*19*), was designed to amplify the promoter of the *gpd* gene (GenBank accession no. DQ984142).

**Table 1.** Primers Used to Amplify Monacolin K-Related Genes Fragments by RT-PCR

| primers[a] | sequence | position[b] | PCR product (bp) |
|---|---|---|---|
| mokA/F | 5′-CCTCAGCGGTGACATCGTGG (20-mer) | 4115 to 4134 (*mokA*) | 350 |
| mokA/R | 5′-GGTGACGACCTTCGTGAGCGT (21-mer) | 4444 to 4464 (*mokA*) | |
| mokB/F | 5′-AAGCCAACTATGCGGCTGCA (20-mer) | 7363 to 7382 (*mokB*) | 321 |
| mokB/R | 5′-CGTTTGTGAAGTGAGGGCCA (20-mer) | 7664 to 7683 (*mokB*) | |
| mokC/F | 5′-GGCCTGAGCCGAAGAAGTAC (20-mer) | 1413 to 1432 (*mokC*) | 305 |
| mokC/R | 5′-TCAGAGATCTTCGTCCCGAC (20-mer) | 1698 to 1717 (*mokC*) | |
| mokD/F | 5′-CAGAACCCCAGCATCCCCAT (20-mer) | 361 to 380 (*mokD*) | 290 |
| mokD/R | 5′-ACCATCTTCGAACCCGCCAG (20-mer) | 631 to 650 (*mokD*) | |
| mokE/F | 5′-AATGGTCACCGCCGACTGGA (20-mer) | 961 to 980 (*mokE*) | 246 |
| mokE/R | 5′-TTCTCTCCCGACAACTGCCC (20-mer) | 1187 to 1206 (*mokE*) | |
| mokF/F | 5′-GAGTGGATCTACGGCGCCAA (20-mer) | 796 to 815 (*mokF*) | 354 |
| mokF/R | 5′-CATCAAGTCCACGGTCTCGG (20-mer) | 1130 to 1149 (*mokF*) | |
| mokG/F | 5′-CCTCGCTCTGAATATGACCC (20-mer) | 803 to 822 (*mokG*) | 217 |
| mokG/R | 5′-TCGGATCGGCTTCTCAAACC (20-mer) | 1000 to 1019 (*mokG*) | |
| mokH/F | 5′-CCTTCATGCACGCCCTATCC (20-mer) | 1109 to 1128 (*mokH*) | 334 |
| mokH/R | 5′-CGGGCCAGGGAAAAGATCTC (20-mer) | 1423 to 1442 (*mokH*) | |
| mokI/F | 5′-GCACAATGCCTGCTCCCAGA (20-mer) | 966 to 985 (*mokI*) | 358 |
| mokI/R | 5′-ACGTCCTTCACGGCCTGGAA (20-mer) | 1304 to 1323 (*mokI*) | |
| 18SrRNA/F | 5′-TGCCAGCAGCCGCGGTAATT (20-mer) | | 332 |
| 18SrRNA/R | 5′-AGCTGAATACTGACGCCCCC (20-mer) | | |

[a] F, forward primer; R, reverse primer. [b] Sites corresponding to those of the *M. pilosus* BCRC38072 monacolin K gene cluster (GenBank accession no. DQ176595).

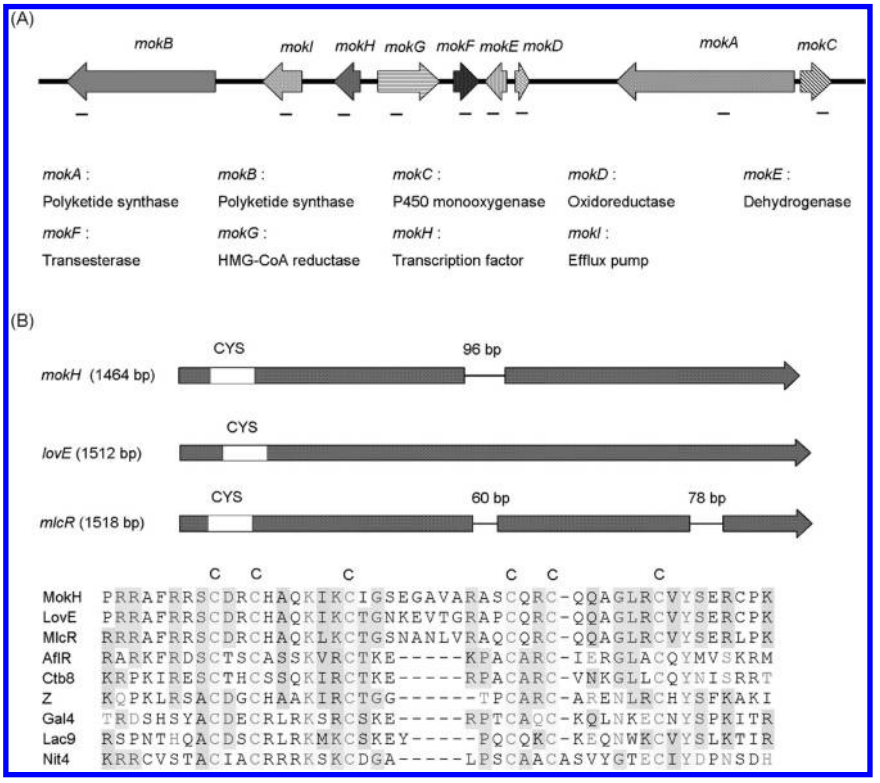


**Figure 1.** (**A**) Monacolin K biosynthetic gene cluster of *M. pilosus* BCRC38072. The small black bars indicate the sequences assayed by RT-PCR analyses. (**B**) The structures of the Zn(II)2Cys6-type transcription factors encoded by the *mokH* gene from *M. pilosus*, *lovE* from *A. terreus*, and *mlcR* from *P. citrinum* and the multiple alignment of the Zn(II)2Cys6 binuclear DNA binding domain of the *mokH* gene with related organisms. The abbreviation CYS represents the cysteine-rich region. The line indicates the intronic region. The following are the accession numbers for the comparisons of the cysteine-rich regions used: MokH (*M. pilosus*, DQ176595), LovE (*A. terreus*, AF141925), MlcR (*P. citrinum*, AB072893), AflR (*Aspergillus pseudotamarii*, AAM02990), Ctb8 (*Cercospora nicotianae*, ABK64185), Z (*Trichoderma virens*, ABV48713), Gal4 (*Aspergillus fumigatus*, XP_754932), Lac9 (*Pichia stipitis*, ABN67063), and Nit4 (*Neurospora crassa*, AAA33602).

This promoter triggers expression of *mokH*. The pMSmokH expression vector was constructed and included a fusion of the hygromycin B resistance gene (HPH) with enhanced green fluorescent protein (EGFP). pMSmokH was further utilized to achieve overexpression of *mokH* for the genetic transformation in *M. pilosus* BCRC38072.

**Characterization of Transformants with *mokH* Overexpression.** To assess the effects of *mokH* overexpression on the transcriptional regulation of monacolin K biosynthetic genes, three transformants of *M. pilosus* BCRC38072 were obtained following the transformation of the plasmid pMSmokH containing the *mokH* gene under the transcriptional control of the *gpd* promoter. All of these

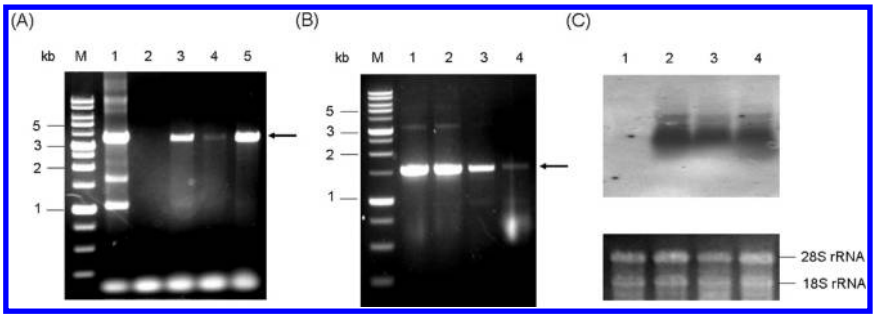


**Figure 2.** (**A**) Electrophoretic analysis of the PCR product for the *mokH* gene containing the *gpd* promoter and the *trpC* terminator with the primer set pgpdF and ptrpR (pgpdF: 5′-GAAGATCTGATATCAATGAGTACAACTATATC and ptrpR: 5′-TCGAGTGGAGATGTGGAGTGGG). Lane 1: expression vector, pMSmokH. Lane 2: wild-type strain, *M. pilosus* BCRC38072. Lane 3: T-mokH1. Lane 4: T-mokH2. Lane 5: T-mokH3. The abbreviation "M" indicates the 1 kb ladder marker. (**B**) Electrophoretic analysis of the nested-PCR product obtained for the *mokH* gene with the pmHF and pmHR primer set (pmHF: 5′-CTCGCTCACAAACAATGCAC and pmHR: 5′-AAGGGCCTTGACCGTAGCAT). Lane 1: expression vector, pMSmokH. Lane 2: T-mokH1. Lane 3: T-mokH2. Lane 4: T-mokH3. (**C**) Northern hybridization analysis of the *mokH* gene present in DNA from the wild-type strain (Lane 1), T-mokH1 (Lane 2), T-mokH2 (Lane 3), and T-mokH3 (Lane 4) hybridized with the *mokH* probe.

transformants showed 2-fold greater monacolin K production than the wild-type strain after 14 days of cultivation. The three transformants containing the complete *gpd* promoter, *mokH* gene, and *trpC* terminator were verified by PCR, nested-PCR (**Figure 2A**,**B**), and sequencing. Furthermore, the transcripts from the *mokH* gene in these transformants were expressed at a higher level than those of the wild-type strain, as verified by Northern blot analysis (**Figure 2C**).

The copy number of the *mokH* gene in the wild-type strain (BCRC38072) and three transformants (T-mokH1, T-mokH2, and T-mokH3) was determined by Southern hybridizations using the restriction enzymes *Sal*I, *Xho*I, *Hin*dIII, and *Nar*I (**Figure 3**A). *Sal*I and *Xho*I cut at one site in the pMSmokH plasmid, whereas *Hin*dIII and *Nar*I did not cut within the plasmid. The 3.2 kb *Sal*I and 2.5-kb *Xho*I fragments corresponded to the single copy of *mokH* in the wild-type strain BCRC38072. The transformants contained two integration events (two copies of *mokH*) using *Sal*I and *Xho*I restriction enzymes. The 3.4 kb *Hin*dIII and 5 kb *Nar*I fragments corresponding to the single copy of *mokH* were found in the wild-type strain BCRC38072. Two transformants, T-mokH1 and T-mokH3, also showed two integration events when cut with the *Hin*dIII and *Nar*I restriction enzymes. However, only one fragment corresponding to the transformant T-mokH2 was detected. These results suggested that the plasmid (pMSmokH) integrated as a tandem repeat in T-mokH2 (**Figure 3B**), while it randomly integrated into the ectopic locations of the chromosome in the transformants T-mokH1 and T-mokH3. These results indicate that all three of the transformants contained two copies of *mokH*.

Expression of EGFP was detected by fluorescence microscopy and shown in **Figure 3C**,**D**. Fluorescence was clearly observed in these transformants when excited with a red filter. These results indicated that the EGFP protein was extensively expressed in the mycelia and spores. It was also stably inherited through the next generation after several rounds of cultivation.

**Analysis of Monacolin K Production.** In a time course study, the transformant T-mokH1 was used for further determination of monacolin K production and for RT-PCR analyses. In previous studies, monacolin K production in *Monascus* gradually increased with the duration of cultivation (*20*). The amounts of monacolin K produced from the wild-type strain and transformant were determined by HPLC after 15 days of cultivation without the addition of the antibiotic hygromycin B. Although the initiation of monacolin K yield was not altered, the constitutive overexpression of *mokH* led to a higher accumulation of monacolin K after 8 days of cultivation (**Figure 4A**). Monacolin K production in the T-mokH1 transformant was 1.7-fold higher than the corresponding values

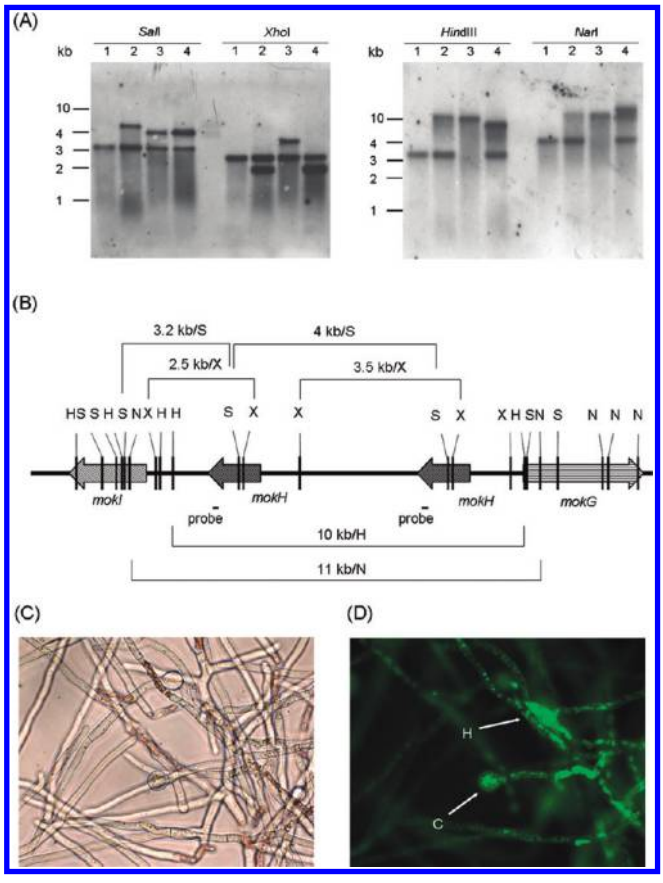


**Figure 3.** (**A**) Southern hybridization analyses of *mokH* copy number in *M. pilosus* BCRC38072 (Lane 1) and the transformant genomes of T-mokH1 (Lane 2), T-mokH2 (Lane 3), and T-mokH3 (Lane 4). Chromosome DNAs extracted from the wild-type strain and transformants were digested with *Sal*I, *Xho*I, *Hin*dIII, and *Nar*I, separated by electrophoresis gel and hybridized to the *mokH* probe. (**B**) Extra copy of *mokH* inserted into the monacolin K gene cluster in the transformant T-mokH2. S: *Sal*I restriction enzyme. X: *Xho*I restriction enzyme. H: *Hin*dIII restriction enzyme. N: *Nar*I restriction enzyme. Enhanced green fluorescent protein (EGFP) in transformed *M. pilosus* was analyzed by bright field (**C**) and fluorescence (**D**) microscopy. The arrows "H" and "C" indicate the hyphae and conidia of the transformant, respectively. The photograph was taken at a magnification of 1000×. The exposure time was 1−2 s.

for the wild-type strain, while the cultured cell mass revealed no difference from wild type (**Figure 4B**). The amount of red pigment

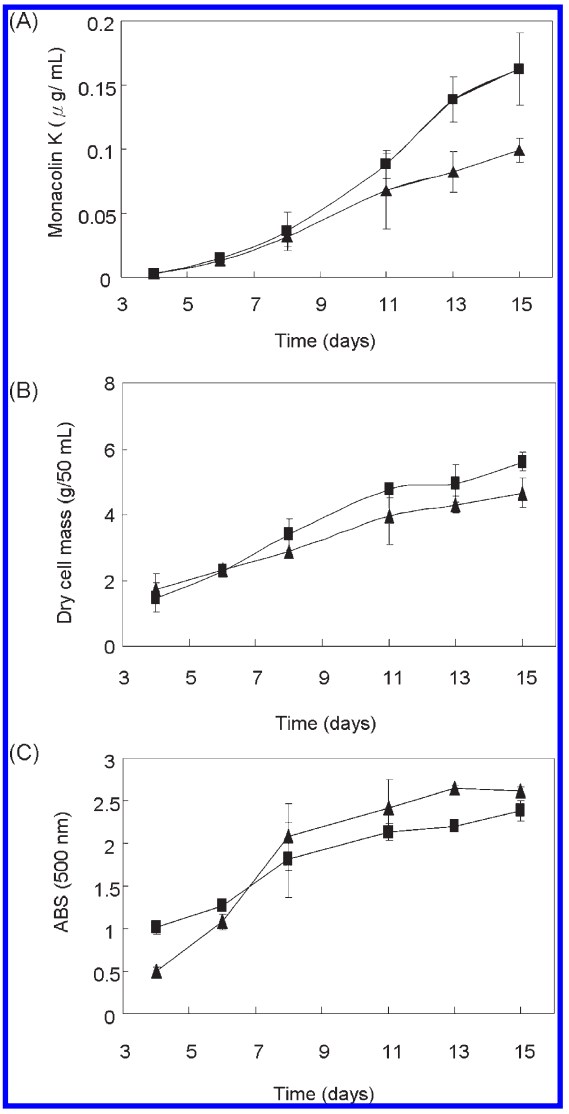


**Figure 4.** Submerged cultures of the wild-type strain BCRC38072 (▲) and the transformant T-mokH1 (■) were incubated on a 200 rpm rotary shaker for 15 days at 25 °C for the analyses of monacolin K (**A**), cell mass (**B**), and red pigment (**C**).

was also determined as a measure of the effect of *mokH* transcription factor overexpression. The results showed that the production of red pigment in T-mokH1 was similar to that in the parent strain, suggesting that the altered *mokH* expression does not affect the production of red pigment (**Figure 4C**).

**Transcription of Monacolin K Biosynthesis Genes.** To confirm the effect of *mokH* overexpression on the transcriptional regulation of the monacolin K biosynthetic genes, several sets of oligonucleotide primers were designed to analyze the expression of the nine genes (*mokA*−*mokI*) by RT-PCR (**Table 1**). The expression of the *mokH* gene was higher in the transformant T-mokH1 than that in the wild-type strain BCRC38072. Monacolin K biosynthetic gene expression in the transformant T-mokH1 appeared earlier than that in the wild-type strain (**Figure 5**). These results were consistent with the monacolin K productivities of the transformant described above and demonstrated that *mokH* upregulated the transcription of the monacolin K biosynthetic genes.

## DISCUSSION

Monacolin K synthesized by the polyketide gene clusters from *Monascus* is used to reduce serum cholesterol levels in humans.

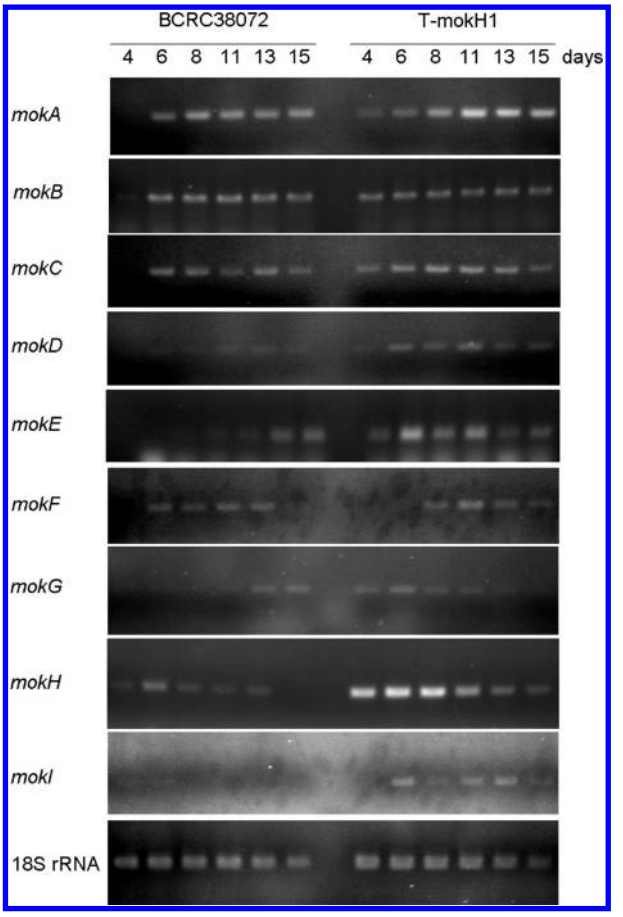


**Figure 5.** Time course of monacolin K biosynthetic gene expression profiles in the wild-type strain BCRC38072 and the transformant T-mokH1 determined by RT-PCR analysis. The primer sets used are listed in **Table 1**.

Thus, there is enormous value in increasing the production of monacolin K (*20*). A great deal of effort has been directed at optimization of the nutrient parameters for monacolin K production in *Monascus* species (*20*−*25*). Previous studies have shown that the mediation of the pH value and ethanol addition can increase the monacolin K level by 47% and decrease the citrinin level by 54% (*21*). In addition, carbon and nitrogen sources regulate monacolin K biosynthesis in *Monascus* (*23*−*25*). However, no study has yet tried to improve monacolin K production by genetic regulation in *Monascus*. Our previous study revealed that monacolin K is synthesized by polyketide synthase in *M. pilosus* BCRC38072 (*8*). This study identified the transcription factor Zn(II)2Cys6 binuclear DNA binding protein as a monacolin K biosynthesis contributor (**Figure 1**A). The functional analysis of *mokH*, a regulatory gene for monacolin K biosynthesis, was further explored.

The regulation of clustered secondary metabolite genes is complex (*26*, *27*). According to the model of sterigmatocystin polyketide (ST, the penultimate precursor to aflatoxin) or aflatoxin in *A. nidulans*, the biosynthesis signal transduction pathway G-protein, a methyltransferase (LaeA) and a transcription factor (AflR) are involved in fungal secondary metabolism (*26*−*28*). Over the past few years, several attempts have been made to demonstrate that the transcription factor within the polyketide biosynthetic gene cluster plays an important role as an activator for the production of polyketide (*28*). The *aflR* gene, which encodes a Zn(II)2Cys6 binuclear DNA binding protein, appears to be a transcriptional regulator of aflatoxin pathway genes. Disruption of *aflR* results in the loss of aflatoxin production,

whereas *aflR* overexpression leads to increased aflatoxin production in *Aspergillus* (*29*, *30*). Likewise, *ctnA*, which encodes a Zn(II)2Cys6 binuclear DNA binding protein, is a major activator of citrinin biosynthesis in *Monascus purpureus*. A strain of *M. purpureus* in which *ctnA* has been disrupted also exhibits a decrease in citrinin production (*31*), whereas heterologous expression of an additional copy of *ctnA* in *Aspergillus oryzae* results in 400-fold higher citrinin production than that of the parental transformant (*32*). In the present study, we found that the *mokH* gene shared 54% and 49% similarity with the *lovE* gene of the lovastatin gene cluster in *A. terreus* and the *mlcR* gene of the compactin gene cluster in *P. citrinum*, respectively. Transformants containing two copies of the *mokH* gene-encoded transcription factor were obtained. After several rounds of cultivation without addition of the antibiotic hygromycin B, the ectopic *mokH* gene of the transformants was stably inherited through the next generation, as shown in the conidia (**Figure 3C**,**D**). Moreover, increased monacolin K was observed in the transformant (**Figure 4A**). This finding was more consistent with the earlier expression of monacolin K biosynthetic genes in the transformant than in the wild-type strain (**Figure 5**). These results were in agreement with the findings of Kennedy et al. (*9*, *10*), who demonstrated that an extra copy of the regulatory *lovE* gene can improve the lovastatin yield. A similar regulation effect on compactin biosynthesis was found in *P. citrinum*, with increases of 10−15% (*11*).

The sequence alignment of *mokH* showed homology with the *lovE* and *mlcR* genes (**Figure 1B**). This alignment showed that they are all Zn(II)2Cys6 binuclear cluster proteins and possess DNA-binding and activation domains typical of the Gal4 family of positive regulatory proteins (*33*). The N-terminal conserved region was $CX_2CX_6CX_{11}CX_2CX_6C$, which represents a common arrangement of cysteines observed in *mokH*, *lovE*, and *mlcR* (**Figure 1B**). Furthermore, regulatory proteins, including AflR, Ctb8, Z, Gal4, Lac9, and Nit4, contained the consensus sequence, $CX_2CX_6CX_nCX_2CX_nC$, with a variable-length spacer between the cysteines. The Gal4-type transcription factor binds to a palindromic consensus sequence, such as 5′-CGGNxCCG-3′ or 5′-CCGNxCGG-3′, where Nx represents variable spacing (*34*). In contrast to the nucleotide-binding sequence of Gal4, AflR binds to the partially palindromic consensus sequence 5′-$TCGN_5$-CGR-3′. Here, within the region of the monacolin K biosynthetic gene cluster in *M. pilosus* BCRC38072, several consensus sequences, 5′-$TCGN_5CGR$-3′, were also found in the promoter regions of different genes. Thus, it was suggested that the expression levels of monacolin K biosynthetic genes were influenced by the regulatory gene, *mokH*, which contains the same nucleotide-binding sequence. In addition, the results demonstrated that the *mokH* gene was required for increasing monacolin K production. Further study of the transcription factor binding sequence, including the electrophoretic mobility shift assay (EMSA) and footprinting, will clarify the individual regulation of each gene in monacolin K biosynthesis.

Overexpression of *aflR* using the fusion *gpd*(P)::*aflR* in *Aspergillus flavus* increases the high production of aflatoxin which was not altered in the initial time (*29*). In this study, the transformant T-mokH1 harboring *gpd*(P)::*mokH* exhibited an initial monacolin K accumulation profile similar to that of the wild-type strain (**Figure 4A**). Nevertheless, the monacolin K yield in the transformant T-mokH1 contributed to the increase, exhibiting a trend different from that observed for the wild-type strain. It is known that monacolin K production by *Monascus* species is dependent on the culture conditions (*24*, *25*). In this study, the profile of monacolin K production was consistent with the finding that modulating the pH value significantly increases monacolin K production in later days but not during the initial days of the measurement (*20*, *21*). This result suggested that the regulation of monacolin K production may occur in the decelerated growth phase. Moreover, rather than increasing the production of the red pigment (**Figure 4C**), MokH represented a point of direct regulation of monacolin K biosynthesis. It is well documented that the biosynthesis of a polyketide usually contains a specific transcription factor within the gene cluster (*16*). The monacolin K biosynthetic genes of *M. pilosus* and the lovastatin biosynthetic genes of *A. terreus* are othologs. They are related to compactin biosynthesis in *Penicillium citrinum*. Although the efficiencies of lovastatin, monacolin K, or compactin production through the overexpression of transcription factor are quite different, this disparity might have been caused by the genetic divergence and the complex regulation of each transcription factor due to the lower amino acid similarities and different growth rates of the fungal species (*8*, *10*, *11*).

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**Received for review September 6, 2009. Revised manuscript received November 16, 2009. Accepted November 17, 2009.**