The Journal of Microbiology (2011) Vol. 49, No. 3, pp. 473-480
DOI 10.1007/s12275-011-0362-4

Isolation and Characterization of a Reducing Polyketide Synthase Gene from the Lichen-Forming Fungus *Usnea longissima*

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(Received September 8, 2010 / Accepted February 9, 2011)

The reducing polyketide synthases found in filamentous fungi are involved in the biosynthesis of many drugs and toxins. Lichens produce bioactive polyketides, but the roles of reducing polyketide synthases in lichens remain to be clearly elucidated. In this study, a reducing polyketide synthase gene (UlPKS3) was isolated and characterized from a cultured mycobiont of *Usnea longissima*. Complete sequence information regarding UlPKS3 (6,519 bp) was obtained by screening a fosmid genomic library. A UlPKS3 sequence analysis suggested that it contains features of a reducing fungal type I polyketide synthase with β-ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase (ER), ketoacyl reducatse (KR), and acyl carrier protein (ACP) domains. This domain structure was similar to the structure of ccRads1, which is known to be involved in resorcylic acid lactone biosynthesis in *Chaetomium chiversii*. The results of phylogenetic analysis located UlPKS3 in the clade of reducing polyketide synthases. RT-PCR analysis results demonstrated that *UlPKS3* had six intervening introns and that *UlPKS3* expression was upregulated by glucose, sorbitol, inositol, and mannitol.

***Keywords*:** lichen forming-fungi, mRNA expression, reducing polyketide syntheses, *Usnea longissima*

Polyketides have long been recognized as one of the most important classes of secondary metabolites (Cox, 2007) and represent a class of over 7000 known structures, more than 20 of which are commercial drugs (Weissman and Leadlay, 2005). Polyketides are synthesized from small carbon precursor acids whose successive condensation is catalyzed by polyketide synthase (PKS) (Cox, 2007). Fungal polyketide synthases (PKSs) can generate an amazingly diverse array of chemical compounds from simple aromatics (e.g., orsellinic acid and 6-methylsalicylic acid) to highly modified complex reduced-type compounds (e.g., T-toxin) (Cox, 2007; Fujii, 2010). According to the variations in their optional processing domains, the type I fungal PKSs are divided into three classes: non-reducing (NR-PKS), partially-reducing (PR-PKS), and highly-reducing PKSs (HR-PKS) (Kroken *et al*., 2003; Cox, 2007). Among these PKS genes, the HR-PKSs found in filamentous fungi are the most interesting, but the least understood (Cox, 2007; Chiang *et al*., 2010).

Many secondary metabolites of lichens or lichen-forming fungi (LFF) evidence profound anti-fungal, anti-bacterial, anti-viral, and anti-oxidant bioactivities (Boustie and Grube, 2007; Stocker-Wörgotter, 2008). Accordingly, lichens and LFF have huge potential value in new drug discovery (Huneck, 1999; Müller, 2001; Boustie and Grube, 2007; Shukla *et al*., 2010). However, lichens grow quite slowly in nature, and thus it is difficult to analyze confidently the biosynthesis of their secondary metabolites and to achieve the large-scale extraction of these metabolites (Balaji and Hariharan, 2007). Moreover, it is quite challenging to synthesize bioactive compounds under culture conditions (Miao *et al*., 2001). Therefore, Miao *et al*. (2001) previously mentioned the use of genetic approaches for harvesting lichen products. The successful isolation and functional analysis of PKSs will be the first step in the production of lichen products via genetic approaches. Although many studies of lichen PKSs have been conducted (Chooi *et al*., 2008; Brunauer *et al*., 2009; Valarmathi *et al*., 2009), only one study has been performed on reducing-PKSs in lichens to date, namely SocPKS1 of *Solorina crocea* (Gagunashvili *et al*., 2009).

In this study, a full-length reducing PKS gene (*UlPKS3*) was isolated from an *Usnea longissima* mycobiont by screening a genomic library. The isolated clone has the obvious characteristics of a reducing PKS gene including the KS, AT, DH, ER, KR, and ACP conserved domains. Its domain structure was similar to the structure of ccRads1, which is involved in resorcylic acid lactone biosynthesis in *Chaetomium chiversii*. Finally, UlPKS3 expression under a number of culture conditions is briefly described.

Materials and Methods

Lichen sample collection and lichen-forming fungus separation

Fresh thalli of *U. longissima* were collected from northwestern Yunnan Province, China (N26°37'44.3" E99°43'51.9"; altitude: 3966 m) in 2005. Voucher samples were deposited with the lichen herbarium of Kunming Botanical Institute, CAS, and duplicates were deposited with the Korean Lichen Research Institute at Sunchon National University. The LFFs of *U. longissima* were isolated from the thalli of the lichens via the tissue culture method (Yamamoto *et al*., 1985). A voucher sample of *U. longissima* LFF was deposited with the Korea Lichen and Allied Bioresearch Center, Sunchon National University. The LFF was grown and maintained in malt-yeast (MY) medium

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(Difco, USA). To confirm the identity of the isolated LFF, the total DNA of the LFF and the original thalli of *U. longissima* were isolated with a DNeasy Plant Mini kit (QIAGEN GmbH, Germany). PCR was then conducted using the primers ITS5 (5′-GGAAGTAAAAGT CGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) to specifically amplify the fungal ITS regions. The PCR products were subsequently purified using a PCR Product Purification kit (Intron Biotechnology, Korea), after which they were sequenced using the same primers.

PCR amplification of the KS domain

Total DNA of *U. longissima* LFF was isolated with a DNeasy Plant Mini kit (QIAGEN GmbH). A pair of CODEHOP primers predicted to bind to regions conserved within the clade III NR-PKSs (NR3KS-F: 5′-CTACGACGCCTTCGACCAYMRNTTYTT-3′ and NR3KS-R: 5′-GGGGTGCCGGTGCCRTGNGCYTC-3′) was used for KS fragment amplification (Chooi *et al*., 2008). The PCR process was conducted in accordance with touchdown PCR cycle conditions as recommended by Rose *et al*. (1998) for CODEHOP primers. The PCR product was subcloned into the pGEM-T vector (Promega, USA). Six white colonies with the inserted fragment were selected for sequencing.

Southern blotting

A specific pair of primers (Fnrks26: 5′-GCTTATGAGGCGTTGGAG AA-3′ and Rnrks26: 5′-TTCAAGATAGCGACTGCGGT-3′) were designed according to the KS domain fragment sequence amplified by NR3KS-F and NR3KS-R. The DIG-labeled DNA probe NRks26 was generated from the cloned KS domain PCR product using DIG-High Prime Labeling Mix (Roche Applied Sciences, Switzerland). Approximately 20 μg of genomic DNA was digested with restriction enzyme (TaKaRa, Japan) and incubated overnight at 37°C to ensure complete digestion. The digested genomic DNA was then electrophoresed on a 0.7% (w/v) agarose gel and transferred onto positively charged nylon membranes (GE Healthcare, UK). The membranes were hybridized with the DIG-labeled probe NRks26 at 42°C and detected with the DIG Luminescent Detection System (Roche Applied Sciences).

Genomic library construction and screening

For genomic library construction, the genomic DNA of *U. longissima* LFF was prepared with a DNeasy Plant Maxi kit (QIAGEN). The genomic libraries were constructed using a CopyControl™ HTP Fosmid Library Production kit (Epicentre Biotechnologies, USA), in accordance with the manufacturer's instructions. The *U. longissima* genomic library colonies (3×10^3) were transferred onto Nytran membranes (GE Healthcare) and fixed for hybridization in accordance with the manufacturer's instructions. The membranes were hybridized with the DIG-labeled probe NRks26 at 42°C, and positive signals were detected using a Digoxigenin (DIG) Detection Starter kit I (Roche).

Sequencing and gene cluster analysis

Two positive clones (FoNRks2625 and FoNRks2662) were obtained. Sequencing with Fosmid sequencing primers (FP: 5′-GTACAACGA CACCTAGAC-3′; RP: 5′-TAATACGACTCACTATAGGG-3′) was conducted to ensure that there was no PKS sequence at the end of the insertion fragment. Sequencing with the probe primer (Fnrks26) was used to determine that the insertion fragment included the PKS fragment. One clone including a complete PKS fragment (FoNRks2662) was selected for sequencing from the NRks26 probe. The sequencing was conducted by Genotech Co. Ltd. (Korea). The potential open reading frame (ORF) of FoNRks2662 was scanned with FGENESH (Berg *et al*., 2008) using *Aspergillus* as the matrix (http://linux1.softberry.com). The putative function of these ORFs was determined using a basic local alignment search tool (BLAST). The conserved U1PKS3 domain was verified using the CDD-Search/ PRS-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The putative PKS in FoNRks2662 was designated *UlPKS3*.

Multiple alignment and phylogenetic analyses

The UlPKS3 KS domain was aligned with 24 fungal PKS sequences (reducing and non-reducing types) retrieved from GenBank. The alignment was analyzed using the CLUSTAL W application embedded in the MEGA 4.0.2 program (http://www.megasoftware.net/). To obtain the confidence value for the aligned sequence dataset, bootstrap analysis of 100 replications was conducted using the MEGA 4.0.2 program. The phylogenetic tree was constructed via the minimum evolution method. The resultant phylogenetic tree was rooted with the animal fatty acid synthases (*Gallus gallus* FAS), as described by Kroken *et al*. (2003).

Reverse transcription-polymerase chain reaction (RT-PCR) of *U1PKS3*

The mycelia of *U. longissima* LFF (100 mg) were ground into powder after freezing in liquid nitrogen. The RNA was then extracted with an RNeasy Plant Mini kit (QIAGEN). Next, SuperScript II reverse transcriptase (Invitrogen, USA) was used for first-strand cDNA synthesis. The full cDNA of *U. longissima* PKS1 was amplified with primers designed in accordance with the predicted mRNA sequence of *U1PKS3*: FRT UlPKS3 located at the start-codon of *UlPKS3* (5′-ATGCCCATCGCTCTAGGCAA-3′) and RRT UlPKS3 located at the end-codon of *UlPKS3* (5′-CTAATCCGTCATATTCCCCCCCCT-3′).

Culture treatments and RT-PCR detection

The *U. longissima* LFF were ground to homogeneity and incubated on MY solid medium. After 2 months of culture on MY basal medium at 15°C, the mycelia were harvested as seeds, which were then transferred into MY basal medium containing 2% or 10% (w/v) inositol, mannitol, sorbitol, sucrose, glucose, or fructose. Amino acid media were also prepared via the addition of 0.2% and 1% (w/v) glutamine, asparagine, glycine, or alanine to the MY basal medium. The *U. longissima* LFF were cultured for 1 month and 3 months on the various media. The RNA of samples was then extracted with an RNeasy Plant Mini kit (QIAGEN). One microgram of total RNA from each sample was employed for first-strand cDNA synthesis, which was conducted using SuperScript II reverse transcriptase (Invitrogen). The following primers were employed for the detection of *U1PKS3* gene expression using tubulin as a control: Fnrks26: 5′-GCTTATGAGGC GTTGGAGAA-3′ and Rnrks26: 5′-TTCAAGATAGCGACTGCGGT-3′. All PCRs were conducted using the following reaction mixtures: 1 μl cDNA, 2 μl of primer mixture, 1 μl of Accupower PCR PreMix (Bioneer, Korea), and 16 μl of distilled water. The RT-PCR experiments were conducted 3 times, and 3 independent replications were employed each time.

Results

Isolation of *U. longissima* LFF

The LFF of *U. longissima* was isolated successfully via the methods described previously by Yamamoto *et al*. (1985) then confirmed by comparing the ITS and 5.8 rDNA sequences

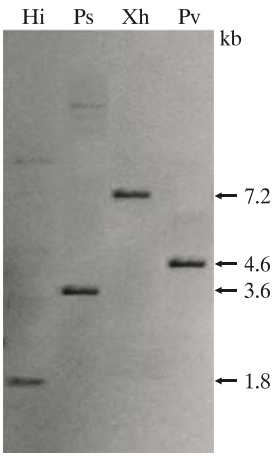

Fig. 1. Southern blot analysis of genomic DNA from the *Usnea longissima* mycobiont. Hi, *Hin*dIII digest; Ps, *Pst*I digest; Xh, *Xho*I digest; Pv, *Pvu*II digest. Southern blot detected by the NRks26 digoxigenin-labeled probe.

of the LFF with those of the original lichen thalli. The ITS sequence of the LFF evidenced 100% similarity with that of the thallus deposited in GenBank with the accession number DQ001304. This fungus also exhibited a typical mycelium with a callous-like morphology and a brown color (data not shown).

Isolation of the ketosynthase (KS) domain using degenerate primers

PCR of the LFF genomic DNA with degenerate primers (NR3KS-F and NR3KS-R) yielded an approximate 750 bp band, which was subsequently cloned and sequenced. Among the clones, one harbored a partial KS domain sequence (NRks26). Based on this sequence, a new primer (Fnrks26 and Rnrks26) was designed for the 445 bp NRks26 probe fragment. The closest NRks26 homologue found in the GenBank database was a putative reducing PKS KS domain fragment from *Magnaporthe oryzae* (57% identity, GenBank accession no. XP_361278.2), followed by the KS domain fragment of *Sordaria macrospora* (57% identity, GenBank accession no. CBI53318.1). Both sequences in the GenBank database were similar to the NRks26 probe fragment and belonged to the reducing PKSs, as defined by Kroken *et al*. (2003).

Detection of *UlPKS3* gene copy number

The *U. longissima* genomic DNA was acquired using DNeasy Plant Maxi columns (QIAGEN). Southern hybridization with the DIG-labeled NRks26 probe revealed a single band for the entire genomic DNA digest (Fig. 1), with a 1.8 kb band observed following the *Hin*dIII digest, a 3.6 kb fragment obtained from the *Pst*I digest, a 7.2 kb fragment from the *Xho*I digest, and a 4.6 kb fragment from the *Pvu*II digest. These findings demonstrated that the *UlPKS3* gene was present as a single copy in *U. longissima*.

Domain organization of the *UlPKS3* gene

An initial *U. longissima* LFF genomic library was constructed in pEpiFOS (Epicentre Biotechnology). Two positive FoNRks 2625 and FoNRks2662 clones were obtained by screening the genomic library. The clone, which included a complete PKS fragment (FoNRks2662), was selected for further sequencing. The sequencing of FoNRks2662 demonstrated that 13,845 bp of UlPKS3 information had been acquired. There were two genes presumably clustered with UlPKS3 on the sequenced fragment. They were putatively identified as heterokaryon incompatibility protein and serine hydrolase (Fig. 2A).

UlPKS3 was a 6519 bp gene encoding a 2172 amino acid protein. The presence of six introns (positions 264-312, 1353-1399, 3579-3788, 5751-5848, 5922-6050, and 6169-6222 from the start codon) was confirmed via reverse transcription (RT)-PCR (Fig. 2B). Five catalytic domains were identified in UlPKS3 based on the presence of conserved motifs. These domains were the β-ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase (ER), ketoacyl reducatse (KR), and acyl carrier protein (ACP) domains, in the order KS/AT/DH/ER/KR/ACP (Fig. 2C). The comparison of

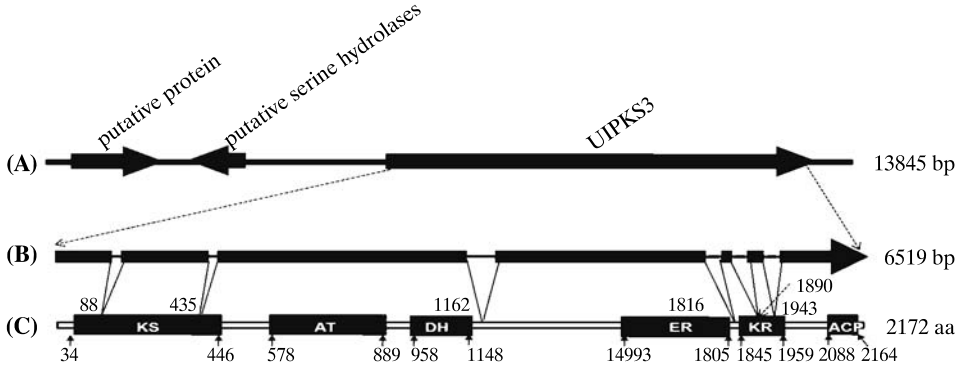

Fig. 2. *UlPKS3* gene cluster structure and domain organization. (A) *U1PKS3* gene cluster structure in *U. longissima*. Three open reading frames encoding a putative heterokaryon incompatibility protein, a putative serine hydrolase, and *UlPKS3*, respectively. (B) *UlPKS3* gene structure. The six introns are represented by lines and the exon is represented by a block. C Schematic organization of the domains. β-Ketoacyl synthase (KS; 34-446), acyltransferase (AT; 578-889), dehydratase (DH; 958-1148), enoyl reductase (ER; 1493-1805), β-ketoacyl reductase (KR; 1845-1959), and acyl carrier protein (ACP; 2088-2164). The approximate domain boundaries were determined using the iterative PKS domain search program (http://linux1.nii.res.in/~pksdb/ITRDB/query2.html).

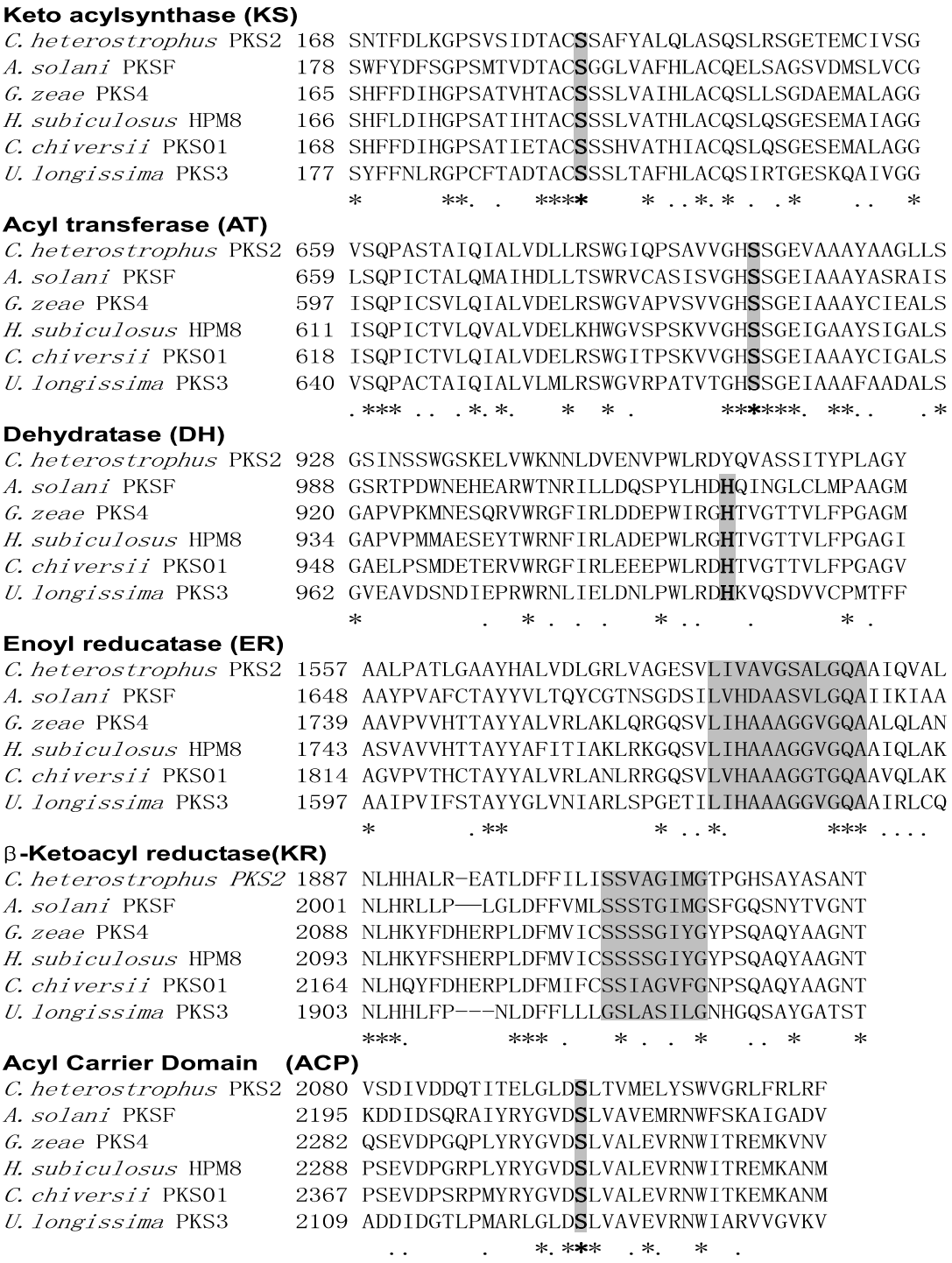

Fig. 3. The alignment of *U. longissima* predicted polyketide synthase (UlPKS3) active regions with five closely-related fungal PKSs. Alignment of the putative catalytic motifs found in the deduced amino acid sequence of *U. longissima* PKS3 with those of *Cochliobolus heterostrophus* PKS2 (ABB76806.1); *Alternaria solani* PKSF (BAE80697.1); *Gibberella zeae* PKS4 (ABB90283.1); *Hypomyces subiculosus* HPM8 (ACD39767.1); *Chaetomium chiversii* PKS01 (ACM42406.1). Amino acid residues conserved among all sequences are marked with an asterisk; the variability between two amino acid residues is marked with a dot. Highlighted regions indicate the catalytic amino acid residue in the active site.

UlPKS3 with the corresponding domains of other fungal PKSs showed the conservation of active site residues in KS (DTACSSSL), AT (GHSSGEI), DH (LRDHKVQ), ER (LIH AAAGGVGQA), KR (GSLASILG), and ACP (LDSLVA) (Fig. 3). The UlPKS3 sequence was deposited in the NCBI GenBank database under the accession number HQ824546.

Phylogenetic analysis of U1PKS3

The full amino acid sequences of the UlPKS3 KS domain and several fungal reducing/non-reducing PKSs were employed to generate multiple alignments and phylogenetic trees (Fig. 4). UlPKS3 generated an independent clade with very strong bootstrap values (93%; Fig. 4) that were close to the clade-I-B and clade-I-C fungal reducing PKSs. The PKSs genes of clade-I-C are involved in the synthesis of resorcylic acid lactones such as radicicol, hypothemycin, and zearalenone.

Expression patterns of *U1PKS3* transcripts

Mycobionts of *U. longissima* propagated in MY solid medium were transferred to media containing different carbon sources and amino acids, and *U1PKS3* gene expression was evaluated via RT-PCR under different culture conditions (Fig. 5). The results demonstrated that *UlPKS3* was expressed at a very low level in the basic MY medium. The amino acids asparagine and alanine had very slight effects on basal *UlPKS3* expression, but glutamine and glycine upregulated *UlPKS3* expression somewhat. Furthermore, inositol, mannitol, sorbitol,

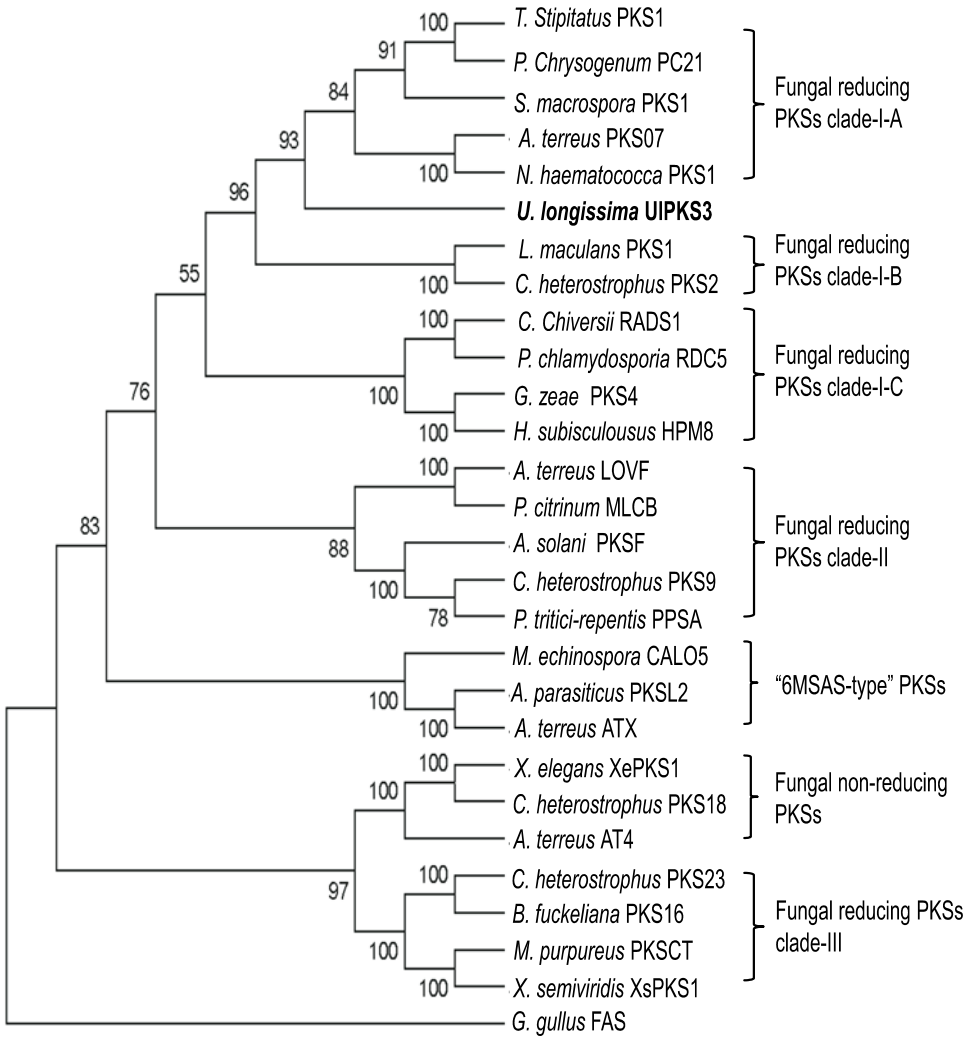

Fig. 4. Phylogenetic relationship of UlPKS3 with other fungal polyketide synthases (PKSs). The available genomic sequence of *UlPKS3* was translated using FGENESH. The translated UlPKS3 (KS, AT, DH, ER, KR, and ACP domains) was aligned with 23 fungal PKS sequences (reducing and non-reducing types) retrieved from the GenBank database. *Aspergillus parasiticus* PKSL2 (AAC23536), *Aspergillus terreus* PKS07 (XP_001209753.1), *Aspergillus terreus* AT4 (BAB88689), *Aspergillus terreus* ATX (BAA20102), *Aspergillus terreus* lovF (AAD34559), *Botryotinia fuckeliana* PKS16 (AAR90252), *Chaetomium chiversii* RADS1 (ACM42406.1), *Cochliobolus heterostrophus* PKS2 (ABB76806.1), *Cochliobolus heterostrophus* PKS9 (AAR90264.1), *Cochliobolus heterostrophus* PKS18 (AAR90272), *Cochliobolus heterostrophus* PKS23 (AAR90277), *Gallus gallus* FAS (AAB46389), *Gibberella zeae* PKS4 (ABB90283.1), *Hypomyces subiculosus* HMP8 (ACD39767.1), *Leptosphaeria maculans* PKS1 (CBX90731.1), *Micromonospora echinospora* CALO5 (AAM70355), *Monascus purpureus* PKSCT (BAD44749), *Nectria haematococca* PKS1 (EEU37129.1), *Penicillium citrinum* MLCB (BAC20566.1), *Penicillium chrysogenum* PC21 (XP_002567530.1), *Pochonia chlamydosporia* RDC5 (ACD39774.1), *Pyrenophora tritici-repentis* PPSA (XP_001930443.1), *Sordaria macrospora* PKS1 (CBI53318.1), *Talaromyces stipitatus* PKS1(XP_002342001.1), *Usnea longissima* UlPKS3 (HQ824546), *Xanthoparmelia semiviridis* XsePKS1 (ABS58604), and *Xanthoria elegans* XePKS1 (ABG91136). The obtained *Usnea longissima* PKS3 (UlPKS3) clade is marked in bold.

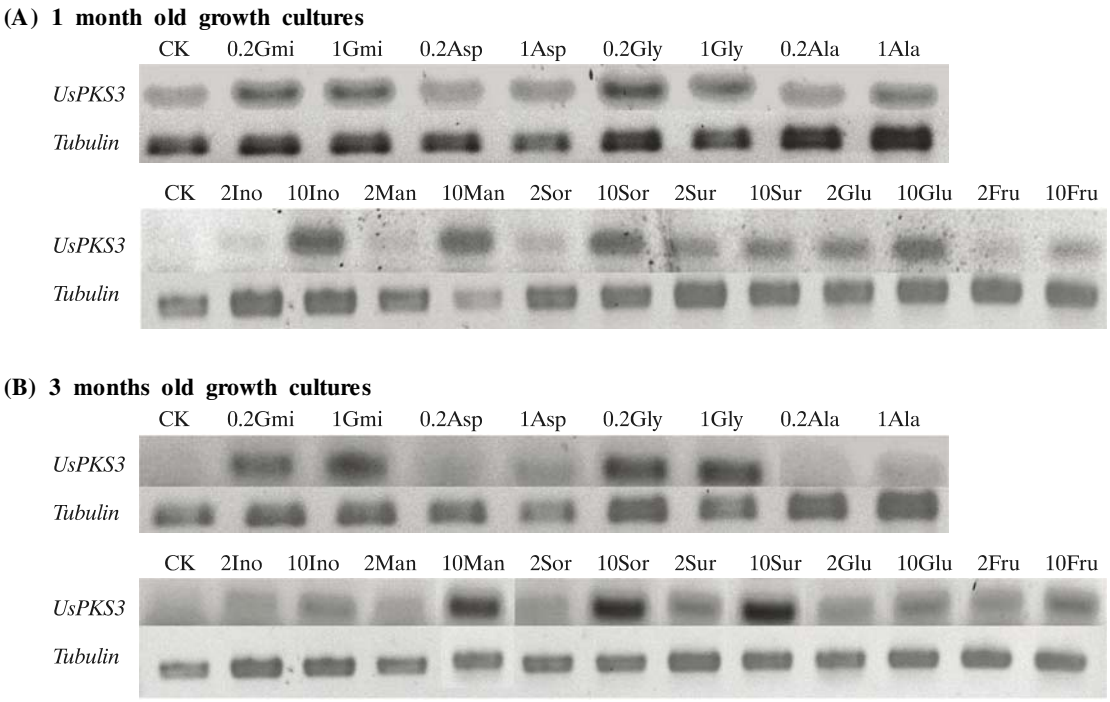

Fig. 5. *UlPKS3* gene expression in 1-month-old growth cultures (A) and 3-month-old growth cultures (B) after treatment with several amino acids (upper panel) and carbon sources (lower panel). RT-PCR analysis of *U1PKS3* gene expression following treatment with different amino acids. CK (control, MY), 1Gmin (1% glutamine), 0.2Gmin (0.2% glutamine), 1Asp (1% asparagine), 0.2Asp (0.2% asparagine), 1Gly (1% glycine), 0.2Gly (0.2% glycine), 1Ala (1% alanine), and 0.2Ala (0.2% alanine), 10Ino (10% inositol), 2Ino (2% inositol), 10Man (10% mannitol), 2Man (2% mannitol), 10Sor (10% sorbitol), 2Sor (2% sorbitol), 10Sur (10% sucrose), 2Sur (2% sucrose), 10Glu (10% glucose), 2Glu (2% glucose), 10Fru (10% fructose), and 2Fru (2% fructose). The different amino acids and carbon sources were added to basic MY medium. The lichen-forming fungi *Usnea longissima* was cultured for 1 month (A) and 3 months (B) at 15°C. RNA was isolated from *U. longissima* grown in various solid media, and RT-PCR was performed.

sucrose, fructose, and glucose slightly induced *UlPKS3* gene expression at low concentrations (2%) and strongly induced *UlPKS3* gene expression at higher concentrations (10%). Although strong pigmentation was observed in the media after 3 months of culture, no difference in *UlPKS3* gene expression was observed during the growth stages of the fungus (Figs. 5A and B).

Discussion

Usnea longissima has a long history of use as a traditional medicine in China, India, Turkey, Canada, and Europe (Brij and Upreti, 1995; Lans *et al*., 2007). Recently, anti-tumor, anti-inflammatory, anti-ulcerogenic, and nematocidal bioactivities have been reported for extracts of *U. longissima* (Nishitoba *et al*., 1987; Yamamoto *et al*., 1995; Halici *et al*., 2005; Muhammad *et al*., 2005; Bayir *et al*., 2006; Odabasoglu *et al*., 2006). Like other lichens, *U. longissima* engages in stable and self-supporting symbioses between LFF and photoautotrophic algal partners, and their secondary metabolites are produced principally by the fungal component. However, in nature, lichens are composed not only of LFF, algae, and cyanobacteria, but also endolichenic-fungi and lichenicolous fungi (Petrini *et al*., 1990; Honegger, 2000; Lawrey and Diederich, 2003; Li *et al*., 2007). Mixed symbiotic organisms may provide imprecise information during genetic analyses of LFF. Therefore, we isolated and cultured the LFF of *U. longissima* via the Yamamoto method (Yamamoto *et al*., 1985) to obtain genetic information.

In this study, we investigated a reducing PKS gene in the LFF of *U. longissima*. Additionally, a 40 kb fosmid clone was screened using a cloned 445 bp DNA fragment corresponding to the KS domain isolated from *U. longissima*. The sequence contained the entire PKS gene (6,519 bp). Southern blotting analysis using the KS domain as conserved and specific probes showed that one fragment was generated following different *U. longissima* genomic DNA restriction digests. Thus, the *UlPKS3* gene had a single copy in the *U. longissima* genome.

The UlPKS3 domain analysis revealed the presence of the KS, AT, DH, ER, KR, and ACP domains. The domain architecture of the UlPKS3 protein also existed in other non-lichenized fungi, such as *Chaetomium chiversii* CcRADS1, which is involved in radicicol synthesis (Wang *et al*., 2008) and the *Gibberella zeae* PKS4 involved in zearalenone synthesis (Kim *et al*., 2005). All of these genes had the domain organization KS-AT-DH-ER-KR-ACP.

Phylogenetic analyses using the amino acid sequence of the KS domains revealed that UlPKS3 were most closely related with the putative PKS from *Sordaria macrospora* (39% se-

quence identity; accession number CBI53318.1), and *Nectria haematococca* (37% sequence identity; accession number EEU37129.1). Additionally, U1PKS3 comprised a novel clade with strong (93%) bootstrap support, including a sister clade in the fungal reducing PKS clade I (Fig. 4). These homologous clones also contained the same domain organization of KS-AT-DH-ER-KR-ACP in the type I PKS genes.

The PKS KS-AT-DH-ER-KR-ACP domain structure exists universally in fungi. Thus far, these types of PKSs have been shown to be involved in the biosynthesis of resorcylic acid lactone (RAL), T-toxin, aslanipyrone, and aslaniol (Baker *et al*., 2006; Kasahara *et al*., 2006; Wang *et al*., 2008). The results of phylogenetic analysis demonstrated that UlPKS3 was more closely related to the PKS involved in RAL biosynthesis, relative to other known PKSs. RAL biosynthesis generally requires a non-reducing PKS (SAT-KS-AT-PT-ACP-TE), a reducing PKS (KS-AT-DH-ER-KR-ACP), a halogenase, and other cluster genes (Reeves *et al*., 2008; Wang *et al*., 2008; Zhou *et al*., 2008). These genes presumably clustered with UlPKS3 on the sequenced fragment did not evidence a gene cluster similar to that seen in RAL biosynthesis. For example, similar serine hydrolases encoded by *ORF2* and heterokaryon incompatibility protein encoded by *ORF1* were not detected in the cluster genes for RAL biosynthesis. A similar gene cluster structure (a serine hydrolase and a reducing PKS) existed in *Sordaria macrospora* (accession number CABT01000015.1) and *Aspergillus terreus* (accession number NT_165933.1).

U1PKS3 expression differed in different media. Asparagine and alanine were not found to alter basal *U1PKS3* expression, but glutamine and glycine induced a slight upregulation of *UlPKS3* expression. Inositol, mannitol, sorbitol, sucrose, glucose, and fructose stimulated *U1PKS3* expression. In particular, high concentrations of inositol, mannitol, sorbitol, sucrose, or glucose (10%) strongly induced *UlPKS3* expression. These findings demonstrated that carbon supplementation stimulates polyketide production. This is similar to the role of sucrose in the osmotic activation of the acetate-polymevalonate pathway that leads to polyketide production (divaricatic acid) in *Evernia esorediosa* and *Ramalina subbreciuscula* (Hamada, 1993).

In this study, a reducing PKS (*UlPKS3*) was isolated from *U. longissima*, which harbored the KS-AT-DH-ER-KR-ACP domain. Southern blot analysis identified *UlPKS3* as a single copy PKS gene. Furthermore, *UlPKS3* was upregulated by glucose, sorbitol, inositol, and mannitol. Although there is no apparent evidence regarding the relationship between secondary metabolites and *UlPKS3*, the UlPKS3 domain organization characteristic implies that it provides a side chain in depsone biosynthesis or is involved in macrolide biosynthesis. Future studies should focus on the heterologous expression of *UlPKS3* and confirm its functions. Such studies are expected to provide a basis for the sustainable application of lichen secondary metabolites in the pharmaceutical and perfume industries.

Acknowledgements

This work was supported by a grant from the Korea National Research Resource Center Program (2010-0000660) through the National Research Foundation of Korea (NRF).

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