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Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*

Markus Veen, Ulf Stahl, Christine Lang *

Technische Universität Berlin, Institut für Biotechnologie, FG Mikrobiologie und Genetik, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany

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Abstract

Genes of the post-squalene ergosterol biosynthetic pathway in *Saccharomyces cerevisiae* have been overexpressed in a systematic approach with the aim to construct yeast strains that produce high amounts of sterols from a squalene-accumulating strain. This strain had previously been deregulated by overexpressing a truncated HMG-CoA reductase (*tHMG1*) in the main bottleneck of the early ergosterol pathway. The overexpression of the gene *ERG1* (squalene epoxidase) induced a significant decrease of the direct substrate squalene, a high increase of lanosterol, and a small increase of later sterols. The overexpression of the *ERG11* gene encoding the sterol-14α-demethylase resulted in a decrease of lanosterol and an increase of downstream sterols. When these two genes were simultaneously overexpressed, later sterols from zymosterol to ergosterol accumulated and the content of squalene was decreased about three-fold, indicating that these steps had limited the transformation of squalene into sterols. The total sterol content in this strain was three-fold higher than in a wild-type strain.

Keywords: Ergosterol biosynthesis; Sterol precursor; Combined overexpression; Truncated *HMG1*; Lovastatin; Deregulation

1. Introduction

Sterols are essential structural and regulatory components of eukaryotic cell membranes [1–3]. Ergosterol, the main sterol in yeast, is responsible for structural membrane features such as fluidity and permeability in a similar way as cholesterol is in mammalian cells [2]. Additionally, ergosterol is economically important as a precursor of vitamin D_2 [4].

In yeast, free ergosterol is incorporated in membranes, mainly the plasma membrane. Ergosterol and some other sterols can be stored as fatty acid esters in lipid particles [5]. The enzymes which catalyze the reaction of esterification are Are2p and Are1p [6]. The biosynthesis of sterols in yeast is complex. The early sterol biosynthetic pathway provides intermediates for the synthesis of essential cellular constituents like heme, quinones and dolichols [7]. The enzymes of the later sterol pathway (Fig. 1) catalyze the transformation of squalene, the polyisoprene precursor, to ergosterol. This way is now well defined and all the structural genes have been cloned. The regulation of sterol biosynthesis in yeast is, however, not well understood (for a review see [8]).

As a first step towards a sterol-overproducing yeast strain, we have previously analyzed the deregulated expression of the HMG-CoA reductase, encoded by the gene *HMG1*. It is the main bottleneck enzyme of the early ergosterol biosynthesis. For deregulation the gene was truncated and overexpressed in a soluble, non-membrane-bound form. It was shown that a large amount of squalene is accumulated, but the sterol content shows only small changes. Squalene is the first specific intermediate on the way to ergosterol. The squalene pool is stored in lipid particles [9,10].

Additional bottlenecks have to be assumed in the post-squalene pathway. With the aim to identify such bottlenecks we have now focused on the genes of this section of the pathway. All the relevant genes which include the 12 genes encoding the enzymes which transform squalene into ergosterol have been identified by now (for a review see [11,12]).

* Corresponding author. Tel.: +49 (30) 31472751; Fax: +49 (30) 31472922.
E-mail address: c.lang@lb.tu-berlin.de (C. Lang).

Abbreviations: GC, gas chromatography; TLC, thin layer chromatography

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Here we report on the systematic overexpression of the genes of this post-squalene biosynthetic pathway in the strain AH22*tH3ura8* which is deregulated in the HMG-CoA reductase, and the application of these data to find additional bottlenecks and to increase the transfer of squalene into sterols. Overexpression of at least two of the genes, *ERG1* and *ERG11*, led to an increased content of sterols in the yeast *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Escherichia coli *strain and growth condition*

E. coli K12 strain JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44* λ^- *relA1* Δ(*lac-proAB*)/F′ *traD36 proA*$^+$-*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*] was used as host in plasmid construction [13]. *E. coli* was grown at 37°C in Luria–Bertani broth containing 100 μg ml^{-1} ampicillin.

2.2. E. coli *transformation*

Bacterial cultures were transformed as described [9].

2.3. *Yeast strains*

The yeast strain *S. cerevisiae* AH22*tH3ura8* [*MAT*a *leu2-3 leu2-112 his4-519 can1 ura3*::*tHMG1*] has been described [9]. The strain *S. cerevisiae* S288c (ATCC No.: 26108) was used for amplification of genomic DNA. Metabolic complementation experiments were performed using deletion strains *erg24*, *erg6*, *erg2*, *erg3*, *erg5*, *erg4* of the strain *S. cerevisiae* BY4741 [*MAT*a *leu2Δ0 his3Δ1 ura3Δ0 met15Δ0*] [14].

2.4. *Yeast transformation*

Yeast strains were transformed by the lithium acetate method [17]. The transformation rate in some deletion strains (*erg6Δ*, *erg3Δ*, *erg5Δ*, *erg4Δ*) was very low under these conditions, due to the abnormal sterol composition in the membranes. An increase of the amount of plasmid DNA to 15 μg per sample led to a number of transformants comparable to that in the wild-type.

2.5. *Growth and culture conditions for sterol analysis*

For sterol analysis, yeast strains were grown at 28°C for 3 days in 250-ml baffled shake flasks containing 50 ml minimal medium WMVIII [15] supplemented with 20 μg ml^{-1} L-histidine-HCl and 100 μg ml^{-1} uracil when appropriate. Cultures were supplemented with different concentrations of lovastatin (10 μg ml^{-1} up to 80 μg ml^{-1}) (Calbiochem, Germany) for lovastatin treatment. Lovastatin was solubilized in ethanolic NaOH [16].

2.6. *Plasmid construction*

To construct plasmid pFlat3, the plasmid YEp24 [18] was linearized with *Sph*I, and a 900-bp *Sph*I fragment containing the *ADH1* promoter and the *TRP1* terminator spaced by a multiple-cloning site of pUC19 plasmid [13] was inserted from plasmid pPT2B [15]. The multiple-cloning site was extended by inserting a polylinker containing the restriction sites for *Not*I and *Xho*I. The resulting plasmid pFlat1 which carries a *URA3* gene for selection was linearized by *Nco*I restriction, blunted by Klenow polymerase, and a blunt-ended *Bam*HI fragment of YDpL [19] containing the yeast *LEU2* gene was integrated. The resulting vector was pFlat3.

The genes (*ERG1* through to *ERG4*, Fig. 1) were amplified from yeast chromosomal DNA of the strain *S. cerevisiae* S288c by PCR under standard conditions using a proof-reading Takara Ex polymerase (BioWhittaker, Belgium). The sequence-specific primers used are listed in Table 1. They include *Not*I and *Xho*I (*Mlu*I for *ERG27*) restriction enzyme recognition sequences at the 5′ end and at the 3′ end, respectively, to facilitate the subsequent cloning of the amplified DNA into the *Not*I (5′) and *Xho*I (*Mlu*I) (3′) sites of pFlat3. The *Not*I/*Xho*I-restricted PCR product of *ERG1* was cloned into the pFlat1 vector in addition, to be able to select for two different vectors. All the cloned genes of the post-squalene biosynthetic pathway were expressed under the control of a constitutive *ADH1* promoter and the *TRP1* terminator [15]. Genes were sequenced by M. Meixner, Humboldt-University, Berlin, Germany.

2.7. *Metabolic complementation*

Deletion strains were transformed with the plasmids carrying the corresponding complementing genes to control the activity of the overexpressed genes and restauration of the wild-type sterol pattern. The sterol content of the transformants was analyzed using gas chromatography (GC) analysis.

2.8. *Transcript analysis*

To analyze the transcriptional level of *ERG* genes a nylon filter array method was used: 0.3 μg of PCR products of individual *ERG* genes were spotted on a nucleobond n$^+$ filter membrane (Amersham, USA). The DNA was crosslinked to the membrane using a crosslinker (Stratagene, USA). Total RNA was isolated at different time points from yeast cells using an RNA isolation kit (Qiagen, Germany) and quantified using photometric determination and agarose gel analysis to normalize the amounts of RNA of different samples. Digoxigenin (Roche, Germany) dCTP-labeled single-strand DNAs were synthesized from 0.5 μg of RNA with first-strand cDNA synthesis kit (Roche, Germany). Efficiency of labeling was checked with

dot blot analyses. Hybridization of labeled cDNA to the DNA filters was carried out at 65°C for 16 h, with Dig easy Hyb buffer (Roche, Germany) and filters were washed twice with 2×SSC/0.1% SDS for 20 min at 25°C and twice with 0.2×SSC/0.1% SDS for 20 min at 65°C. The filters were incubated with an anti-Dig-AP antibody (Roche, Germany) and luminescence was detected with a chemoluminescence detection kit (NEN, USA). Expression profiles of *ERG* genes on filter arrays were validated by Northern blot analysis.

2.9. Analysis of sterols and intermediates

Total sterols (free and esterified) were extracted and quantified from yeast cells as described [20,21]. Yeast cells were grown in 50 ml of minimal medium for 72 h at 28°C with reciprocal shaking at 250 rpm. Cells (OD_{600} of 30) were harvested, washed in water and broken by vortexing five times for 1 min each in the presence of glass beads and chloroform/methanol (4:1). The chloroform phase containing the extracted sterols was recovered, dried under nitrogen and sterols were resuspended in 200 μl chloroform/methanol (4:1) and 10 μl was applied to thin layer chromatography (TLC) 60 F_{254} precoated silica gel plates (E. Merck, Germany). TLC was performed in petrol ether:diethyl ether:acetic acid (90:10:1). Sterols were detected by UV light. Quantification of free and esterified sterols was performed with a Camag TLC scanner system (Camag, Switzerland). Free sterols were reisolated from the TLC plates and eluated by chloroform/methanol (4:1).

To quantify total sterols, steryl esters were saponified prior to GC analysis. Cells (OD_{600} of 125) were treated for 20 min at 100°C in 0.5 N HCl and allowed to cool to room temperature. After that 3 g of KOH and 12.5 ml of methanol with pyrogallol (2 g l^{-1}) were added. For saponification the mixture was incubated for 2 h at 70°C in a water bath. Hydrolyzed esters were extracted in *n*-hexane. Sterol fractions were resuspended in 2 ml of *n*-hexane. Sterols were quantified by GC with cholesterol as an internal standard. Sterols were separated on a Hewlett-Packard 5890 gas chromatograph with a capillary column (25 m by 0.25 mm by 0.25 μm (film thickness); Chrompack CPSil5) programmed from 150 to 250°C. The temperature was initially 150°C for 2 min; it was then increased at 15°C min^{-1} to a final temperature of 250°C at which it was held for 20 min. The linear velocity was 30 cm s^{-1}, helium was used as the carrier gas, and injections were run in the splitless mode. The injection volume was 1 μl. The area of each peak was calculated and related to 1 g of cell dry weight. Each sample was measured in duplicate. All strains were analyzed in at least two independent experiments and the average error of the samples was not greater than 10%. Standards of ergosterol and squalene were used for identification. Other sterols were identified via their specific retention times and by GC–MS [9].

2.10. Isolation of lipid particles

Yeast cells were grown in 50 ml of WMVIII minimal medium for 72 h at 28°C with reciprocal shaking at 250 rpm. Cells were harvested by centrifugation and lipid particles were isolated and purified according to [22]. For sterol analysis lipid particles were either saponified for 16 h in 30% methanolic KOH at room temperature for total sterol quantification by GC, or sterols were directly extracted with chloroform/methanol (4:1) and analyzed by TLC to distinguish between free and esterified sterols or by GC for quantification of free sterols.

3. Results

3.1. Overexpression of genes of the post-squalene biosynthetic pathway in the squalene-accumulating yeast strain *AH22*tH3ura8

We have previously shown that high amounts of squalene are accumulated in the strain *S. cerevisiae* AH22-*tH3ura8* which expresses a truncated form of the HMG-CoA reductase Hmg1p, while sterol precursors are only slightly increased in this strain [9]. We concluded that additional bottlenecks are to be opened in the post-squalene pathway of the ergosterol biosynthesis to significantly increase the sterol content of yeast cells. To identify those steps we have inserted each gene of the post-squalene biosynthetic pathway in the yeast multicopy expression vector pFlat3 and overexpressed the genes under the control of a constitutive version of the *ADH1* promoter using the

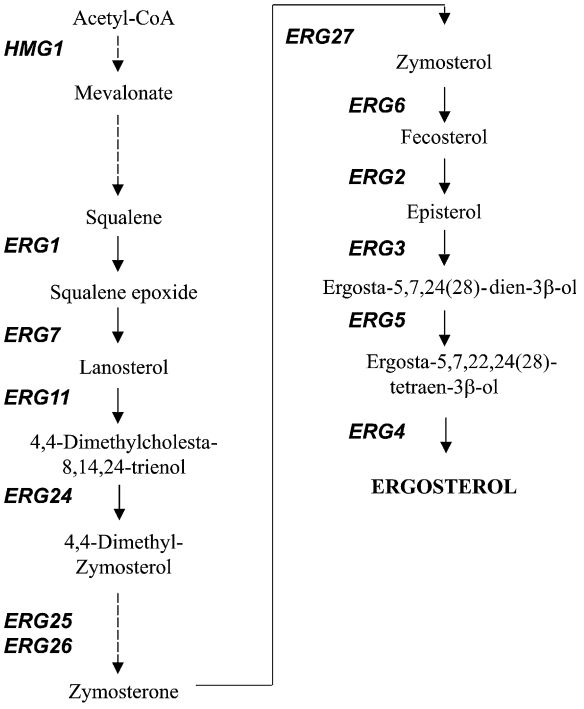

Fig. 1. The pathway of ergosterol biosynthesis in yeast [11,12].

LEU2 gene as a selection marker. The activity of the overexpressed enzymes was checked either by metabolic complementation and restauration of the wild-type sterol pattern of the overexpressed genes in corresponding deletion strains (*ERG24*, *ERG6*, *ERG2*, *ERG3*, *ERG5* and *ERG4*) or by checking for significant changes in the sterol composition (*ERG1* and *ERG11*).

The other genes (*ERG7*, *ERG25*, *ERG26* and *ERG27*) were sequenced to confirm sequence identity with the sequence in public databases.

The influence of the transcriptionally deregulated overexpression of single genes on the sterol content and the sterol composition was analyzed by GC (Table 2). The most significant effect was seen when *ERG1*, the gene for squalene epoxidase, was overexpressed. In this strain, the content of lanosterol was strongly increased by a factor of 3.6 compared to the control strain and the amount of some later sterols from 4,4-dimethylzymosterol to episterol was slightly elevated. The concentration of the end product ergosterol was nearly unchanged. In total, the overexpression of *ERG1* led to a 1.5-fold increase of sterols in the cell and squalene, the substrate for Erg1p, was at the same time decreased by 52% (Table 4).

Interestingly, overexpression of Erg1p did not lead to the accumulation of squalene epoxide, the direct product of the Erg1p enzyme reaction, but of lanosterol. This suggests that the enzyme reaction of the endogenous Erg7p, the lanosterol synthetase, was not a limiting step in the *ERG1*-overexpressing strain. This assumption was underlined by the fact that the strain which overexpressed *ERG7* (Table 2) did not show any significant change in its sterol content or sterol composition compared to the control strain (pFlat3).

Beside the *ERG1*-overexpressing strain, the strain which overexpressed *ERG11* also showed a significant change in its sterol composition. When the gene *ERG11* was overexpressed, the lanosterol content decreased and the amount of all later sterols from 4,4-dimethylzymosterol to ergosterol increased. The conversion of the substrate of the Erg11p enzyme reaction (lanosterol) to later sterols was clearly enhanced in this strain. However, the total sterol content did not rise more than 1.2-fold relative to the control strain (pFlat3). The squalene content was not influenced (Table 4).

The overexpression of any of the genes downstream of *ERG11* did not influence the total sterol content (see Table 2: relative factor [total sterols]), but changes in the sterol composition were seen in almost all strains. Significant changes were seen when e.g. *ERG27* was overexpressed. In this strain, the amount of the early sterols lanosterol and 4,4-dimethylzymosterol was increased, while all other sterols remained more or less unchanged. The content of lanosterol and 4,4-dimethylzymosterol was also increased in the *ERG3*- and *ERG4*-overexpressing strains, but here the content of episterol was strongly decreased. The overexpression of *ERG6* led to a strong decrease of zymosterol, the substrate of the methyl transferase reaction of Erg6p, but there was no increase of any product detectable. When *ERG5* was overexpressed, a strong decrease of episterol was detected, while a yet unidentified sterol intermediate accumulated in relatively high amounts. The content of squalene remained unchanged in all strains except the *ERG1*-overexpressing strain (data not shown).

The GC analysis had shown that mainly sterol precursors accumulated in the strains which overexpressed *ERG1* or *ERG11*. To analyze whether the accumulation of these intermediates occurred as free or esterified sterols, we analyzed sterols by TLC, without prior saponification. Using TLC it is possible to discriminate free and esterified sterols. Esterified sterol precursors are not directly available for transformation into downstream intermediates. Only free sterols can be used as substrates for the ergosterol biosynthetic pathway [11]. As seen in Table 3, changes of the sterol content almost exclusively occurred in the steryl ester fraction while the cellular concentration of free sterols remained unchanged. An exception to this observation was seen in the strain which overexpressed *ERG1*. Here an increased content of free sterols was detected (Table 3, *ERG1*). GC analysis of the substances reisolated from the TLC plates showed that the content of lanosterol was increased in the fraction of free sterols (data not shown). When lipid particles, the compartment in which sterols are stored in an esterified form [22], were isolated and purified from the same strain, lanosteryl esters were found in this compartment. No free lanosterol

Table 1
PCR primers used in this study

Primer	Sequence
ERG1*Not*If	5′-CTGCGGCCGCATCATGTCTGCTGTTAACGTTGC-3′
ERG1*Xho*Ir	5′-TTCTCGAGTTAACCAATCAACTCACCAAAC-3′
ERG7*Not*If	5′-CTGCGGCCGCAAGATGACAGAATTTTATTCTGACAC-3′
ERG7*Xho*Ir	5′-TACTCGAGCTTAAAGCGTATGTGTTTCATATG-3′
ERG11*Not*If	5′-CTGCGGCCGCAGGATGTCTGCTACCAAGTCAATCG-3′
ERG11*Xho*Ir	5′-ATCTCGAGCTTAGATCTTTTGTTCTGGATTTCTC-3′
ERG24*Not*If	5′-AGGCGGCCGCAGGATGGTATCAGCTTTGAATCCC-3′
ERG24*Xho*Ir	5′-AGCTCGAGTTAATAAACATATGGAATGATCTTGTAAG-3′
ERG25*Not*If	5′-CTGCGGCCGCAAGATGTCTGCCGTTTTCAACAACG-3′
ERG25*Xho*Ir	5′-TTCTCGAGTTAGTTAGTTAGTCTTCTTTTGAGC-3′
ERG26*Not*If	5′-CTGCGGCCGCAATATGTCAAAGATAGATTCAG-3′
ERG26*Xho*Ir	5′-ATCTCGAGTTACAAACCTTCGTCCATCC-3′
ERG27*Not*If	5′-CTGCGGCCGCAGGATGAACAGGAAAGTAGCTATCG-3′
ERG27*Mlu*Ir	5′-AGACGCGTTTAAATGGGGGTTCTAGTTTC-3′
ERG6*Not*If	5′-CTGCGGCCGCAAGATGAGTGAAACAGAATTGAG-3′
ERG6*Xho*Ir	5′-TTCTCGAGTTATTGAGTTGCTTCTTGGG-3′
ERG2*Not*If	5′-CTGCGGCCGCACCATGAAGTTTTTCCCACTCC-3′
ERG2*Xho*Ir	5′-TTCTCGAGTTAGAACTTTTTGTTTTGCAAC-3′
ERG3*Not*If	5′-CTGCGGCCGCAATATGGATTTGGTCTTAGAAGTCG-3′
ERG3*Xho*Ir	5′-AACTCGAGTCAGTTGTTCTTCTTGGTATTTG-3′
ERG5*Not*If	5′-CTGCGGCCGCAAAATGAGTTCTGTCGCAGAAAA-3′
ERG5*Xho*Ir	5′-TTCTCGAGCCATTATTCGAAGACTTCTCCAG-3′
ERG4*Not*If	5′-CTGCGGCCGCACTATGGCAAAGGATAATAGTGAG-3′
ERG4*Xho*Ir	5′-TTCTCGAGCTAGAAAACATAAGGAATAAAG-3′

Recognition sites for restriction enzymes *Not*I and *Xho*I[a] are underlined.
[a] *Mlu*I for ERG27*Mlu*Ir.

Table 2
Sterol content of *S. cerevisiae* AH22*tH3ura8* strains overexpressing genes of the post-squalene pathway, in peak area per gram dry matter and factor of change in relation to the empty vector (pFlat3)

Overexpressed gene	Lanosterol	4,4-Dimethylzymosterol	Zymosterol	Fecosterol	Episterol	Ergosterol	Unidentified sterol[a]	Relative factor (total sterols)
pFlat3	1.1	0.8	1.2	1.2	1.0	3.8	–	
(ref)	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**		**1.0**
ERG1	3.8	1.3	1.5	1.6	1.3	3.4		
	3.6	**1.6**	**1.3**	**1.4**	**1.4**	**0.9**		**1.5**
ERG7	0.9	0.7	1.1	1.1	0.8	3.4		
	0.8	**0.9**	**0.9**	**0.9**	**0.9**	**0.9**		**1.0**
ERG11	0.7	1.6	1.8	2.0	1.4	4.5		
	0.7	**2.0**	**1.5**	**1.7**	**1.5**	**1.2**		**1.2**
ERG24	1.5	0.9	1.2	1.7	1.1	3.4		
	1.4	**1.1**	**1.0**	**1.4**	**1.2**	**0.9**		**1.1**
ERG25	1.4	1.3	1.1	1.1	0.7	3.4		
	1.3	**1.7**	**0.9**	**0.9**	**0.7**	**0.9**		**1.0**
ERG26	0.8	1.0	1.0	0.9	0.7	3.4		
	0.8	**1.2**	**0.8**	**0.8**	**0.7**	**0.9**		**0.9**
ERG27	2.1	1.4	1.3	1.2	0.9	4.6		
	2.0	**1.8**	**1.1**	**1.0**	**0.9**	**1.2**		**1.2**
ERG6	1.0	0.9	0.4	0.8	0.8	3.8		
	0.9	**1.1**	**0.3**	**0.7**	**0.8**	**1.0**		**0.9**
ERG2	0.8	0.9	0.8	0.9	0.8	3.1		
	0.8	**1.1**	**0.7**	**0.8**	**0.8**	**0.8**		**0.8**
ERG3	2.0	1.4	1.2	1.3	0.5	4.6		
	1.9	**1.8**	**1.0**	**1.1**	**0.5**	**1.2**		**1.2**
ERG5	1.5	0.9	1.6	1.4	0.2	3.8	2.6	
	1.4	**1.1**	**1.3**	**1.2**	**0.2**	**1.0**		**1.3**
ERG4	1.9	1.2	1.4	0.8	0.5	4.2		
	1.8	**1.5**	**1.2**	**0.7**	**0.5**	**1.1**		**1.1**

Each single gene was overexpressed on the expression vector pFlat3. Data are given as peak area per gram dry matter (small letters) and as the factor of change (bold letters) of individual sterol relative to the reference strain, carrying the empty vector (pFlat3). The relative factor (total sterols) corresponds to the added peak areas of all individual sterols in comparison to the reference strain. Values represent the mean value of four experiments. The standard deviation for each sample was not greater than 10%.
[a]Only detectable in the *ERG5*-overexpressing strain.

was found, indicating that the free lanosterol was located in other membrane compartments.

3.2. *Overexpression of* ERG1 *in combination with any other gene*

Since the overexpression of *ERG1* led to a highly increased conversion of squalene into lanosterol and only moderate elevations of other sterols (Table 2), additional bottlenecks beside the squalene epoxidase reaction (Erg1p) had to be postulated later in the pathway.

We tested this hypothesis by combining the overexpression of *ERG1* with other genes of the post-squalene pathway. For this experiment, yeast was cotransformed with the vector pFlat1-*ERG1* and one of the pFlat3 vectors carrying the downstream genes (*ERG7*, *ERG24*, *ERG25*, *ERG26*, *ERG27*, *ERG6*, *ERG2*, *ERG3*, *ERG5* or *ERG4*). The sterol composition and the content of individual intermediates in these transformed strains were analyzed by GC (Fig. 2).

While the lanosterol content did not change in most combination strains in relation to the reference strain which only overexpressed *ERG1*, there was one combina-

Table 3
Relative amount of free and esterified sterols in the strain AH22*tH3ura8* given as factor of change in relation to the empty vector (pFlat3)

Overexpressed gene	Free sterols	Steryl ester
pFlat3	1.00	1.00
ERG1	1.21	1.43
ERG7	0.96	0.97
ERG11	1.03	1.31
ERG24	1.02	1.04
ERG25	1.00	0.93
ERG26	0.95	0.99
ERG27	1.06	1.23
ERG6	1.03	1.02
ERG2	0.97	0.91
ERG3	0.99	0.92
ERG5	1.02	1.31
ERG4	1.04	1.11

Analysis was done using TLC. Values represent relative amounts of sterols in comparison to the empty vector strain (pFlat3) which is set to 1.00. Ten microliters of a sterol extract was applied to a thin layer plate and sterols were quantified with a TLC scanner (Camag). Values represent the mean value of three experiments. The standard deviation for each sample was not greater than 4%.

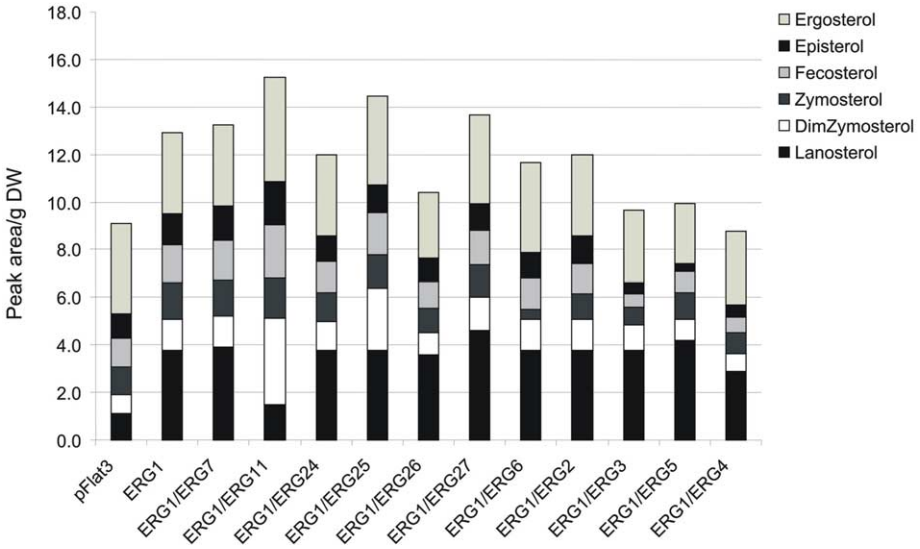

Fig. 2. Combined overexpression of *ERG1* and downstream genes in the yeast strain AH22*tH3ura8*. Data are given as peak area per gram dry matter. *ERG1* was overexpressed on the plasmid pFlat1 and each other gene on plasmid pFlat3. Cells were grown for 3 days and sterols were analyzed by GC. Additionally, data for the strain AH22*tH3ura8* carrying the empty vector pFlat3 or only overexpressing the *ERG1* gene are given. Values represent the mean value of three experiments. The standard deviation for any sample was not greater than 10%. DimZymosterol = 4,4-dimethylzymosterol.

tion where the content of lanosterol decreased significantly (by a factor of 0.4 relative to the reference strain which only overexpressed *ERG1*), and this was the coordinated overexpression of *ERG1* and *ERG11*. In this strain, the content of all downstream sterols from 4,4-dimethylzymosterol to ergosterol was significantly increased, leading to a total sterol content which was 1.9-fold higher than in the strain which was deregulated in the *tHMG1* alone (see Fig. 2: pFlat3) and three-fold higher than in a wild-type strain [9]. This coordinated effect of the combined overexpression could not be seen in any other combination. While the impact of the *ERG1* overexpression leading to an increased lanosterol content was seen in all combination strains, no conversion into later sterols occurred. Instead, some additional, but apparently independent effects of the second genes were detectable. Most of these additional effects had also been seen in strains which only overexpressed the corresponding second genes (see Table 2). It can be stated that an additive effect with respect to sterol accumulation occurred only by simultaneously overexpressing *ERG1* and *ERG11*.

When absolute changes of the peak area per gram dry mass were calculated from Table 2 and Fig. 2, it became evident that the sterol precursors were not converted up to the end product ergosterol in the *ERG1* overexpression strain. The overexpression of *ERG11* led to an increase of all sterols from 4,4-dimethylzymosterol to ergosterol in a similar range (concerning their peak areas). The combined overexpression of *ERG1* and *ERG11* resulted in a more or less equal increase of all individual sterols from zymosterol to ergosterol. This elevation was more than two-fold higher than in the *ERG11* overexpression strain.

3.3. Transcript analysis

To confirm that changes in the sterol content are based

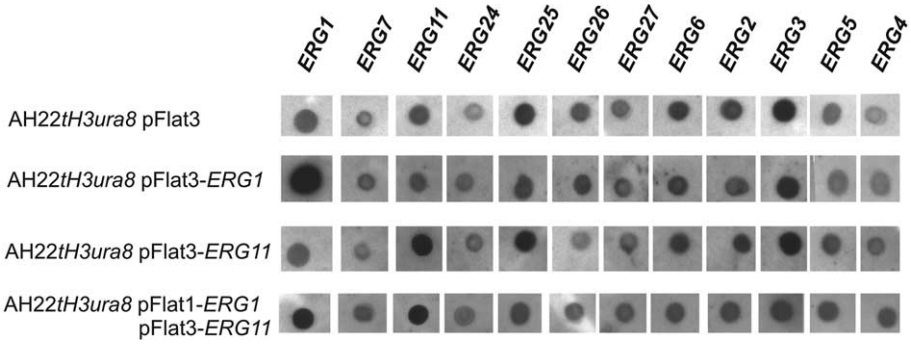

Fig. 3. Filter array analysis of the transcriptional level for the yeast strain AH22*tH3ura8* overexpressing either single genes *ERG1* and *ERG11* or a combination of both. Total RNA was isolated after 48 h of growth and hybridized to PCR products of individual *ERG* genes. The filter represents data of one of three identical experiments.

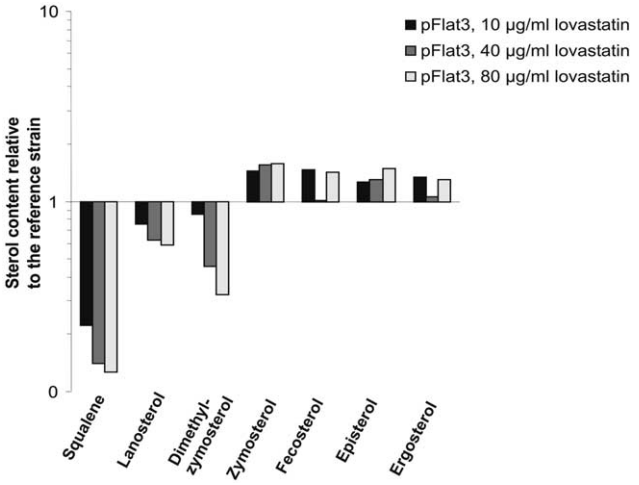

Fig. 4. Effect of lovastatin treatment on sterol content in the strain AH22*tH3ura8* pFlat3 (pFlat3). The HMG-CoA reductase was inhibited with different concentrations of lovastatin. Data are given relative to the corresponding reference strains without lovastatin treatment which corresponds to 1 on the *y*-axis. Cells were grown for 3 days and sterols were analyzed by GC. Values represent the mean value of three experiments. The standard deviation for any sample was not greater than 10%.

directly on the overexpression of *ERG1* and *ERG11*, transcript analyses were done using a nylon filter array. With this method it was not only possible to detect changes of the transcriptional level of the corresponding overexpressed genes, but of all tested genes. Data in Fig. 3 show that after 48 h of cultivation in all cases, either single overexpression of *ERG1* and *ERG11* or combined overexpression of both, the transcriptional level of the overexpressed genes was significantly increased compared to that of the empty vector strain. However, combined overexpression of *ERG1* and *ERG11* was not as high as in the single-overexpressing strains. All other wild-type copy genes were not significantly influenced. An exception can be seen in the *ERG1*/*ERG11* combination strain. Overexpression of both genes simultaneously led to a decreased transcriptional level of *ERG25* and *ERG3*, which gave a relatively high signal in the empty vector strain and the single-overexpressing strains (Fig. 3). This decreased transcriptional level could be an effect of the high sterol content in this yeast strain, leading to a downregulation of the transcription of these genes. Transcript analyses at different time points have demonstrated that overexpression of *ERG1* and *ERG11* under the control of the constitutive copy of the *ADH1* promoter is constant over 4 days (data not shown).

Table 4
Content of squalene in the strain AH22*tH3ura8* overexpressing either *ERG1*, *ERG11* or the combination of both, in peak area per gram dry matter

Strain	Squalene[a]
AH22*tH3ura8*, pFlat3	10.6
AH22*tH3ura8* pFlat3-*ERG1*	5.1
AH22*tH3ura8*, pFlat3-*ERG11*	10.1
AH22*tH3ura8*, pFlat1-*ERG1*, pFlat3-*ERG11*	3.2

[a]The content of squalene is given in peak area per gram dry matter. Values represent the mean value of four experiments. The standard deviation for each sample was not greater than 10%.

3.4. Lovastatin treatment

The data from the overexpression experiments suggest that the accumulation of squalene in strain AH22*tH3ura8* is the consequence of the unregulated activity of the truncated HMG-CoA reductase and the too low activity of the wild-type copy of squalene epoxidase. It was, however, not known if the large squalene pool in this strain influences the flow through the post-squalene biosynthetic pathway. We therefore blocked the activity of the unregulated HMG-CoA reductase by the specific inhibitor lovastatin [16] and assayed the effect on the pool of squalene and on sterols.

Different concentrations of lovastatin from 10 µg ml^{-1} up to 80 µg ml^{-1} were used and the content of squalene and sterols was analyzed by GC. The growth of the cells was not influenced in any tested concentration of lovastatin. Fig. 4 shows that lovastatin treatment indeed led to a strong decrease of squalene, but the squalene concentration was still higher than in a wild-type, where squalene is only detectable in traces [9]. Lovastatin treatment also reduced the content of the sterols lanosterol and 4,4-dimethylzymosterol to different extents, dependent on the lovastatin concentration. The later sterols from zymosterol to ergosterol were slightly increased or were left unchanged.

In a further step, we treated the strains which overexpressed either *ERG1* or the combination of *ERG1* and *ERG11* with 10 µg ml^{-1} lovastatin (data not shown). Lovastatin effected the same changes in these strains as in the strain which carries the empty vector (Fig. 4), and the pools of squalene, lanosterol and 4,4-dimethylzymosterol decreased significantly. The amount of these intermediates was still higher than in the wild-type strain, which might explain why the amount of later sterols from zymosterol to ergosterol was not affected.

4. Discussion

With the aim to increase the content of sterols in yeast, a strategy was established to overexpress the genes of the post-squalene biosynthetic pathway in a squalene-overproducing yeast strain which is deregulated in the main bottleneck of the early ergosterol biosynthetic pathway, the HMG-CoA reductase. The overexpression of *ERG1* alone or the combination of *ERG1* and *ERG11* led to a significant decrease of the squalene pool and a concomitant increase in the amount of sterols. Both genes are known to be transcriptionally regulated. The native promoter of

ERG1 contains a regulatory element that mediates ergosterol-dependent regulation [23]. The promoter region of *ERG11* contains two sterol-dependent upstream activation sites which control transcription of this gene [24]. Our study shows that the deregulated overexpression of these genes leads to a changed activity, resulting in an increased flow of substrate to product.

The storage of squalene or sterols in the cell does not influence the growth or viability of the strains described here, as all the strains which produce increased amounts of squalene or sterols show a growth behavior comparable to that of the wild-type strain. This is contrary to the observation by Donald et al. [25] who have described a decreased growth rate of strains that accumulate squalene.

Ergosterol and some of its precursors are stored as steryl esters [5]. Our data show that all the changes in the total amount of sterols occur in the steryl ester fraction, while free sterols remain constant in all cases except for lanosterol in the *ERG1*-overexpressing strain (Table 3). The data presented here suggest that the esterification of sterols which is catalyzed by the enzymes Are2p and Are1p in yeast compensates for the excess of substrate when sterol precursors accumulate as a result of gene overexpression. This effectively protects the physiology of the membranes. The esterification reaction might be regarded as an overflow for surplus sterols which cannot be directly converted to the end product.

Normally sterols are stored in lipid particles in the esterified form [5]. The *tHMG1*/*ERG1* overexpression strain accumulates lanosterol in both the esterified and the free form. The free lanosterol is not localized in the lipid particles. Why this accumulation of a free intermediate occurs and why this intermediate is only partially esterified is not known. The explanation could be a combination of three factors: (i) a limited activity of the Erg11p reaction in this strain leading to the accumulation of lanosterol with regard to the *ERG1* overexpression; (ii) a reduced affinity of the main steryl-acyltransferase Are2p to lanosterol; and (iii) the low activity of the isoenzyme Are1p [10] which is known to have a higher affinity to lanosterol but a lower activity than Are2p [26]. These three factors together could result in a non-quantitative conversion of free lanosterol into its fatty acid ester leading to an accumulation of free intermediate.

The squalene accumulation is significantly reduced in the *ERG1*-overexpressing strain (Table 4). This finding suggests that the squalene pool is formed due to the deregulation of the HMG-CoA reductase and a limited activity of the enzyme Erg1p when present at its wild-type level. A regulatory role of the squalene epoxidase (*ERG1*) in the post-squalene pathway is suggested as the overexpression of the corresponding gene removes this limitation and consequently leads to an elevated transfer of squalene into sterols.

To analyze the influence of the deregulated HMG-CoA reductase in the strain AH22*tH3ura8* on the formation of intermediate pools, the activity of this enzyme was reduced using the competitive inhibitor lovastatin [16]. The data of this experiment have shown that the pools of squalene, lanosterol and 4,4-dimethylzymosterol are strongly decreased dependent on the concentration of lovastatin. All later sterols (zymosterol to ergosterol) are either slightly increased or uninfluenced under these conditions. Due to the deregulation of the HMG-CoA reductase the substrate pressure in the strain AH22*tH3ura8* is very high. This pressure is lowered due to the limitations at the squalene epoxidase and the sterol-14α-demethylase in the later part of the post-squalene biosynthetic pathway. This leads to an overflow of predominantly early intermediates into pools. Lovastatin treatment reduces the substrate pressure due to the decreased HMG-CoA reductase. As a result the amount of surplus substrates is also decreased. These changes only influence the early pools of squalene, lanosterol and 4,4-dimethylzymosterol. Due to the limitations in the pathway the pools of the downstream intermediates are more or less uninfluenced. These results are in concert with results from [16], who have shown that treatment of a wild-type strain with 10 μg ml^{-1} of lovastatin dramatically decreased the amount of sterols in the cell. Treatment with higher concentrations reduces the production of steryl ester to a minimum level and the content of free sterols is also decreased.

In a wild-type strain the substrate pressure is much lower than in the HMG-CoA reductase-deregulated strain and pools of intermediates are not accumulated. The only sterol which accumulates is the end product ergosterol [27]. As the substrate pressure is lowered due to the lovastatin treatment in the wild-type strain, the content of ergosterol is directly influenced.

In summary, our data demonstrate that in yeast at least three steps regulate the synthesis of surplus sterols. These steps are the HMG-CoA reductase, the squalene epoxidase Erg1p and the sterol-14α-demethylase Erg11p. The combined overexpression of the corresponding genes leads to a significant increase in the amount of total sterols by a factor of three in comparison to a wild-type strain. The HMG-CoA reductase controls the entering of intermediates on the pre-squalene part of the pathway and Erg1p and Erg11p seem to control the transfer of intermediates into the post-squalene part.

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