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Effect of Expression of Genes in the Sphingolipid Synthesis Pathway on the Biosynthesis of Ceramide in *Saccharomyces cerevisiae*

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Ceramide is important not only for the maintenance of the barrier function of the skin but also for the water-binding capacity of the stratum corneum. Although the exact role of ceramide in the human skin is not fully understood, ceramide has become a widely used ingredient in cosmetic and pharmaceutical industries. Compared with other microorganisms, yeast is more suitable for the production of ceramide because yeast grows fast and is non-toxic. However, production of ceramide from yeast has not been widely studied and most work in this area has been carried out using *Saccharomyces cerevisiae*. Regulating the genes that are involved in sphingolipid synthesis is necessary to increase ceramide production. In this study, we investigated the effect of the genes involved in the synthesis of ceramide, *lcb1*, *lcb2*, *tsc10*, *lac1*, *lag1*, and *sur2*, on ceramide production levels. The genes were cloned into pYES2 high copy number vectors. *S. cerevisiae* was cultivated on YPDG medium at 30°C. Ceramide was purified from the cell extracts by solvent extraction and the ceramide content was analyzed by HPLC using ELSD. The maximum production of ceramide (9.8 mg ceramide/g cell) was obtained when the *tsc10* gene was amplified by the pYES2 vector. Real-time RT-PCR analysis showed that the increase in ceramide content was proportional to the increase in the *tsc10* gene expression level, which was 4.56 times higher than that of the control strain.

Keywords: *Saccharomyces cerevisiae*, ceramide, biosynthesis, gene expression, HPLC

Sphingolipids were first discovered more than a hundred years ago in brain tissue and since then their chemical and biophysical properties have been extensively characterized [3]. They are abundant components of many membranes in eukaryotic cells. In particularly, the sphingolipid of *Saccharomyces cerevisiae* comprises approximately 30 mol% and 7 mass% of the phospholipids in the plasma [22]. *S. cerevisiae* has been extensively used in the study of sphingolipids, and owing to this extensive amount of work, a lot is known about the genes that are involved in sphingolipid metabolism [2]. Interest in ceramides has been increasing relative to other sphingolipids, since it is the backbone of all sphingolipids and is known to play important roles in many cellular processes. Most studies have focused on understanding the role of ceramides in different cells when subjected to different stress conditions and in the presence of biological factors [4, 20]. Ceramide signals are known to respond to cellular stress such as cytokines, ultraviolet light, heat, and chemotherapeutic agents. However, it is not fully understood how ceramides activate different signal transduction pathways. However, it is clear that the pathways control many cellular processes such as apoptosis, immune responses, and cell–cell interactions [3, 12].

In yeast, sphingolipid synthesis begins with the condensation of serine and palmitoyl-CoA to form 3-ketodihydrosphingosine by serine palmitoyl transferase in the endoplasmic reticulum (ER) [19]. Two gene products are necessary for the formation of serine palmitoyl transferase, designated as *lcb1* and *lcb2*. These genes encode for an enzyme that forms a heterodimeric complex [9]. In the second step of sphingolipid synthesis, 3-ketodihydrosphingosine is converted to dihydrosphingosine by 3-keto reductase, which is encoded by *tsc10* [6]. The next step in yeast sphingolipid synthesis is hydroxylation of dihydrosphingosine at C4 to generate phytosphingosine. This reaction is catalyzed by an enzyme encoded by *sur2* [2]. The next reaction step is the conversion of phytosphingosine to ceramide, which is catalyzed by ceramide synthase. This enzyme is encoded by *lag1* and its homolog *lac1* [6]. Ceramide is then incorporated into three types of complex sphingolipids. A choline phosphate in the head group is

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replaced by an inositol phosphate to produce inositol phosphorylceramide (IPC). IPC is then mannosylated to yield mannose-inositol-P-ceramide (MIPC). The last step in sphingolipid synthesis is the transfer of an inositol phosphate from phosphatidylinositol onto MIPC to form mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C) (Fig. 1) [19].

In this study, *S. cerevisiae* was transformed with the expression vector pYES2, which contains *GAL1* as the promoter and *URA3* as the selection marker. The objective of this study was to examine the effect of the expression of genes involved in the sphingolipid pathway on the production of ceramide in *S. cerevisiae*. Six genes that are directly related to the synthesis of ceramide were cloned in the pYES2 vector, and their individual effect on the synthesis of ceramide was investigated by measuring the cellular ceramide content and gene expression level.

MATERIALS AND METHODS

Strains and Plasmid

The yeast strain *Saccharomyces cerevisiae* KCCM 50515 (*Mat*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) was obtained from the Korean Culture Center of Microorganisms. *Escherichia coli* XL1-blue (Stratagene, U.S.A.) was used for transformation of plasmid DNA. Yeast and bacterial strains were stored in 20% glycerol at −70°C. The pYES2 (Invitrogene, U.S.A.) expression vector, which contains a 2 μ origin, *GAL1* promoter, and *URA3* selectable marker, was used in this study.

Media and Cultivation

E. coli was grown in Luria–Burtani medium. Ampicillin (Sigma, U.S.A.) at 50 μg/ml was added to the medium when required. *S. cerevisiae* KCCM 50515 was cultivated in YPD medium (10 g of yeast extract, 20 g of Bacto-peptone, and 20 g of glucose per liter). For the selection of strains containing the plasmid, SDC (A, T) medium was used. The SDC (A, T) media contained 6.7 g/l Bacto-yeast nitrogen base (without amino acids), 20 g/l glucose, 5 g/l casamino acids with 20 mg/l adenine sulfate, and 20 mg/l L-tryptophan [27]. Solid media were prepared by the same method with 1.5% agar. YPDG (10 g of yeast extract, 20 g of Bacto-peptone, 15 g of glucose, and 5 g of galactose per liter) was used as the culture medium. Yeast cells were routinely cultivated at 30°C in Erlenmeyer flasks closed with cotton plugs and shaken at 200 rpm.

Genomic DNA Preparation

S. cerevisiae was grown overnight at 30°C in YPD and collected by centrifugation at 2,000 rpm for 5 min. Cells were resuspended in 0.5 ml

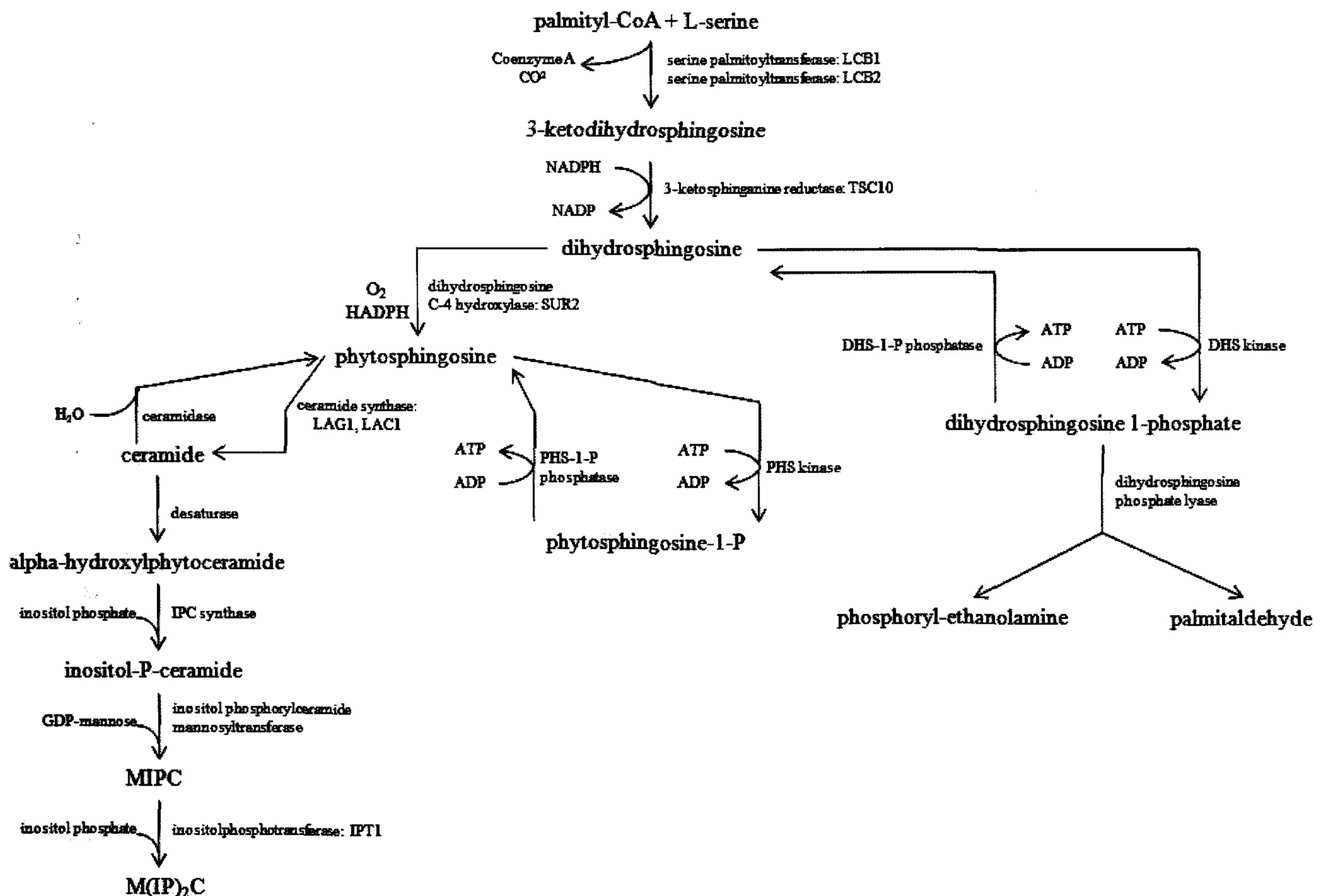

Fig. 1. Pathway for sphingolipid synthesis in *S. cerevisiae* [25].

of 1 M sorbitol, 0.1 M Na_2EDTA (pH 7.5), and 0.02 ml of a 2.5 mg/ml solution of zymolase 60,000 was added. The mixture was incubated at 37°C for 60 min and cells were recovered by centrifuging the cell suspensions. Cells were resuspended in 0.5 ml of 50 mM Tris-Cl (pH 7.4) and 20 mM Na_2EDTA. Then, 10% SDS (0.05 ml) was added, mixed well, and incubated at 65°C for 30 min. A 5 M concentration of potassium acetate (0.2 ml) was added and the cell suspension was placed in ice for 1 h. Cells were centrifuged for 5 min and the supernatant was transferred to a fresh centrifuge tube. The supernatant was mixed with one volume of 100% isopropanol and incubated at room temperature for 5 min. After centrifugation, the supernatant was poured off and the pellet was dried in air. The pellet was resuspended in 0.3 ml of TE (pH 7.4) buffer and mixed after adding 0.03 ml of 3 M sodium acetate. DNA was precipitated with 0.2 ml of 100% isopropanol and the solution was centrifuged to collect the pellet. The supernatant was discarded and the DNA pellet was resuspended in 0.3 ml of TE (pH 7.4) buffer after drying [10].

Construction of Plasmid

The target genes *lcb1*, *lcb2*, *tsc10*, *lac1*, *lag1*, and *sur2* were obtained from *S. cerevisiae* KCCM 50515. *Taq* polymerase was purchased from Novagen (KOD hot start DNA polymerase; Germany) and the primers used for PCR were purchased from Cosmo Genetech (Korea). PCR products and pYES2 were linearized by restriction enzymes (NEB, U.S.A.). The restriction enzymes used in the study are shown in Table 1. After ligation for 12 h at 40°C, the ligation mixture was transformed into *E. coli* XL1-blue, and cells containing the plasmid were selected by ampicillin resistance. Confirmation that the sequence of each gene was contained within the plasmid was determined using the LaboPass Plasmid Mini Purification Kit (Cosmo Genetech, Korea). The LiAc/SS-Carrier DNA/PEG method [7] was used to transform the plasmid containing genes into yeast. Yeast transformants were selected by growing cells on SDC (A, T) plates.

Extraction of Lipids

After cultivation, cells were harvested and dried in a freeze-dryer (Ilshin Lab. Co., Korea). Cells were reconstituted with 100 ml of Sol. I [chloroform:methanol=1:2 (v/v)]. Two grams of acid-washed glass bead (Sigma, U.S.A.) was added to the solution and the mixture was vortexed for cell disruption. The broken cells were separated by centrifugation at 10,000 rpm for 10 min using a centrifuge 5403 (Eppendorf Co., Germany). After centrifugation, disrupted cells were suspended in 100 ml of Sol. I [chloroform:methanol=1:2 (v/v)] and the supernatant was mixed with four volumes of Sol. II [chloroform:methanol=2:1 (v/v)]. Both fractions were stirred for 30 min at room temperature. The residual cell debris suspension was filtered through a 0.45-μm RC filter (Sartorius, Germany) and cell debris was suspended in 100 ml of Sol. II and stirred for 30 min. The stirring and filtering process was repeated four times. After mixing the supernatant and Sol. II mixture, the bottom layer was separated using a separatory funnel. The cell extracts and upper layer were combined and the solvent was discarded by evaporation. After addition of a chloroform:methanol:water mixture (8:4:3, volume ratio), the residue was washed in order to remove the polar components and non-lipid contaminants [24]. The lower lipid phase was concentrated to 10 ml using a rotary evaporator.

Table 1. Restriction enzymes used for the cloning of genes involved in the sphingolipid pathway.

Gene	Restriction enzyme
lcb1	*Hin*dIII, *Bam*HI
lcb2	*Bam*HI, *Eco*RI
tsc10	*Hin*dIII, *Bam*HI
lac1	*Hin*dIII, *Xho*I
lag1	*Hin*dIII, *Xho*I
sur2	*Bam*HI, *Xho*I

Isolation and Purification of Ceramide

Total lipids were subjected to mild alkaline hydrolysis to obtain a sphingolipid fraction [24]. The samples were mixed with 30 ml of methanol:carbon tetrachloride [5:1 (v/v)]. After addition of 1 volume of 0.2 M methanolic NaOH, alkaline hydrolysis was carried out for 1 h at room temperature. Then, a 0.8 volume of water was added and the mixture was neutralized with 1 M acetic acid.

HPLC

The cell extracts were concentrated and mixed with 10 ml of chloroform:methanol (2:1). The cell extracts were filtered through a 0.2-μm RC filter (Sartorius, Germany). HPLC was performed using an Acme 9000 HPLC system (Younglin Instrument, Korea). Autochro 3000 (Younglin Instrument, Korea) was used for data collection, and ELSD Sedex 75 (Sedere, France) was used as the detection system. The drift temperature of ELSD was set at 40°C and the flow rate of the nebulizer gas (nitrogen) was 3.5 bar. The normal-phase column used in this work was Waters Spherisorb 5 μm Silica (Waters, U.S.A.) and the dimension of the column was 4.6×250 mm [11, 14]. The mobile phase was prepared in an isocratic mode with chloroform:methanol (96:4) and the flow rate was 1.0 ml/min.

Quantification of Glucose

The culture broth was filtered through a 0.2-μm cellulose acetate filter (Satorius, Germany) and analyzed using a HPLC 10A system (Shimadzu, Japan) equipped with RI detector LC-10AD (Shimadzu, Japan). The HPLC column was an Aminex HPX87H (Bio-Rad, U.S.A.), and 0.5 M sulfuric acid was used as the mobile phase at a flow rate of 0.5 ml/min.

Total Cellular Lipid Extraction

The total lipid was obtained from freeze-dried yeast cells using the method developed by Kaneko *et al.* [13], with slight modifications. 0.3 g of the dried yeasts was suspended in 30 ml of a solvent [chloroform: methanol, 1:2 (v/v)] with two volumes of glass beads (Sigma, U.S.A.). Cells were disrupted by vortexing the cell suspension for 30 min at room temperature. The disrupted cells were filtrated through a 0.45-μm filter (Satorius, Germany). The cell debris was suspended and filtered with 30 ml of solvent, two times. The solvent filtrate was evaporated at 65°C and transferred to a glass vial. After evaporation of the solvent in the glass vial at 65°C, the lipid amount was estimated by the weight differences of the vials before and after evaporation.

Real-Time RT-PCR

RNA from *S. cerevisiae* was prepared using the RNeasy Mini Kit (Qiagen, U.S.A.). The extracted RNA was treated with DNase I (Takara, Japan) at 37°C for 3 h to remove DNA. The reverse transcription for the synthesis of cDNA was performed using a

RNA PCR Kit (Takara, Japan); $MgCl_2$ 2 µl, 10× RT buffer 1 µl, RNase-free dH_2O 3.75 µl, dNTP mixture 1 µl, RNase inhibitor 0.25 µl, AMV reverse transcriptase XL 0.5 µl, random 9 mers 0.5 µl, and experimental sample 1 µl. The reverse transcription reaction was performed for 10 min at 30°C followed by 30 min at 60°C, and the reverse transcriptase was inactivated by heating the mixture for 5 min at 95°C.

Real-time PCR was performed using SYBR Premix EX *Taq* (Takara, Japan). Primers for the target gene were generated (Cosmo Genetech, Korea) and optimized such that the annealing temperature was 60°C (Table 3). The PCR reaction mixture was SYBR Premix Ex *Taq* 10 µl, PCR forward primer 0.4 µl, PCR reverse primer 0.4 µl, ROX reference dye 0.4 µl, template 2 µl, and dH_2O 6.8 µl, and performed using the real-time chromo4 System (Bio-Rad, U.S.A.). The real-time PCR experimental run was performed as follows: denaturation program (95°C for 15 min), amplification and quantification program repeated 45 times (95°C for 15 sec, 60°C for 20 sec, 72°C for 60 sec, with a single fluorescence measurement), melting curve program (72–95°C with a heating rate of 0.2°C per second and a continuous fluorescence measurement), and finally a cooling step to 40°C [1, 23].

RESULTS AND DISCUSSION

Saccharomyces cerevisiae containing pYES2 was cultured in YPD with galactose to induce expression through the *GAL* promoter. Cells were grown in medium containing a total glucose and galactose concentration of 2% as a carbon source to determine the proper quantity of galactose needed for *GAL* promoter induction (data not shown). A vector containing the *lacZ* gene in the pYES2 vector was prepared and β-galactosidase activity was measured at various glucose to galactose ratios. Based on these experiments, β-galactosidase activity was found to be highest when the glucose concentration was 15 g/l and the galactose concentration was 5 g/l. Thus, all experiments were performed in YPDG containing 10 g/l yeast extract, 20 g/l Bacto-peptone, 15 g/l glucose, and 5 g/l galactose.

Six genes in the sphingolipid synthesis pathway, *lcb1*, *lcb2*, *tsc10*, *lac1*, *lag1*, and *sur2*, were extracted from the genomic DNA of *S. cerevisiae* and amplified by PCR. The result of PCR is shown in Fig. 2. After PCR, the sequences of the target genes *lcb1, lcb2, tsc10, lac1, lag1,* and *sur2* were verified by comparing the gene sequence with the sequence in the *Saccharomyces* Genome Database [25]. For insertion of each target gene into the cloning sites of pYES2, the PCR products and plasmids were digested with restriction enzymes (Table 1). The presence of the cloned genes was verified by gel electrophoresis after double digestion with restriction enzymes. The band for pYES2 (5.9 kb) and bands for the 6 genes (*lcb1*: 1,667 bp; *lcb2*: 1,686 bp; *tsc10*: 963 bp; *lac1*: 1,257 bp; *lag1*: 1,236 bp; *sur2*: 1,050 bp) are shown in Fig. 3.

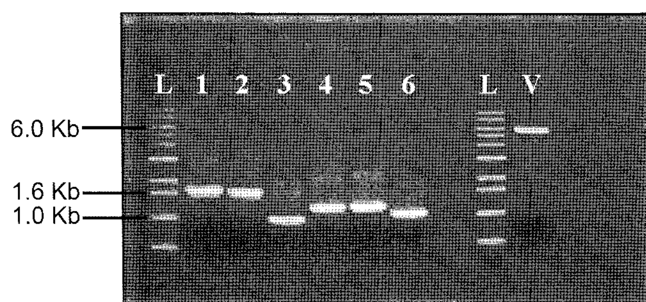

Fig. 2. Gel electrophoresis of the cloned target genes and the pYES2 vector.
Lane L: 1-Kb ladder (Bioneer, Korea); Lane 1: *lcb1*; Lane 2: *lcb2*; Lane 3: *tsc10*; Lane 4: *lac1*; Lane 5: *lag1*; Lane 6: *sur2*; and Lane V: linear pYES2.

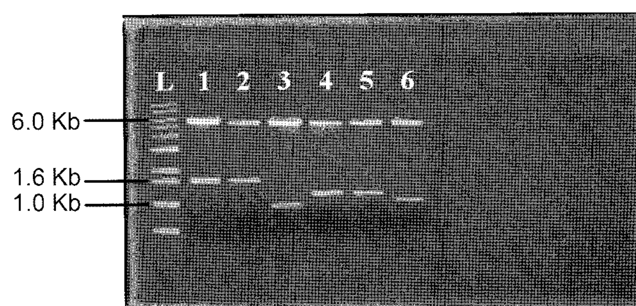

Fig. 3. Gel electrophoresis after insertion of the genes into the pYES2 vector using a restriction enzyme.
Lane L: 1-Kb ladder (Bioneer, Korea); Lane 1: pYES2 and *lcb1*; Lane 2: pYES2 and *lcb2*; Lane 3: pYES2 and *tsc10*; Lane 4: pYES2 and *lac1*; Lane 5: pYES2 and *lag1*; and Lane 6: pYES2 and *sur2*.

S. cerevisiae containing the constructed pYES2 plasmid was selected by plating the transformants on SDC (A, T). The six strains containing the genes involved in ceramide synthesis were cultivated in YPDG (10 g/l yeast extract, 20 g/l Bacto-peptone, 15 g/l glucose, 5 g/l galactose) medium. The strains that were used in this study are described in Table 2. Cells were recovered at the mid-stationary phase, which occurred approximately 12 h after depletion of glucose in the medium [15].

As shown in Fig. 4, the production of ceramide in the control strain *S. cerevisiae* SCY was 2.08 mg ceramide/g cell. The highest production of ceramide was obtained from *S. cerevisiae* SCYT10 (9.79 mg ceramide/g cell),

Table 2. Description of strains used in this study.

Strains	Constructed plasmid	Gene
SC	None (native *S. cerevisiae* KCCM50515)	
SCY	pYES2	
SCYL1	pYES2/*lcb1*	*lcb1*
SCYL2	pYES2/*lcb2*	*lcb2*
SCYT10	pYES2/*tsc10*	*tsc10*
SCYC1	pYES2/*lac1*	*lac1*
SCYG1	pYES2/*lag1*	*lag1*
SCYS2	pYES2/*sur2*	*sur2*
SCYLZ	pYES2/*lacZ*	*lacZ*

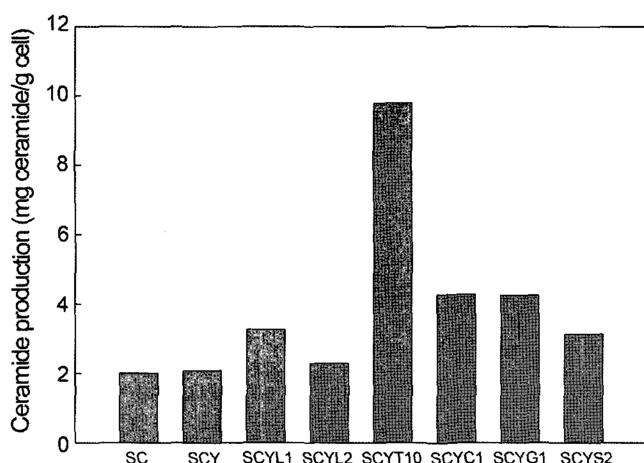

Fig. 4. Ceramide production in cells containing genes involved in the sphingolipid pathway.

which contained the gene for 3-keto reductase, and the concentration of ceramide was 4.7 times higher than that of *S. cerevisiae* SCY. More ceramide was obtained from *S. cerevisiae* SCYT10 than the strains that contained the genes for the enzyme palmitoyl transferase, which is involved in the first reaction, SCYL1 or SCYL2. This most likely occurred because there are two subunits for the palmitoyl transferase and *S. cerevisiae* SCYL1 and SCYL2 only contained a gene encoding for one of the subunits (*lcb1* or *lcb2*) [17]. However, it seems that the *lcb1* gene product was the limiting component in this reaction step since the overexpression of the *lcb1* gene resulted in an increase in ceramide content. The enzymes expressed by *lac1* and *lag1* are involved in the ceramide synthesis reaction and it has been reported that they have a homolog structure [8, 26]. This might result in relatively high levels of ceramide production in *S. cerevisiae* SCYC1 and SCYG1 (4.28 mg ceramide/g cell and 4.24 mg ceramide/g cell,

Table 3. The sequence of primers used for real-time RT–PCR.

gene	Primer	sequence
act1	Forward	GTT ACG TCG CCT TGG ACT TC
	Reverse	CGG ACA TCG ACA TCA CAC TT
lcb1	Forward	TAC GGT AAC CAG GAC GTT CA
	Reverse	GCG AAT TCA TAT CGT TGT GG
lcb2	Forward	TTC AAT CTG GTG GTC CAA GA
	Reverse	CAG CAC CAG AAA GCC TAA CA
tsc10	Forward	AAA CCA AGG AAC ACC ACC T
	Reverse	ATG CTT GTT TGC ATG GGA TA
lac1	Forward	ATT TGG GTC AAG CTG CAT TT
	Reverse	AT CCA CGC AAC AAC AAA GA
lag1	Forward	ATG CGT GTA TAG CGC GTA CT
	Reverse	GAC GAT GCT CGG AAG TAA CA
sur2	Forward	GGA ATG TCC GCT TTG AAG AT
	Reverse	TTC ATG GTG GCA AAG GTA AA

Table 4. Total lipid content and ceramide content of strains containing genes involved in the sphingolipid pathway.

Strain	Total lipid/DCW (mg/g)	Ceramide/Total lipid (%)
SCY	117.03	1.79
SCYL1	85.18	4.02
SCYL2	102.39	2.26
SCYT10	74.01	13.29
SCYC1	75.71	5.81
SCYG1	68.10	6.23
SCYS2	71.23	4.49

respectively), even though two genes together encode for ceramide synthase. The productivity of *S. cerevisiae* SCYS2 was 3.13 mg ceramide/g cell, which was approximately 50% higher than that of the control.

The total lipid concentration of *S. cerevisiae* was measured to determine the effect of gene expression on lipid production (Table 4). The ceramide content was not high in *S. cerevisiae* SCYL2; however, the total lipid content (102.39 mg total lipid/g cell) was much higher than that of the other strains. No direct relationship between the ceramide content and the total lipid concentration was observed. This occurred because various kinds of lipids exist in *S. cerevisiae* and ceramide comprises only a small fraction of the total lipid in *S. cerevisiae*.

Real-time RT–PCR was conducted to examine the relationship between the expression of genes associated with the sphingolipid pathway and ceramide concentration. Real-time RT–PCR is a rapid, convenient, and relatively accurate method to quantitatively analyze mRNA expression. Relative quantification is used to determine changes in the expression level of a target gene relative to a reference gene. In this study, the actin gene *act1* was used as a reference gene, since the expression level is not regulated in the cell [18]. The coefficients of variation (CV) of C(t) for the six strains were 0.79, 0.13, 1.31, 1.39, 0.12, and 0.31, respectively. These values were very low (±1%) and the average standard deviation was 0.12 (Table 5). It is very difficult to attain maximum (2) PCR efficiency because of variations in the reaction and the presence of inhibition factors such as the dilution component or GC content [21]. The PCR efficiency of the strains was estimated, since

Table 5. Results of real-time RT–PCR for strains containing genes involved in the sphingolipid pathway.

Description	C(t) (mean±SD)	CV (%)	Efficiency
SCYL1	16.54±0.13	0.79	1.77
SCYL2	15.67±0.02	0.13	1.88
SCYT10	17.56±0.23	1.31	1.85
SCYC1	18.75±0.26	1.39	1.85
SCYG1	16.28±0.02	0.12	1.86
SCYS2	16.32±0.05	0.31	1.85

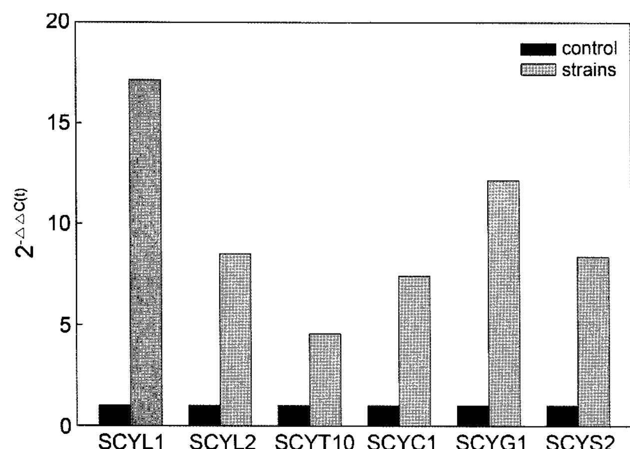

Fig. 5. mRNA expression level of strains containing genes involved in the sphingolipid pathway.

small difference in PCR efficiency would result in large differences in amplicon product after many cycles [5]. The PCR efficiency of each strain was determined to be 1.77, 1.88, 1.85, 1.85, 1.86, and 1.85, respectively. Based on these results, the PCR efficiency was relatively constant between the various strains except for *S. cerevisiae* SCYL1 (Table 5). To examine variations in the expression level of specific gene targets by real-time RT–PCR, a standard curve was prepared for the reference gene and target genes. The level of amplification was estimated based on the standard curve. Since errors are easily compounded by variations in the amount of starting material between the samples, RNA values were normalized to reduce errors using the $2^{-\Delta\Delta C(t)}$ method (Fig. 5.) [1, 16]. All transformed strains containing any of the genes involved in the sphingolipid pathway showed higher expression levels compared with the control strain (Fig. 5). The expression of the *tsc10* gene in *S. cerevisiae* SCYT10 was 4.56 times higher compared with *S. cerevisiae* SCY. This value was very similar to the increase in ceramide production, which was 4.7 times higher relative to the control. However, the change in ceramide production in the other strains was not proportional to the gene expression level. Consequently, it was found that the *tsc10* gene had a greater effect on ceramide production in *S. cerevisiae* than the other 5 genes tested.

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