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Engineering the *Saccharomyces cerevisiae* isoprenoid pathway for *de novo* production of aromatic monoterpenes in wine

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Abstract

Grape musts contain a variety of terpenols that significantly affect wine aroma. The amounts of these metabolites depend on the grape variety, and many cultivars are non-aromatic. Yeasts like *Saccharomyces cerevisiae* cannot produce and excrete monoterpenes efficiently, mainly due to their lack of monoterpene synthases. By metabolic engineering we have modified the isoprenoid biosynthesis pathway in a wine yeast strain of *S. cerevisiae* expressing the *Clarkia breweri* S-linalool synthase gene. Under microvinification conditions, without compromising other desirable and useful fermentative traits, the recombinant yeast efficiently excreted linalool to levels exceeding the threshold of human perception. Bearing in mind the possibility of (co-)expressing other genes that encode enzymes leading to the production of various aroma compounds and the feasibility of controlling the levels of their expression, the potential of this achievement for future genetic manipulation of wine varietal aroma or for use in other alcoholic drinks seems very promising.
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Keywords: *Saccharomyces cerevisiae*; *Clarkia breweri*; Wine aroma; Monoterpenes; Linalool synthase; Isoprenoid pathway; Metabolic engineering

1. Introduction

One of the most important characteristics of a quality wine is its aromatic fragrance. Several hundred volatile components have been identified in grapes and wine, and many of these are important for wine aroma and flavor (Schreier, 1979). Among these, floral monoterpene alcohols such as geraniol, nerol, linalool and α-terpineol are important in defining the aroma and flavor of young Muscat-related wines, whereas low levels of these compounds are present in juice from non-aromatic cultivars of *Vitis vinifera* (Ribèrau-Gayon et al., 1975; Marais, 1983; Sefton et al., 1993; Falque et al., 2002). Geraniol and linalool are considered to be the most important monoterpene alcohols as they are present in greater concentrations and have lower flavor thresholds than other major wine monoterpenes (Ribèrau-Gayon et al., 1975); moreover, linalool is important for the flavor of other alcoholic beverages (Steinhaus et al., 2003). Furthermore, some monoterpenes are important due to their antimicrobial activity (Pattnaik et al., 1997) and potential health benefits (Crowell, 1999).

Different biotechnological strategies have been developed, with varying degrees of success, to improve wine flavor through enhancement of terpenoid content. Since monoterpenes are present in the grape as free volatile forms as well as glycosidically bound flavorless precursors (Williams et al., 1982; Günata et al., 1985), enzymatic hydrolysis of grape must monoterpenyl–glycoconjugates gave positive results in stimulating the volatilization of aglycons. This was achieved either by adding exogenous enzyme preparations (Günata et al., 1993; van Rensburg and Pretorius, 2000) or by using transgenic wine yeasts that produce such activities (Pretorius and Bauer, 2002; Manzanares et al., 2003; Ramón et al., 2005; Schuller and Casal, 2005). However, these strategies are less useful for those musts from non-aromatic grape varieties having low contents of free and bound monoterpenes. In these cases, a good alternative would be that the yeast carrying out the vinification process be able to produce these aromatic compounds through its own metabolism.

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Yeasts like *Saccharomyces cerevisiae* do not efficiently excrete monoterpenes (Carrau et al., 2005) but synthesize the phosphorylated form of geraniol, geranyl diphosphate (GDP), as an intermediate of farnesyl diphosphate (FDP) synthesis, a key molecule in the isoprenoid pathway that leads to the synthesis of dolicols, ubiquinones and sterols (Lees et al., 1999). It has been reported that leaky FDP synthase mutant laboratory strains of *S. cerevisiae* are able to produce geraniol, linalool and citronellol (Chambon et al., 1990, Javelot et al., 1991) probably through the action of unknown phosphatases on accumulated GDP, and subsequent bioconversions. Monoterpene producing ability in wine yeasts was introduced by sexual crosses made between those mutant strains and an industrial wine strain (L116). Progeny strains were able to produce considerable amounts of monoterpenes; however, their ability to consume glucose and produce ethanol was much lower to that of L116 (Javelot et al., 1991). Because most winemaking characteristics are probably under multigenic control and industrial yeasts are usually polyploid or aneuploid, the above outcome illustrates the difficulty in genetically manipulating wine yeast strains through 'classic' breeding programs. In contrast, recombinant DNA techniques provide the means to construct novel yeasts with improved qualities in a more defined way.

Monoterpenes also determine the scent of many flowers and over the last 10 years many genes have been characterized that encode plant monoterpene synthases, a family of enzymes, which specifically catalyze the synthesis of these bioactive metabolites. Such is the case of the linalool synthase gene (*LIS*) of *Clarkia breweri* (Dudareva et al., 1996), a strongly fragrant annual flowering plant native to California that is a member of the Evening-Primrose Family (*Onagraceae*). Since all monoterpenes are produced from the ubiquitous C_{10} intermediate GDP, it is possible to engineer host plants (Mahmoud and Croteau, 2002) as well as microorganisms for *de novo* production of specific monoterpene(s). Indeed, microorganisms have frequently been used to functionally test complementation by novel monoterpene synthase encoding genes, and more recently their utility as cell factories to produce monoterpenes has been shown (Carter et al., 2003; Reiling et al., 2004; Hernández et al., 2007; Oswald et al., 2007). In this work, we evaluate the possibility of producing plant monoterpene synthases, and consequently aromatic monoterpenes, in *S. cerevisiae* under vinification conditions.

2. Materials and methods

2.1. Strains and culture conditions

Escherichia coli strain DH5α [*endA1*, *hsdR17*, *gyrA96*, *thi-1*, *relA1*, *supE44*, *recA1*, *ΔlacU169* (*Φ80 lacZΔM15*)] was used as the recipient strain for cloning experiments and plasmid amplification. The *S. cerevisiae* wine strain T_{73}-4 [*ura3*::470/*ura3*::470] (Puig et al., 1998) was used for functional expression of plasmids bearing the $TDH3_p$::*LIS*::$PGK1_t$ expression cassette. The industrial wine yeast strain T_{73} (CECT1894, commercialized by Lallemand Inc., Montreal, Canada) was used as a control for microvinification experiments.

E. coli was maintained in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with or without 100 μg ml^{-1} ampicillin. *S. cerevisiae* strains were maintained in YPD-rich medium (1% yeast extract, 2% bacteriological peptone, 2% glucose) or SD-minimal supplemented medium (0.17% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, USA), 2% glucose, 0.5% ammonium sulfate, 20 mg l^{-1} adenine, 20 mg l^{-1} histidine and 30 mg ml^{-1} leucine) with or without 20 mg l^{-1} tryptophan or 20 mg l^{-1} uracil. For solid media, 1.5% agar was added. The production of linalool was determined under the following growth conditions: selected transformants were cultured overnight in SD supplemented medium, lacking uracil, and the cells were then used to inoculate (10^6 cells ml^{-1}) 100 ml cultures, in 500 ml flasks, of complete (YPD) medium. Yeast cultures were grown with continuous shaking (200 rpm) at 30 °C. To test the ethanol tolerance of controls and *LIS*-transformed yeasts, yeast cells that had been grown in selective SD medium for 16 h were washed twice with sterile distilled water (DW), suspended in sterile DW to an OD_{600} of 1.0, serially diluted to OD_{600} of 0.1, 0.01, 0.002 and 0.001, and then 3 μl drops of each dilution were spotted on YPD plates containing 0%, 5%, 8% and 10% (v/v) ethanol (van Voorst et al., 2006). Growth was conducted at 30 °C for 2 days before plates were photographed.

2.2. DNA manipulations, transformations and RT-PCR

E. coli plasmid isolation and general DNA manipulations were performed following standard protocols (Sambrook and Russell, 2001). Transformation of the T_{73} strains was done using lithium acetate to permeabilize the cells as previously described (Gietz et al., 1995; Puig et al., 1998). Transformants were selected and maintained on SD plates without uracil. Restriction enzymes, Expand High Fidelity, Klenow fragment, T4 DNA ligase and Shrimp alkaline phosphatase (SAP) were purchased from Roche Diagnostics and used as recommended by the supplier. Reverse transcription-PCR (RT-PCR) was used to confirm the presence of *LIS* mRNA. Total RNA samples were treated with RNase-free DNase I (Roche) to eliminate contaminating DNA. MMLV (USB) and oligo-dT_{18} primer were used to synthesize the first-strand cDNA (10 μg of RNA per 50 μl reaction mixture). A 1 μl from the previous reaction was added to the PCR mixtures (19 μl). The *LIS* primers used were 5′-CTACCTTAGGTTATCTTGC-3′ and 5′-TTGGGGCTGATGAGGTGG-3′. The *S. cerevisiae SUS1* primers 5′-GGATACTGCGCAATTAAAGAG-3′ and 5′-GTGTATCTACAATCTCTTCAAG-3′ were used as internal positive control (data not shown). A negative control (no reverse transcription step) confirmed the absence of DNA in the RNA samples (not

shown). PCR results were analyzed by electrophoresis on a 0.8% agarose gel.

2.3. Construction of yeast plasmids carrying the LIS gene of C. breweri

The plasmid pBS-Lis containing the *C. breweri LIS* cDNA (GenBank accession no. U58314; Dudareva et al., 1996) from 35-bp upstream of the translation start codon to 49-bp downstream of the translational stop codon, was digested with *Xba*I and *Sal*I and the 2.08-kb small fragment, carrying most of the *LIS* cDNA, was isolated from a 0.8% agarose gel. To complete the *LIS* ORF, a 0.65-kb *Bgl*II-*Xba*I fragment was obtained by digesting the PCR product synthesized using the oligonucleotide primers Lis-Bgl2 (5′-CGGAAGATCTGGTACCACCTTAAACAAGAC-3′) and Lis-XbaI (5′-AGGCAATGCTTCTAGAAATAG-3′). Both fragments were ligated between the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase gene promoter ($TDH3_p$) and the yeast phosphoglycerokinase (PGK_t) terminator and polyadenylation signals of plasmid pG-1 (Schena et al., 1991) which had been previously digested with *Bam*HI and *Sal*I. These manipulations placed *LIS* next to the *TDH*3 promoter in a high copy number shuttle vector to allow high-level expression. The plasmid pG1-Lis obtained in this way contained the *S. cerevisiae TRP*-1 selectable gene. In order to transfer the *LIS* gene to the wine yeast strain T_{73}-4, a 1.5-kb PCR fragment containing the *S. cerevisiae URA*3 gene was obtained by PCR from T_{74} genomic DNA using oligonucleotides URA1 (5′-AATGTGGCTGTGGTTTCAGGG-3′) and URA4 (5′-GGCGAGGTATTGGATAGTTCC-3′). This fragment was subsequently digested with *Hin*dIII, Klenow-filled and cloned into the unique *Sma*I site of PG1-Lis to render pG1-Lis-URA. A control vector, pG1-URA, was also generated after cloning the *URA3* fragment into the SAP-treated *Sma*I site of pG-1. Fragments obtained by PCR were checked by sequencing to ensure the absence of PCR induced mutations. For plasmid stability analyses transformants were grown under both selective (SD) and non-selective (YPD) conditions and the colonies growing under each condition were counted.

2.4. Microvinification experiments

Microvinifications were done in triplicate at 18 °C using 250 ml glass bottles containing 225 ml of Parellada (Villafranca del Penedés, Spain) white grape must. The must was sterilized with 0.2% (v/v) of dimethyl dicarbonate (Velcorin; Bayer, Levercusen, Germany), supplemented with 0.3 g l^{-1} of Lalvin Nutrient Vit (Lallemand Inc.), and inoculated with 10^6 cells ml^{-1} from overnight cultures of the T_{73} strain or its recombinant derivatives T_{73}-4-pG1-URA (YR65) and T_{73}-4-pG1-Lis-URA (YR64). To monitor the progress of the fermentation, reducing sugar concentration was measured refractometrically as BRIX grades in a Carl Zeiss 38189 refractometer; an Echo-Enosys analyzer (Tecnova S.A., San Sebastián de los Reyes, Spain) was used to measure the sugar content of the initial must and to determine the final point of the fermentation, using glucose as standard. At the end of fermentation (sugar concentration below 2 g l^{-1}) the wines were centrifuged to remove yeast cells and subsequently transferred to new bottles, which were kept at −20 °C prior to gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses. Ethanol concentration in the final wines was measured in an Alliance Infrascan infrared Spectrophotometer (Alliance Instruments, Eragny-Sur-Oise, France).

2.5. Metabolite analyses

Monoterpenes produced by recombinant yeasts were quantified by GC analyses and identified by GC and GC-MS. Aliquots of the yeast cultures were taken and analyzed by headspace solid-phase-microextraction sampling (SPME) using poly(dimethylsiloxane) (PDMS) fibers (Supelco, Bellefonte, PA, USA) following a method adapted from Rojas et al. (2001). Aliquots of 3 ml of the samples were placed in 9 ml vials containing 0.6 g of NaCl and 15 μl of 0.005% 2-octanol (Fluka) as internal standard. The vials were subsequently closed with screwed caps with silicone septa (Supelco) and stirred for 2 h at 25 °C to get the required headspace–liquid equilibrium. 100 μm PDMS fibers were injected through the vial septum and exposed to the headspace for 30 min. The fiber was then desorbed for 4 min in a Hewlet-Packard (HP) capillary gas chromatograph, model 5890 series II, equipped with an HP-Innowax column (length, 15 m; inside diameter 0.25 mm; film thickness, 0.25 μm). The injection block and detector temperatures were kept constant at 220 and 280 °C, respectively. The oven temperature program was 60 °C (5 min) to 190 °C at 5 °C min^{-1} and to 250 °C at 20 °C min^{-1} and then kept at 250 °C for 2 min. Linalool and other aromatic compound concentrations were calculated using standard solutions and are given as the average of three independent cultures. Calibration curves were determined using the same protocol by exposure of the PDMS filament to the headspace of YPD spiked with standards of final concentrations of 1, 10, 100, 1000 and 10 000 μg l^{-1}.

Identification of compounds was determined by comparing retention times with those of standard compounds (Sigma St Louis, MO, USA) using an Agilent 6890 gas chromatograph and an Agilent 5973N mass selective detector (Agilent Technologies, Waldbronn, Germany), under the same chromatographic conditions.

3. Results

3.1. Construction of wine yeast strains functionally expressing the LIS gene of C. breweri

The *LIS* gene (encoding S-linalool synthase) from *C. breweri* (Dudareva et al., 1996) was used to evaluate the

possibility of producing plant monoterpene synthases, and consequently monoterpenes, in *S. cerevisiae* under vinification conditions. GDP is the general substrate for monoterpenes and linalool is produced from this compound in a single step (Pichersky et al., 1995). The *LIS* cDNA was cloned under the regulation of the *S. cerevisiae TDH3* strong constitutive promoter (Bitter and Egan, 1984) and the *PGK1* terminator into a binary vector and used to transform the *S. cerevisiae* T_{73}-4 wine strain. The uracil prototrophic transformants YR63 and YR64 (T_{73}-4-Lis) were thus isolated. To probe functional expression of the transgene, supernatants from overnight cultures of these transformants, as well as the control strain YR65 (T_{73}-4 transformed with the empty vector), were analyzed by GC and GC-MS. These results revealed the presence of linalool only in the culture supernatants of the YR63 and YR64 strains while, as expected, linalool was not detected in YR65 (Fig. 1A). RT-PCR (Fig. 1B) and northern (data not shown) analyses revealed that production of linalool was related to the presence of *LIS* mRNA.

3.2. Growth characteristics of engineered wine yeasts expressing C. breweri LIS

Introduction of the linalool synthase gene in *S. cerevisiae* may lead to a redirection of the flux of the isoprenoid precursors DMADP (dimethylallyl diphosphate) and IDP (isopenthenyl diphosphate) towards GDP, competing with FDP formation, which is required to produce sterols. Since depletion of some of these isoprenoids could have a

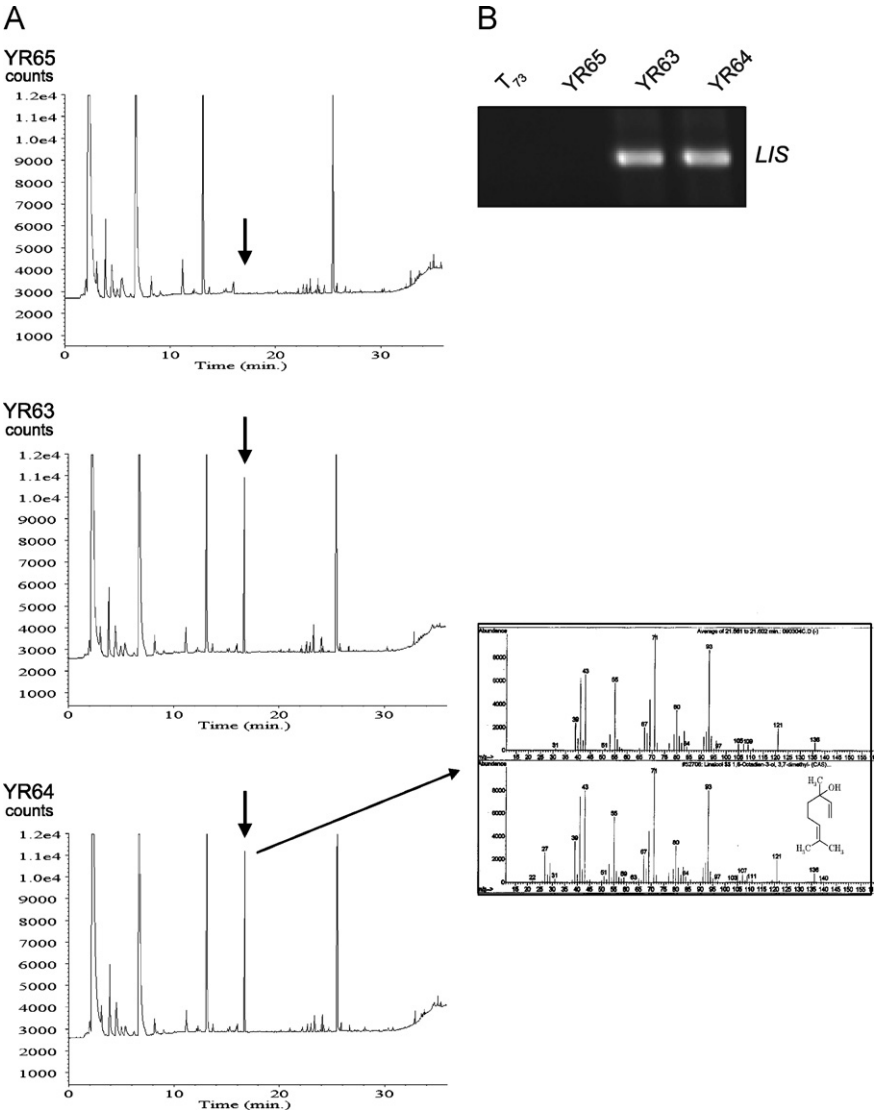

Fig. 1. Functional expression of the *LIS* transgene in *S. cerevisiae*. (A) Presence of linalool in cell cultures of yeast expressing *C. breweri LIS*, analyzed by GC and identified by GC-MS as described in Material and methods. Arrows indicate retention time and peaks of linalool. Bottom right, mass spectrum of the product detected in the recombinant yeast strain YR64 yielding a fragmentation pattern identical to that of linalool. (B) Detection of *LIS* expression by RT-PCR analysis of total RNA from YR63 and YR64 (pG1-Lis-URA) and the negative control strains YR65 (complemented with pG1-URA) and T_{73}.

dramatic effect on the yeast phenotype, and linalool accumulation could induce toxic effects, the consequences of overexpressing the *LIS* gene in *S. cerevisiae* were studied in terms of growth rates and ethanol tolerance. Time course growth curves were made with the two transformants harboring the *C. breweri LIS* expression cassette (YR63 and YR64) and the control complemented strain (YR65). The strains were cultured overnight in synthetic minimal selective medium and used to inoculate 100 ml cultures of complete (YPD) medium. The growth rates of the yeast strains expressing *LIS* were identical to that of the control strain (Fig. 2A), indicating that the amount of linalool and the putative reduction of precursors from the isoprenoid pathway do not apparently produce deleterious effects on yeast growth under such conditions. The stabilities of the plasmids in transformants YR63 and YR64 after 58 h growth in non-selective YPD media were calculated to be 97% and 93%, respectively. Thus, plasmid loss cannot explain why growth rates do not differ.

Several studies made on yeasts have correlated ergosterol synthesizing ability with ethanol tolerance (Alexandre et al., 1994; Swan and Watson, 1998) which is a useful phenotypic trait for wine yeasts. Therefore, we investigated whether *LIS* gene expression could have pleiotropic effects on ethanol resistance. The growth of transformant strains under ethanol stress was investigated by adding freshly grown cells to YPD solid medium containing 0–10% (v/v) ethanol, as described in Materials and methods. The transformants expressing the *LIS* gene displayed the same ethanol tolerance as that of the control strain (Fig. 2B).

3.3. Linalool production by engineered wine yeasts in synthetic YPD medium

To evaluate to what extent the wine yeast isoprenoid biosynthetic pathway is accessible for foreign monoterpene production, the yeast transformants' ability to form linalool, and eventually other volatile metabolites, was analyzed over a 58 h period. Excreted linalool was detected 5 h after transfer to YPD medium (Fig. 2C). The highest linalool concentration ($\sim$77 $\mu g\,l^{-1}$) was reached at around 11 h, coinciding with the mid-exponential growth phase (see Fig. 2A), but then decreased slightly. The drop in linalool concentration over time could be due to the loss of the volatile terpene under our experimental conditions. No other significant differences in volatile composition were detectable between transgenic and control strains (data not shown), not even those involving linalool to α-terpineol bioconversions previously described in yeasts (King and Dickinson, 2000). However, as headspace SPME of the yeast cultures was employed to measure volatiles, we cannot rule out that minor side reactions producing linalool derivatives may go undetected as a consequence of evaporation or that total linalool production titers over the 58 h time course could be underestimated.

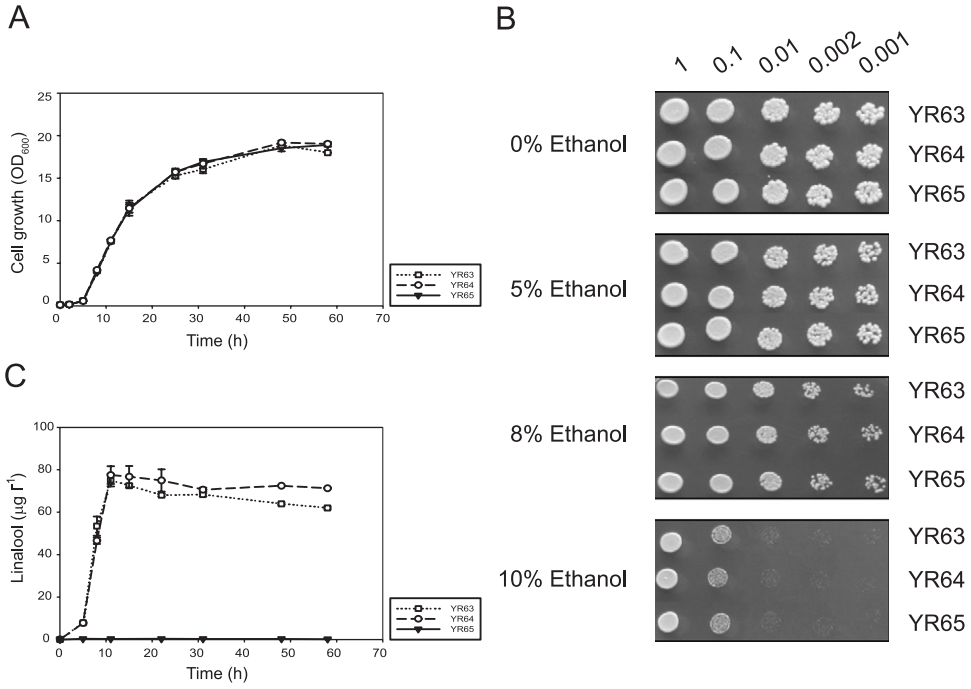

Fig. 2. Growth and linalool production kinetics of recombinant yeasts expressing *LIS*. (A) Growth curves of wine yeast cells transformed with plasmids containing *LIS*, YR63 (□) and YR64 (○), and the complemented control strain YR65 (transformed with pG1-URA) (▼) grown in YPD liquid medium. (B) Growth of the control strain YR65 and the *LIS* transformed strains YR63 and YR64 on ethanol medium. The data shown in panel B are representative of at least two separate experiments. (C) Linalool production in recombinant and control strains during growth in YPD; headspace analysis by GC of the same cultures from the growth curve was performed. Results are presented as the mean and standard deviations of three different experiments.

Very recently, high levels of geraniol production has been reported by reference laboratory strains of *S. cerevisiae* expressing *Ocimum basilicum* geraniol synthase (GES) (Oswald et al., 2007). However, the aforementioned authors did not manage to produce linalool when the same strains were transformed with the *C. breweri LIS* gene. Direct comparison between this result and that reported here is not feasible since the growth and production conditions were different, as were the strains and plasmid constructs used. Nevertheless, a conclusion common to both studies is that *S. cerevisiae* cells (from laboratory and wine strains) contain enough free GDP to be catalytically transformed by monoterpene synthases (such as GES or LIS) into monoterpenes.

3.4. Linalool production by engineered wine yeast under microvinification conditions

To evaluate the industrial utility of this new biotechnological approach for modifying wine aroma, microvinification experiments were carried out with one of the transformants expressing the *LIS* gene (YR64), with the control transformant lacking *LIS* (YR65), and with the untransformed T_{73} industrial strain. Each of the selected strains was inoculated into sterile Parellada white must and the kinetics of fermentations were followed. As can be seen in Fig. 3, fermentation progressed similarly in the microvinifications and all three strains were able to complete fermentation in eight days. The stability of the plasmid at the end of the vinification was 82% in YR64, indicating high maintenance of the *LIS* expression cassette throughout the process. The growth rates of the recombinant strains (YR64 and YR65) were almost identical, although slightly slower than that of T_{73} (Fig. 3A) as observed with other *S. cerevisiae* T_{73} recombinant strains (our unpublished data). This fact could explain, at least partially, the even slighter difference observed in the kinetics of sugar consumption (Fig. 3B), or ethanol production (Fig. 3C). GC analyses revealed the presence of linalool only in fermentations carried out by the YR64 (T_{73}-4-Lis) strain and throughout the whole fermentation process. The highest level ($\sim 26\,\mu g\,l^{-1}$) was reached in two days,

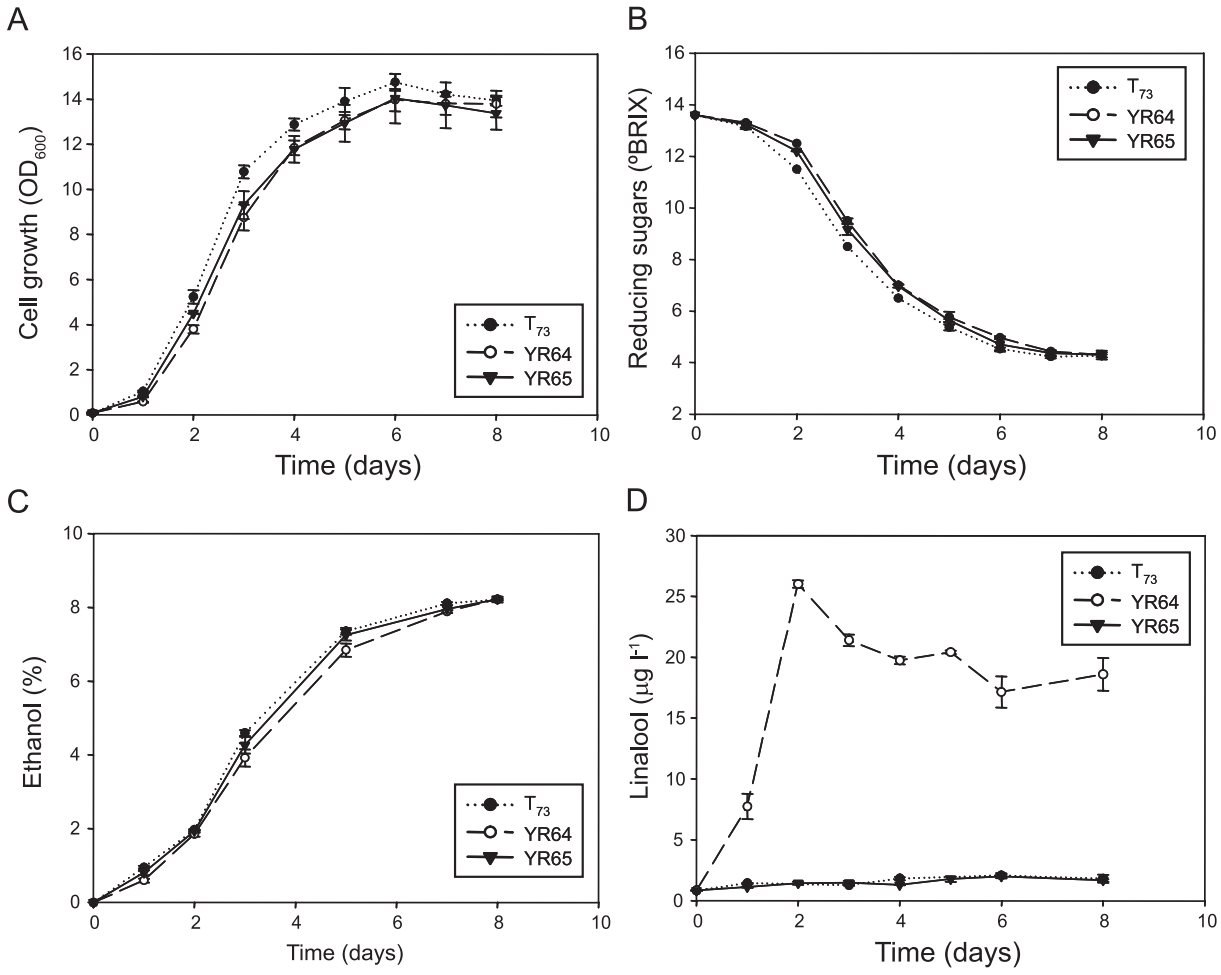

Fig. 3. Analysis of microvinifications. Microvinifications were carried out with the commercial wine yeast strain T_{73} (●), the YR65 control strain transformed with the vector pG1-URA (▼), and the YR64 transformant strain expressing the *C. breweri LIS* gene (○). The growth curves of the inoculated strains (A), the kinetics of reducing sugar consumption (B), ethanol production (C), and linalool accumulation (D) are shown during the course of fermentation. Bars represent standard deviations of duplicate measurements from triplicate microvinifications.

coinciding with the start of the exponential growth phase and later declining by 30% until the end of this phase. After the sixth day of fermentation (the beginning of stationary phase) the amount of linalool remained more or less constant (Fig. 3D). Linalool production was further confirmed using GC-MS to analyze wines (not shown). Linalool has an aroma with a sweet, floral alcoholic note and its aroma threshold is in the range of 4–10 ppb (Fenaroli, 2002). The amount of linalool excreted by the recombinant yeast under microvinification conditions reaches levels of at least twice this value, indicating the biotechnological potential of this engineered wine yeast to modify the sensorial qualities of wine. It is noteworthy that linalool production profiles under microvinification conditions (Fig. 3D) differ from those obtained in aerobic YPD growth (Fig. 2C). Moreover, the amount of linalool produced in natural grape must in relation to cell number is around 30% lower than in YPD medium. The reason for these discrepancies is not clear at present; however, they could be explained by the fact that linalool production totally depends on GDP availability and that the intracellular levels of the first three metabolites of the mevalonate pathway are affected by the carbon source, the growth phase and the specific growth rate (Serker et al., 2006).

3.5. Characterization of wine's volatile profile

One of the possible drawbacks in metabolic engineering is the possibility that the introduction of one trait, such as linalool or geraniol production, could inadvertedly result in unforeseen changes in metabolite profiles, as has been reported for transgenic tomato fruits expressing *LIS* or *GES* (Lewinsohn et al., 2001; Davidovich-Rikanati et al., 2007). To assess whether *LIS* expression could lead to other changes in a wine's volatile profile, a partial metabolomic study of certain volatile compounds of oenological relevance was carried out on both recombinant yeast derived and control wines. Full-scan GC analysis (Fig. 4 and Table 1) shows that apart from the linalool peak there were no other significant differences ($P<0.05$) in the levels of other related monoterpenes, linalool derivatives or other important aromatic compounds

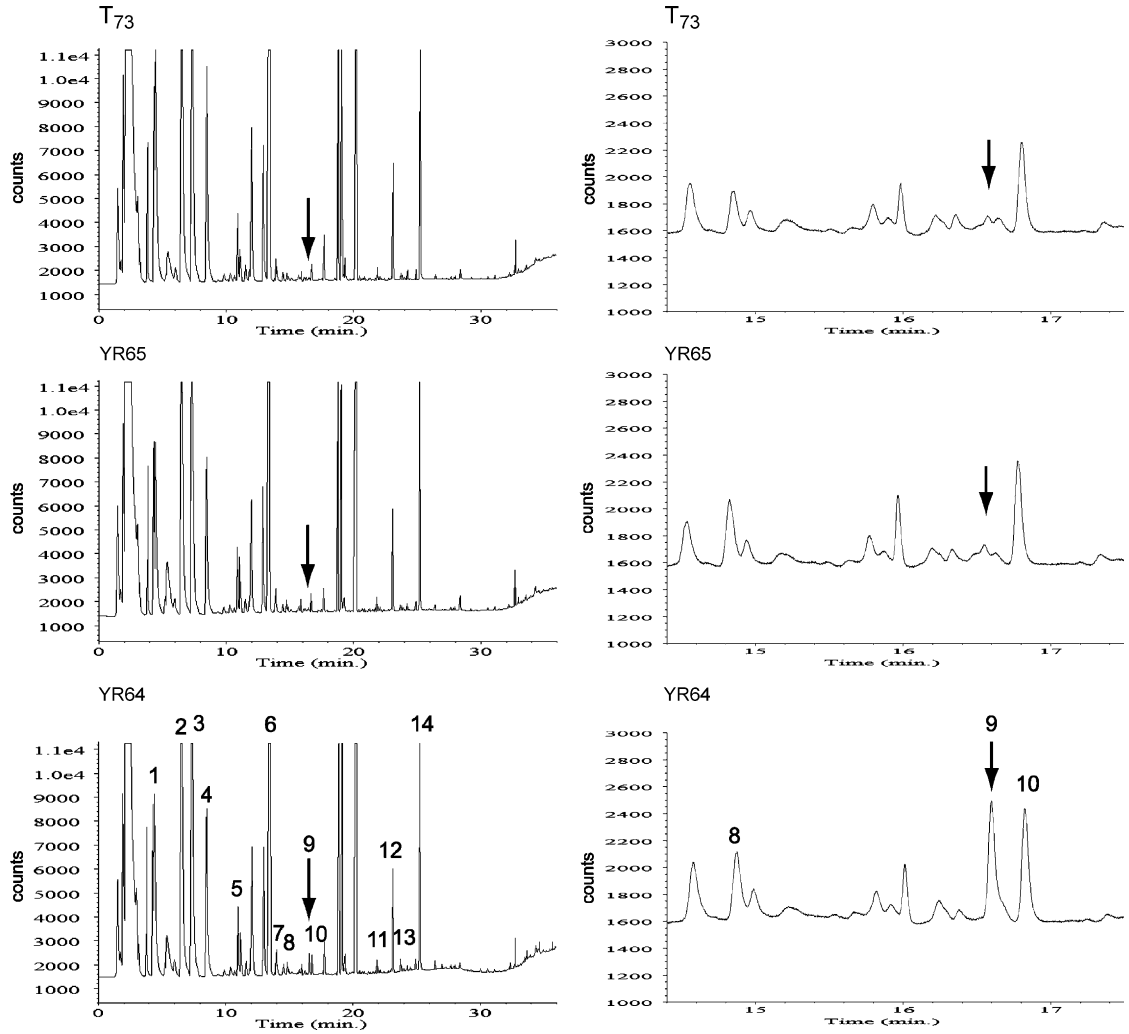

Fig. 4. Partial metabolomic study of wine volatiles. Chromatograms of wines fermented with T_{73}, YR65 and YR64 strains. Peak numbers refer to the aromatic compounds listed in Table 1. Arrows indicate retention time and peaks of linalool.

Table 1
Concentrations ($\mu g\,l^{-1}$) of a selected subset of aromatic compounds in Parellada wines fermented with T_{73}, YR65 and YR64 strains

Compounds	Yeast strain		
	YR64	YR65	T_{73}
Terpenes			
Cis-linalool oxide	nd	nd	nd
Trans-linalool oxide	nd	nd	nd
Linalool (9)	18.60±1.33[a]	1.55±0.22	1.57±0.48
α-Terpineol	nd	nd	nd
β-Citronellol (11)	1.69±0.15	1.74±0.03	1.59±0.22
Nerol	nd	nd	nd
Geraniol (13)	5.85±0.82	6.24±1.77	5.18±0.28
Alcohols			
3-Methyl-1-butanol (2)	86 963±5514	84 533±1096	88 036±4004
1-Hexanol (5)	713.58±60.94	705.11±41.34	642.08±19.82
1-Heptanol (7)	56.16±3.16	57.33±2.96	51.15±5.38
2-Ethyl-1-hexanol (8)	10.97±4.96	10.45±4.14	11.31±4.41
1-Octanol (10)	13.77±0.46	13.45±2.47	11.10±0.33
2-Phenylethyl alcohol (14)	17 149±3248	14 871±1644	16 727±2107
Esters			
Isoamyl acetate (1)	727.67±115.74	675.26±101.29	754.17±70.38
Ethyl caproate (3)	354.50±29.35	388.81±64.67	326.42±25.05
Hexyl acetate (4)	132.57±23.13	127.07±25.01	134.88±17.72
Ethyl caprylate (6)	315.73±45.95	279.51±43.97	340.84±40.11
2-Phenylethyl acetate (12)	230.52±3.66	245.64±25.42	233.73±14.10

[a]Indicates a statistically significant difference of the mean comparison by Tukey's HSD procedure ($P<0.05$) between the wines produced by the YR64 strain and controls. nd: not detected. Numbers between brackets refer to the GC peaks in Fig. 4. Values represent the means and standard deviations of three independent microvinifications.

(i.e. alcohols and esters). We can therefore conclude that the transformant strain with the *C. breweri* linalool synthase encoding gene was able to produce wine in the same way as the industrial T_{73} strain except for the quality trait introduced. To our knowledge this is the first report concerning the design of an *S. cerevisiae* industrial wine strain expressing a plant monoterpene synthase, which has the potential to alter the monoterpene profile of wine. The information gained in the past about the nature of the components determining wine aroma provides a good basis for changing aroma in wines. New opportunities for engineering wine aroma are arising as a result of the continuing identification of novel monoterpene synthase encoding genes, as well as other genes whose products are involved in monoterpene modification. The expression of other monoterpene synthases such as geraniol synthase and/or α-terpineol synthase in wine yeast strains could be of great interest. An *à la carte* monoterpene wine yeast strain with such characteristics would be useful for the production of fruity floral wines from non-aromatic raw materials.

The commercial application of genetically modified industrial microorganisms is problematic due to public concern. Very recently, the wine yeast ML01 received GRAS (Generally Recognized as Safe) status from the FDA (http://www.cfsan.fda.gov/~rdb/opa-g120.html) and is the first metabolically engineered transgenic yeast to be commercialized by the wine industry (Husnik et al., 2006). This opens up great possibilities for the genetic manipulation of wine yeast and affords new perspectives to this work. Since monoterpenes are also important aroma components in other alcoholic drinks, the strategy developed here could also be applied to other *Saccharomyces* strains used to carry out alcoholic fermentations. Moreover, application could be made to microorganisms other than *S. cerevisiae*, i.e. non-*Saccharomyces* yeasts and also bacteria, which also contribute to the process.

In summary, we show that by engineering the pathway for monoterpene production in wine yeasts it is possible to achieve the production of free linalool at a concentration above its aroma threshold. Furthermore, fermentative capacity, such as fast growth rate in sugar-rich media, along with high alcohol production and tolerance were apparently unaffected in the engineered yeast.

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