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ORIGINAL ARTICLE

Isoprene hydrocarbons production upon heterologous transformation of *Saccharomyces cerevisiae*

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Keywords
biofuels, isoprene synthase, kudzu vine, methyl-butenol, renewable energy, yeast.

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Abstract

Aims: Isoprene (2-methyl-1,3-butadiene; C_5H_8) is naturally produced by photosynthesis and emitted in the atmosphere by the leaves of many herbaceous, deciduous and woody plants. Fermentative yeast and fungi (Ascomycota) are not genetically endowed with the isoprene production process. The work investigated whether Ascomycota can be genetically modified and endowed with the property of constitutive isoprene production.
Methods and Results: Two different strategies for expression of the *IspS* gene in *Saccharomyces cerevisiae* were employed: (i) optimization of codon usage of the *IspS* gene for specific expression in *S. cerevisiae* and (ii) multiple independent integrations of the *IspS* gene in the rDNA loci of the yeast genome. Copy number analysis showed that *IspS* transgenes were on the average incorporated within about 25% of the endogenous rDNA. Codon use optimization of the *Pueraria montana* (kudzu vine) *IspS* gene (*SckIspS*) for *S. cerevisiae* showed fivefold greater expression of the IspS protein compared with that of nonoptimized *IspS* (*kIspS*). With the strategies mentioned earlier, heterologous expression of the kudzu isoprene synthase gene (*kIspS*) in *S. cerevisiae* was tested for stability and as a potential platform of fermentative isoprene production. The multicopy *IspS* transgenes were stably integrated and expressed for over 100 generations of yeast cell growth and constitutively produced volatile isoprene hydrocarbons. Secondary chemical modification of isoprene to a number of hydroxylated isoprene derivatives in the sealed reactor was also observed.
Conclusion: Transformation of *S. cerevisiae* with the *Pueraria montana var. lobata* (kudzu vine) isoprene synthase gene (*IspS*) conferred to the yeast cells constitutive isoprene hydrocarbons production in the absence of adverse or toxic effects.
Significance and Impact of the Study: First-time demonstration of constitutive isoprene hydrocarbons production in a fermentative eukaryote operated through the mevalonic acid pathway. The work provides concept validation for the utilization of *S. cerevisiae*, as a platform for the production of volatile hydrocarbon biofuels and chemicals.

Introduction

Efforts are currently being made to develop technologies for the renewable generation of fuels and chemicals to meet increasing demands for energy and synthetic chemistry feedstock, without contributing to global warming, environmental degradation or disruption of food supply (Stephanopoulos 2007; Rittmann 2008). Such technologies need to be sustainable, secure, renewable and environmentally friendly. Possible renewable energy carriers include biologically generated hydrocarbon molecules and their derivatives. Hydrocarbons are desirable products because they have a high energy density, both on a mass as well as a volume basis (Fischer *et al.* 2008; Peralta-

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Yahya and Keasling 2010). Isoprene (2-methyl-1,3-butadiene; C_5H_8) is a volatile hydrophobic terpenoid hydrocarbon molecule that meets these requirements, plus it affords a number of practical advantages in terms of generation and separation from the biomass (Lindberg *et al.* 2010; Bentley and Melis 2012). It also serves as feedstock in the synthetic chemistry industry, for example, production of rubber. Isoprene is currently derived from the petroleum refining process, as a by-product of ethylene production (Sharkey and Singsaas 1995).

Isoprene is naturally synthesized in the chloroplast of many herbaceous, deciduous and woody plants species and released in the atmosphere from the leaves in a process induced by heat-stress (Sharkey and Yeh 2001; Sharkey *et al.* 2008). The biological significance of isoprene release by plants is unclear; however, it has been suggested that plants utilize isoprene emission as a thermotolerance mechanism (Singsaas *et al.* 1997; Sharkey *et al.* 2001; Behnke *et al.* 2007; Sasaki *et al.* 2007). Plant isoprene production is not suitable for commercial exploitation, owing to the difficulty of harvesting this volatile product from the extended canopy of leafy plants.

It was recently demonstrated that isoprene could be synthesized in the photosynthetic apparatus of transgenic cyanobacteria (Lindberg *et al.* 2010) and collected in suitably designed photobioreactors (Bentley and Melis 2012). Isoprene emission was also reported from bacteria, such as *Escherichia coli* (Miller *et al.* 2001; Zhao *et al.* 2011) and *Bacillus subtilis* (Julsing *et al.* 2007). However, it is not known whether heterologous transformation of eukaryotic yeast and fungi could also result in stable and constitutive isoprene hydrocarbons production. This is an important question, as many Ascomycota are commercially valuable fermentative microorganisms. However, they are substantially different from photosynthetic plastid and bacterial systems because (i) they are fermentative eukaryotes lacking a plastid structure and the attendant photosynthetic apparatus and (ii) they lack the deoxyxylulose phosphate/methylerythritol phosphate (DXP-MEP) isoprenoid pathway that exclusively operates in isoprene-producing photosynthetic systems.

Plant isoprene synthase genes have been cloned from poplar (*Populus alba* x *Populus tremula*, *Populus alba* and *Populus nigra*) (Miller *et al.* 2001; Sasaki *et al.* 2005; Fortunati *et al.* 2008) and kudzu vine (*Pueraria montana*) (Sharkey *et al.* 2005). The IspS enzyme is localized in the chloroplast of these plants and catalyses the conversion of dimethyl allyl diphosphate (DMAPP) to isoprene (Silver and Fall 1991; Sasaki *et al.* 2005; Shiba *et al.* 2007). DMAPP in the chloroplast of plants is generated via the deoxyxylulose phosphate/methylerythritol phosphate (DXP-MEP) 'bacterial' isoprenoid biosynthetic pathway (Lichtenthaler 1999, 2000), which is responsible for the flux of metabolites, leading to the synthesis of isoprene in isoprene-producing plants. Primary reactants of the DXP-MEP pathway are glyceraldehyde 3-phosphate and pyruvate (Lindberg *et al.* 2010).

Saccharomyces cerevisiae (budding yeast) is a model organism that employs the mevalonic acid (MVA) pathway to generate isoprenoids essential for cell growth, such as geranyl, farnesyl and geranylgeranyl derivatives (Jackson *et al.* 2003; Oswald *et al.* 2007; Shiba *et al.* 2007; Takahashi *et al.* 2007). In the MVA pathway, three molecules of acetyl-coenzyme A (acetyl-CoA) condense successively to form the 6-carbon intermediate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The HMG-CoA is then reduced to MVA, which is subsequently

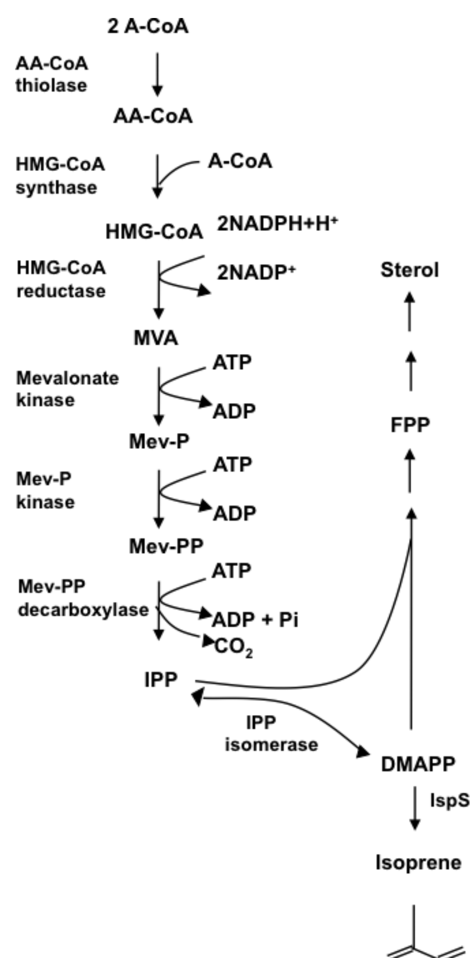

Figure 1 Mevalonic acid pathway for isoprene biosynthesis. Abbreviations used: A-CoA = acetyl-Coenzyme A; AA-CoA = acetoacetyl CoA; HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA; MVA=mevalonic acid; Mev-P = mevalonic acid 5-phosphate; Mev-PP = mevalonic acid pyrophosphate; IPP = isopentenyl pyrophosphate; DMAPP = dimethylallyl pyrophosphate; IspS = isoprene synthase; FPP = farnesyl pyrophosphate. It is noted that yeast cells are not naturally endowed with the *IspS* gene.

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phosphorylated twice and decarboxylated to form isopentenyl diphosphate (IPP). IPP can be isomerized to DMAPP (Kuzuyama 2002) through the action of the IPP isomerase (Fig. 1). Thus, the MVA pathway of yeast cells is substantially different from the DXP-MEP pathway of plant plastids and bacteria. Moreover, the yeast genome does not encode for an isoprene synthase (IspS), and therefore, it is not known whether yeast cells are capable of converting the metabolically critical DMAPP into isoprene upon introduction of the *IspS* transgene in the cells. Unknown also is the ability of yeast cells to tolerate overexpression of the IspS transgenic protein or whether they might be subject to toxicity effects (Withers *et al.* 2007) from the IspS product.

In this work, *S. cerevisiae* were genetically engineered for isoprene production upon heterologous expression of the codon-optimized kudzu vine *IspS* gene. Stability of transgenes and their expression, constitutive production of isoprene and absence of adverse or toxic effects are reported. Thus, it is possible to drive the MVA pathway in the cytoplasm of a eukaryote and to constitutively produce volatile isoprene hydrocarbons from the fermentation metabolism of the host organism. The work provides a new dimension for the potential utilization of yeast (Sudbery 1996; Huang *et al.* 2008), as a platform for the production of volatile hydrocarbon biofuels and other useful chemicals.

Materials and Methods

Strains and culture conditions

Escherichia coli DH5α [F′/*endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{u169}$: (m80Δ*lacZM15*)] (Invitrogen, Carlsbad, CA, USA) was used to amplify plasmid DNA using standard procedures (Ausubel *et al.* 2008). *Saccharomyces cerevisiae* 20B-12, a peptidase mutant (α trp1 pep4-3; ATCC 20626, Manassas, VA) was used as the recipient strain of plasmid pSckIspStg containing *S. cerevisiae* codon-optimized kudzu vine (*Pueraria montana var. lobata*) *IspS* gene and plasmid pkIspStg containing the original sequence of the kudzu vine *IspS* gene, respectively. *Escherichia coli* was cultured on LB medium (Luria-Bertani; 1% tryptone, 0·5% yeast extract, 1% sodium chloride) or LB containing appropriate antibiotics for competent cell preparation and plasmid isolation (Maniatis *et al.* 1989). *Saccharomyces cerevisiae* strain 20B-12 (recipient strain) was grown on YPD medium (1% yeast extract, 2% peptone, 2% glucose). We used YNBD medium (0·67% yeast nitrogen base without amino acids, 2% glucose) as a selective medium for the isolation of yeast transformants (Lopes *et al.* 1989). For measurements of isoprene production, yeast transformants were cultured in YPG medium (2% galactose instead of 2% glucose in YPD). Galactose was chosen as the carbohydrate feedstock in these measurements because the culture supports lower amounts of ethanol relative to glucose, without negatively affecting the growth rate of the yeast cells (unpublished observations).

Construction of plasmids containing the *S. cerevisiae* codon-optimized *IspS* or the nonoptimized kudzu vine *IspS*

Plasmid pNGVF served as the plasmid backbone. The pNGVF was constructed using pAPNGFPVFNB and pGFPV (Hong and Linz 2008). The pAPNGFPVFNB was digested with *Not*I and *Asc*I, and the 3·7-kb fragment from the pAPNGFPVFNB replaced 2·8-kb fragment in pGFPV digested with the same enzymes, resulting in pNGVF. A 0·65-kb *GPD* promoter was generated by PCR with *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), appropriate primers and p424 GPD (ATCC **87357**, Manassas, VA, USA) as a template using standard procedures (Ausubel *et al.* 2008) (see Table S1 for primer sequences). PCR was performed in a PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The reaction conditions were as follows: 94°C for 5 min followed by 35 cycles of 94°C for 1 min, annealing for 1 min (see Table S1 for annealing temperatures) and extension at 72°C (2 min/1 kb). The reaction was completed with a final extension at 72°C for 10 min. The PCR fragment was digested with *Not*I and *Sgf*I and cloned into pNGVF cut with the same enzymes, resulting in pGVF. A 0·8-kb *TRP1d* gene fragment was generated by PCR with *Pfu* DNA polymerase, appropriate primers and p424 GPD plasmid as a template. Similar PCR conditions were used as for pGVF (Table S1). The PCR fragment was digested with *Pac*I and *Not*I and cloned into pGVF cut with the same enzymes, resulting in pTGVF. To subclone a *CYC1* terminator fragment, the 0·26-kb fragment was generated by PCR with *Pfu* DNA polymerase, appropriate primers and p424 GPD as a template. Similar PCR conditions were used as for pGVF (Table S1). The PCR fragment was cloned into the *Sma*I site of pUC18, resulting in pUCCYC1t. To subclone rDNA fragments, the 0·67-kb and 0·59-kb fragments were generated by PCR with *Pfu* DNA polymerase, appropriate primers and genomic DNA from *S. cerevisiae* recipient strain as a template using standard procedures (Ausubel *et al.* 2008) (for primers, see Table S1). Genomic DNA isolation from *S. cerevisiae* was performed according to the method of Philippsen and co-workers with minor modifications (Philippsen *et al.* 1991). Similar PCR conditions were employed as for the construction of pGVF (Table S1). The 0·67-kb ribosomal DNA (rDNA) fragment was subcloned into

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pUCCYC1t cut with *Asi*SI and *Asc*I, resulting in pUCCD. DNA fragments containing the CYC1 terminator and the rDNA were then subcloned from pUCCD into pTGVF cut with *Fse*I and *Asc*I, resulting in pTGVCD. The 0·59-kb rDNA fragment was subcloned into pTGVCD cut with *Sbf*I and *Pac*I, resulting in pDTGVCD.

A 1·7-kb *S. cerevisiae* codon-optimized kudzu vine *IspS* gene (cDNA; *SckIspS*) was designed on the basis of the codon usage table for *S. cerevisiae* (Nakamura *et al.* 2000) using the GeneDesigner software, synthesized by DNA 2·0, Inc. (Menlo Park, CA, USA), and subcloned to generate plasmid pSckIspS. DNA fragments containing the *CYC1* terminator and the 0·67-kb rDNA were then subcloned from pUCCD into pSckIspS cut with *Fse*I and *Asc*I, resulting in pSckIspSCD. Finally, DNA fragments containing the *SckIspS* and the *CYC1* terminator were subcloned from pSckIspSCD into pDTGVCD cut with *AsiS*I(=*Sgf*I), resulting in plasmid pSckIspStg (Fig. 2). To construct the plasmid containing the 1·7-kb original sequence of the kudzu vine *IspS* gene (cDNA; *kIspS*), the fragment was generated by PCR with *Pfu* DNA polymerase, appropriate primers and pBA2kIA2 (Lindberg *et al.* 2010) as a template. Similar PCR conditions were used as for pGVF (Table S1). The 1·7-kb kudzu vine *IspS* fragment was subcloned into the *Sma*I site of pUC18, resulting in pUCkIspS. DNA fragments containing the *CYC1* terminator and the 0·67-kb rDNA were then subcloned from pUCCD into pUCkIspS cut with *Fse*I and *Asc*I, resulting in pUCkIspSCD. Finally, DNA fragments containing the *kIspS* and the *CYC1* terminator were subcloned from pUCkIspSCD into pDTGVCD cut with *AsiS*I(=*Sgf*I), resulting in plasmid pkIspStg.

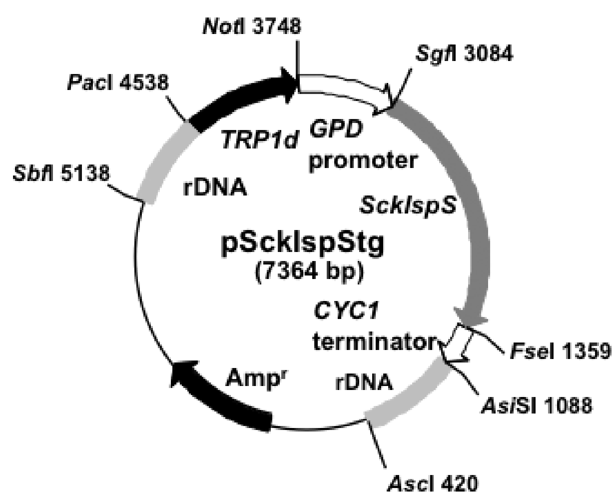

Figure 2 Restriction enzyme map of plasmid pSckIspStg. The 0·65-kb *GPD* promoter was fused to the 1·7-kb *Saccharomyces cerevisiae* codon-optimized *SckIspS*, followed by the 0·26-kb *CYC1* terminator. The 0·8-kb *TRP1d* DNA fragment (for tryptophan prototrophy) was inserted as a selectable marker for the transformation of *S. cerevisiae*. The 0·59-kb and 0·67-kb rDNA fragments were inserted for integration of the *IspS* construct into the yeast chromosome by double homologous recombination. Plasmid pkIspStg, containing the kudzu vine nonoptimized *kIspS* gene, was constructed upon a replacement of the *SckIspS* DNA fragment in the pSckIspStg with the *kIspS* fragment. Abbreviations used: rDNA = ribosomal DNA; TRP1d = N-(5′-phosphoribosyl) anthranilate isomerase (one of the enzymes in the tryptophan biosynthetic pathway); GPD = glyceraldehyde-3-phosphate dehydrogenase; SckIspS = *S. cerevisiae* codon-optimized kudzu vine isoprene synthase; CYC1 = iso-1-cytochrome c; Amp^r = ampicillin resistance gene.

Saccharomyces cerevisiae transformations with plasmid DNA

For yeast transformation, plasmids were digested with restriction enzymes *Sbf*I and *Asc*I and the DNA fragments containing the selectable marker (*TRP1d*), *IspS* gene and rDNA regions were agarose gel-purified. The DNA fragments were targeted for integration into the NTS2 (non-transcribed spacer 2) and 5′ ETS (external transcribed spacer) region in the rDNA. The rDNA of *S. cerevisiae* consists of 100–200 tandemly repeated copies of a 9·1-kb unit on the right arm of chromosome XII (Venema and Tollervey 1999). Each repeated unit contains the genes for 5S, 18S, 5·8S and 25S rRNAs, as well as three types of spacer regions: internal transcribed spacers (ITS1, ITS2), external transcribed spacers (5′ ETS, 3′ ETS) and non-transcribed spacers (NTS1, NTS2) (Fig. S1). Each ribosomal RNA is present in a single copy in an 80S yeast ribosome. 18S rRNA is a component of the 40S ribosomal subunit, and the 5S, 5·8S and 25S rRNAs are components of the 60S subunit (Spahn *et al.* 2001). Yeast transformations were performed by the lithium acetate method with minor modifications (Gietz *et al.* 1995).

Yeast colony PCR

Yeast cells were transferred in 0·2% sodium dodecyl sulphate (SDS) solution and were ruptured by heating at 90°C for 4 min (Akada *et al.* 2000). After centrifugation, 1 μl of supernatant was used for PCR reaction. PCR was performed with primers (see Table S2 for primer sequences) specific to each coding region. The reaction conditions were as follows: 95°C for 2 min followed by 35 cycles of 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 3 min. The reaction was completed with a final extension at 72°C for 10 min.

Southern hybridization and genomic PCR analyses

Genomic DNA was isolated by the method of Philippsen and co-workers with minor modifications (Philippsen

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et al. 1991). Southern hybridization analyses were conducted using standard procedures (Maniatis *et al.* 1989). Approximately 1 μg of genomic DNA cut with *Bgl*II or *Pvu*II were separated by agarose gel electrophoresis and were transferred onto a nylon membrane (Amersham Hybond- N+, GE Healthcare, Little Chalfont, UK) by capillary action. The DNA probe was generated with the Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare) and the 0·57-kb rDNA PCR fragment (see Table S2 for primer sequences) specific to NTS2 (nontranscribed spacer 2) in a 9·1-kb rDNA unit using a procedure provided by the manufacturer. Detection of the DNA fragments was performed using the Amersham Detection System (GE Healthcare). PCR analyses were performed with genomic DNA and primers specific to a set of the NTS2 and *TRP1d* or another set of the *CYC1* terminator and 18S rDNA (for primer sequences, see Table S2) to confirm the integration site of the DNA fragment.

Copy number analysis

Copy number of the integrated DNA fragment containing the selectable marker (*TRP1d*), *IspS* gene and rDNA regions was calculated by comparison of the intensity of the new band with that of the endogenous ribosomal band from each transformant on X-ray film generated by Southern hybridization analyses. The intensity of the bands was measured by Lab Works Image Acquisition and Analysis Software (UVP Bioimaging System, UVP Inc., Upland, CA, USA).

Western blot analysis of IspS proteins

Yeast cells were cultured in 50 ml of YNBD (+Trp) medium at 30°C for 24 h with shaking at 200 rev min^{-1} by 5% inoculation of overnight seed culture in the same medium. Protein from yeast cells was extracted using a standard glass beads procedure (Ausubel *et al.* 2008). Protein concentration in extracts was determined using a DC Protein Assay (Bio-Rad Laboratories). Approximately 25 μg of protein extracts were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunodetection was carried out with a specific polyclonal antibody against the IspS protein (Lindberg *et al.* 2010) as a primary antibody, a goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories) as a secondary antibody, and a Supersignal ECL detection kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) using a procedure provided by the manufacturer. A PageRuler Prestained Protein Ladder (Fermentas Inc., Burlington, Ontario, Canada) was used as a molecular mass marker. The intensity of the bands was measured by Lab Works Image Acquisition and Anaylsis Software (UVP Bioimaging System, UVP Inc., Upland, CA, USA).

RT-PCR

Yeast cells were harvested at OD_{600} = 1·0. Total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was treated with DNaseI (Invitrogen). RT-PCR was performed with 0·5 μg of total RNA, primers specific to the coding region of the *SckIspS* and *kIspS*, respectively (see Table S2 for primer sequences) using the Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA, USA). The reaction conditions were as follows: 50°C for 30 min and 95°C for 15 min followed by 25 cycles of 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 3 min. The reaction was completed with a final extension at 72°C for 10 min.

Measurement of isoprene production

For measurements of isoprene production, yeast cells were cultured in 10 or 50 ml of YPG medium at 30°C with shaking at 200 rev min^{-1}. After 48 or 72 h incubation under aerobic conditions, the flasks were sealed with rubber stoppers. One ml of gas from the headspace of sealed cultures was sampled and analysed by gas chromatography using a Shimadzu 8A GC (Shimadzu, Columbia, MD, USA) equipped with a Porapak N 80/100 column and a flame-ionization detector (FID). This column is designed to optimally separate short-chain hydrocarbons, namely C1 to C5 molecules. Amounts of isoprene produced by yeast transformants were estimated by comparison with an isoprene standard (Acros Organics, Fair Lawn, NJ, USA).

For GC-MS analysis, 200 μl of gas from the headspace of sealed cultures was injected into the GC inlet port of Agilent Technologies 6890 GC/5973 MSD equipped with a DB-XLB column (0·25 mm i.d. × 0·25 μm × 30 m, J &W Scientific). The DB-XLB column is suited for the separation of a wide range of molecules including pesticides, herbicides, PCBs and PAHs. Programming of the GC oven entailed initial setting at 40°C (hold for 4 min), 5°C min^{-1} ramp to 80°C, 12 min of total run time and 260°C of inlet temperature. Size selection conditions were chosen to detect 48–100 Da products and to eliminate smaller or larger molecules from the analysis. This GC-MS size selection range effectively eliminated ethanol signals from the GC-MS output.

Mitotic stability determination

Stability of the multi-copied integrative DNA fragment was determined by the method of Nieto and co-workers (Nieto *et al.* 1999). Transformants were cultured in 5 ml of YPD

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medium at 30°C with shaking at 200 rev min^{-1} starting at OD_{600} = 0·001 by inoculation of an overnight seed culture and were diluted into the same medium to start at the same OD_{600} value each day during the yeast culture until 100 generations. The cells were plated onto YPD agar medium after growth for 100 generations and replica-plated onto YNBD and YNBD+Trp agar media. The copy number of the fragment was analysed as described earlier. The IspS activity was tested by gas chromatography (GC) after transformants were cultured in YPG medium.

Statistical analysis

Statistical analyses were performed by student's *t*-test using Excel statistical software (Microsoft Corp., Redmond, WA, USA). Statistical significance between samples was defined by a *P* value not >0·05 ($P \leq 0{\cdot}05$).

Results

Transformation of *Saccharomyces cerevisiae* recipient strain with the IspS plasmid

For efficient expression of the isoprene synthase gene (*IspS*) in *S. cerevisiae*, a codon-optimized *IspS* cDNA (*SckIspS*) was designed on the basis of the codon usage table for *S. cerevisiae* (Nakamura *et al.* 2000). The *SckIspS* gene was synthesized without the putative transit peptide, consisting of amino acid 45–608 of the kudzu IspS sequence. The *SckIspS* gene was cloned in plasmid pSckIspStg (Fig. 2), in which *TRP1d* and rDNA fragments were used, respectively, for transformant selection and multi-copy integration into the rDNA region via double homologous recombination. To test the effect of the codon-optimization on the efficiency of the *IspS* gene expression in *S. cerevisiae*, a control plasmid was also constructed (pkIspStg, not shown) in which the codon-optimized *SckIspS* gene was replaced with the native kudzu vine *kIspS* cDNA. Transformation of the *S. cerevisiae* recipient strain with plasmids pSckIspStg or pkIspStg generated 20 and 29 colonies, respectively. Yeast colony PCR analysis was performed to screen these transformants for the isolation of *IspS* multi-copy integrants. Four transformant lines (ScT2, ScT11, ScT16 and ScT17) from the pSckIspStg and three transformant lines (KT8, KT16 and KT24) from the pkIspStg showed a high copy number of the integrated plasmid (not shown). These transformants were chosen for further analyses.

Analyses of integration sites of the IspS plasmid in the yeast chromosome

To identify the integration sites of the *IspS* transgenes in the yeast chromosome, Southern hybridization and genomic PCR analyses were performed with the recipient strain and the seven isolated transformants. Southern blot analyses were designed with primers and a probe such that hybridization bands from the transformants would be larger in size compared with that from the endogenous region of the recipient strain, the difference being due to integration of the plasmid *SckIspS* or *kIspS* into the rDNA region (Fig. 3a). This strategy is schematically depicted in the genomic DNA map of the recipient strain (Fig. 3a, upper) and the IspS transformant strains (Fig. 3a, lower). Integration of the *IspS* (*SckIspS* or *kIspS*) expression construct into the yeast rDNA locus is shown in Fig. 3a to occur between the NTS2 and 5′ETS DNA regions. Restriction enzyme sites for *Bgl*II (=B) and *Pvu*II (=P) are indicated. The expected band sizes are also indicated above the yeast chromosome after integration of the *SckIspS* expression fragment and below the chromosome map after integration of the *kIspS* expression fragment (Fig. 3a, lower). Primer positions for genomic PCR analysis are shown as $P_{1,}$ P_2, P_3 and P_4.

Southern blot analysis of the *S. cerevisiae* recipient strain (Fig. 3b, lanes 1 and 6), using the NTS2 (nontranscribed spacer 2) as a probe, showed a single hybridization band at 4·58 kb (lane 1) and 2·06 kb (lane 6). Transformants carrying the *SckIspS* construct generated a new hybridization band at 7·06 kb (Fig. 3b, lanes 2–5) and 4·1 kb (Fig. 3b, lanes 7–10). These results are evidence that plasmid *SckIspS* integrated within the rDNA repeat units in each of the four *SckIspS* transformants.

Similarly, Southern blot analysis of the *S. cerevisiae* recipient strain (Fig. 3c, lanes 1 and 5) showed a single hybridization band at 4·58 kb (lane 1) and 2·06 kb (lane 5). Transformants carrying the *kIspS* construct generated a new hybridization band at 8·04 kb (Fig. 3c, lanes 2–4) and 5·52 kb (Fig. 3c, lanes 6–8). These results also showed that plasmid *kIspS* integrated within the rDNA repeat units in each of the three *kIspS* transformants.

Genomic PCR analysis was independently employed to confirm integration sites of the *IspS* construct in the rDNA locus in the transformants (Fig. 3d). Two sets of primers (P1-P2 or P3-P4, Fig. 3a) specific for the *IspS* construct and rDNA region were employed in this PCR analysis. Recipient strains (Fig. 3d, lanes 1 and 2) showed no PCR product with either of the two sets of primers. All four *SckIspS* and three *kIspS* transformants showed PCR products with both the P1-P2 and P3-P4 sets of primers. These results further strengthened the notion that plasmids *SckIspS* and *kIspS* integrated within the rDNA repeat units in each of the two different types of *IspS* transformants.

Copy number of the integrated *IspS* DNA construct was calculated from the Southern blot analysis results as relative band intensity, by way of comparison of the intensity of the new (transgene) band with that of the

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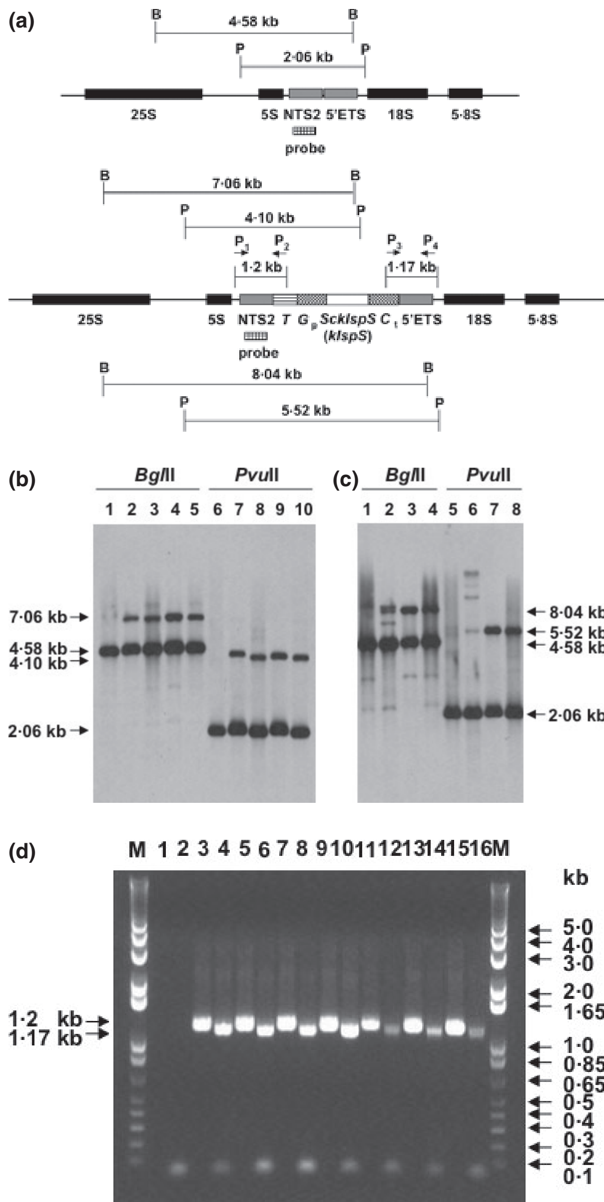

Figure 3 Southern hybridization and genomic PCR analyses of *IspS* integration sites in *Saccharomyces cerevisiae* transformants. (a) Schematic for Southern hybridization and genomic PCR analyses of the integration of the *IspS* (*SckIspS* or *kIspS*) expression fragment into the yeast rDNA locus. Restriction enzyme sites for *Bgl*II (=B) and *Pvu*II (=P) are indicated. The expected band sizes, after integration of the *SckIspS* expression fragment, are indicated in kb above the transformed yeast chromosome, while those resulting upon integration of the *kIspS* expression fragment are shown below the transformed yeast chromosome. Primer positions for genomic PCR analysis are marked as P_1, P_2, P_3 and P_4. (b) Southern hybridization analysis of *SckIspS* integration sites in *S. cerevisiae* recipient strain and transformants carrying the *SckIspS* transgene. Genomic DNA was isolated from *S. cerevisiae*, digested with either *Bgl*II or *Pvu*II and hybridized with an rDNA fragment (NTS2 region) as a probe. Lanes 1–5: *Bgl*II digestion. Lanes 6–10: *Pvu*II digestion. Lane 1 and 6: recipient strain; lane 2 and 7: ScT2; lane 3 and 8: ScT11; lane 4 and 9: ScT16; lane 5 and 10: ScT17. (c) Southern hybridization analysis of *kIspS* integration sites in *S. cerevisiae* recipient strain and transformants carrying the *kIspS* transgene. Lanes 1–4: *Bgl*II digestion. Lanes 5–8: *Pvu*II digestion. Lane 1 and 5: recipient strain; lane 2 and 6: KT8; lane 3 and 7: KT16; lane 4 and 8: KT24. (d) Genomic PCR analysis of *IspS* (*SckIspS* or *kIspS*) integration sites in *S. cerevisiae* recipient strain and transformants. Lanes 1, 3, 5, 7, 9, 11, 13 and 15: PCR reaction in which a primer pair, P_1 and P_2 was used. Lanes 2, 4, 6, 8, 10, 12, 14 and 16: PCR reaction in which a primer pair, P_3 and P_4 was used. Lane 1 and 2: recipient strain; lane 3 and 4: ScT2; lane 5 and 6: ScT11; lane 7 and 8: ScT16; lane 9 and 10: ScT17; lane 11 and 12: KT8; lane 13 and lane 14: KT16; lane 15 and 16: KT24; M: 1-kb plus ladder as a molecular size marker.

endogenous rDNA band on the blot (Fig. 3b and c). Results showed that the intensity of the new (transgene) band was on the average about 25% of that from the endogenous rDNA band in the various lanes of the blot. Given that as many as 100–200 copies of the rDNA unit are present on the yeast chromosome per cell, it was estimated that 25–50 copies of the *IspS* expression construct were integrated into the rDNA repeats in each of the seven transformants.

Expression of the IspS transgenes

Transcription of the *IspS* transgenes was tested upon RT-PCR analysis. This was conducted on the seven transformant lines to test whether the *SckIspS* or *kIspS* genes are transcribed under physiological growth conditions. The RT-PCR reactions were performed with primers specific to the coding regions of the *SckIspS* and *kIspS* constructs, respectively. The result in Fig. 4a showed that the *SckIspS* gene was transcribed, as the respective mRNA accumulated in all four of the *SckIspS* transformants (Fig. 4a, lanes 5, 7, 9 and 11). Similarly, the result in Fig. 4b showed that the *kIspS* gene was transcribed, as the respective mRNA accumulated in all three of the *kIspS* transformants (Fig. 4b, lanes 5, 7 and 9). These results confirm the presence of *IspS* mRNA in all seven *S. cerevisiae* transformants harbouring the *IspS* transgenes. In contrast, no transcript was detected in the negative controls, where the reverse transcriptase was not added (Fig. 4a, lanes 4, 6, 8 and 10 and Fig. 4b, lanes 4, 6 and 8).

Translation of the *IspS* transgenes from the corresponding mRNAs was tested in Western blot analyses with specific polyclonal antibodies raised against the kIspS protein (Lindberg *et al.* 2010). This analysis confirmed the presence of the IspS protein as a 65-kD protein in the transformants (Fig. 5a, lanes 2–8) but not in the recipient strain (Fig. 5a, lane 1). A Coomassie-stained SDS-PAGE of the same samples (Fig. 5b) served as an internal

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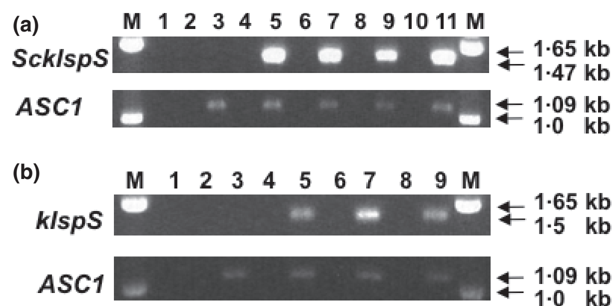

Figure 4 RT-PCR analysis of *Saccharomyces cerevisiae* recipient strain and transformants carrying the *SckIspS* or *kIspS* transgenes. Total RNA was isolated from yeast cells grown in YNBD (+Trp) medium at 30°C with shaking at 200 rev min^{-1} until OD_{600} = 1·0. (a) Transcription in *S. cerevisiae* recipient strain and transformants carrying the *SckIspS* transgene. Lanes 2, 4, 6, 8 and 10: RT-PCR without addition of reverse transcriptase. Lane 1: RT-PCR with ddH_2O instead of an RNA sample as a negative control. Lane 2 and 3: recipient strain; lane 4 and 5: ScT2; lane 6 and 7: ScT11; lane 8 and 9: ScT16; lane 10 and 11: ScT17; M: 1-kb plus ladder as a molecular size marker. *ACT1*, a constitutively expressed gene, is shown as a positive control in the lower panel. (b) Transcription in *S. cerevisiae* recipient strain and transformants carrying *kIspS* transgene. Lanes 2, 4, 6 and 8: RT-PCR without addition of reverse transcriptase. Lane 1: RT-PCR with ddH_2O instead of an RNA sample as a negative control. Lane 2 and 3: recipient strain; lane 4 and 5: KT8; lane 6 and 7: KT16; lane 8 and 9: KT24; M: 1-kb plus ladder as a molecular size marker. *ACT1*, a constitutively expressed gene, is shown as a positive control in the lower panels, respectively.

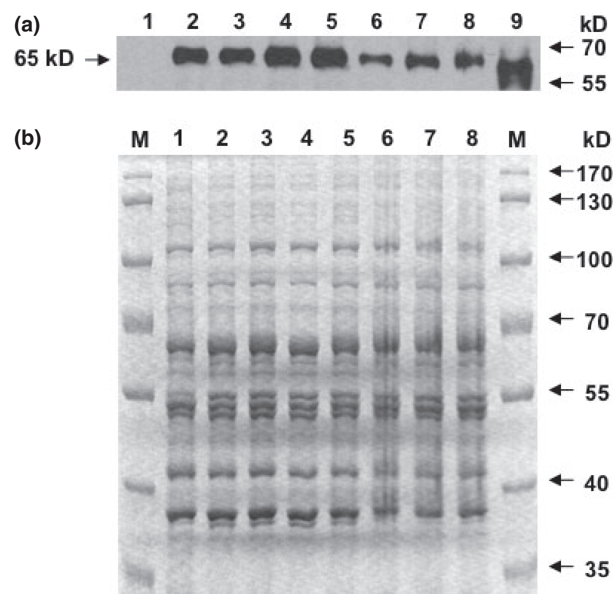

Figure 5 Western blot analysis of proteins from *Saccharomyces cerevisiae* recipient strain and transformants carrying the *SckIspS* or *kIspS* transgenes. Yeast cells were cultured in YNBD (+Trp) medium at 30°C for 24 h with shaking at 200 rev min^{-1}. Approximately 25 μg of proteins were separated by 8% SDS-PAGE, transferred onto PVDF membranes and probed with a specific IspS polyclonal antibodies. (a) Western blot analysis with anti-IspS antibodies. Lane 1: recipient strain; lane 2: ScT2; lane 3: ScT11; lane 4: ScT16; lane 5: ScT17; lane 6: KT8; lane 7: KT16; lane 8: KT24; lane 9: purified IspS. (b) SDS-PAGE stained with Coomassie Brilliant Blue R-250, shown as a control of protein loading. Lane 1: recipient strain; lane 2: ScT2; lane 3: ScT11; lane 4: ScT16; lane 5: ScT17; lane 6: KT8; lane 7: KT16; lane 8: KT24; M: molecular mass marker.

control for this measurement, showing no discernible differences in protein pattern or loading. Interestingly, the intensity of the antibody-kIspS cross-reaction was on the average fivefold greater in the protein extracts from the four transformants carrying the *S. cerevisiae* codon-optimized *SckIspS* construct (Fig. 5a, lanes 2–5) compared with three transformants carrying the original unmodified kudzu vine *kIspS* (Fig. 5a, lanes 6–8), affording evidence for the enhancement in *IspS* gene expression upon codon usage optimization. These results clearly show that the IspS protein is synthesized and accumulates in all seven *IspS* transformants and that transformants carrying codon-optimized *SckIspS* synthesize and accumulate the IspS protein more efficiently than transformants carrying the nonoptimized *kIspS*.

Isoprene production by IspS transformants

Vapour samples from the sealed headspace of recipient and transformant strains carrying the *SckIspS* or *kIspS* transgenes were examined by GC-MS gas chromatography (Agilent Technologies 6890 GC/5973 MSD equipped with a DB-XLB column). In the GC-MS approach, a 48–100 Da size selection was employed, which effectively eliminates ethanol signals from the GC-MS output. The recipient strain headspace showed two peaks with retention times of about 1·55 and 6·1 min (Fig. 6a, recipient strain). MS analysis of peak [1] showed multiple lines of low abundance reflecting unspecific molecules (noise level signal, see Fig. S2a). MS analysis of peak [2] showed a dominant line '57' corresponding to 1,6- hexanediol. This compound is probably generated in the presence of the YPG media as a by-product of the yeast cell metabolism.

GC-MS analysis of vapour from the headspace of *IspS* transformants is shown in Fig. 6b. Peak [3] with retention time of 1·55 min displayed dominant 53, 67 and 68 lines attributed to isoprene (Fig. S2b and c). Peaks [4] through [6] with retention times of 2·2–3·8 min were dominated by lines (55, 56, 57 and 70) indicative of 5-carbon branched alcohols (2-methyl-3-buten-1-ol and 3-methyl-1-butanol), probably derived from the nonspecific hydroxylation of isoprene. Peak [7] with retention time of 5·71–5·74 min displayed a single GC-MS line (96) attributed to 5-hydroxypyrimidine, whereas peak [8] with

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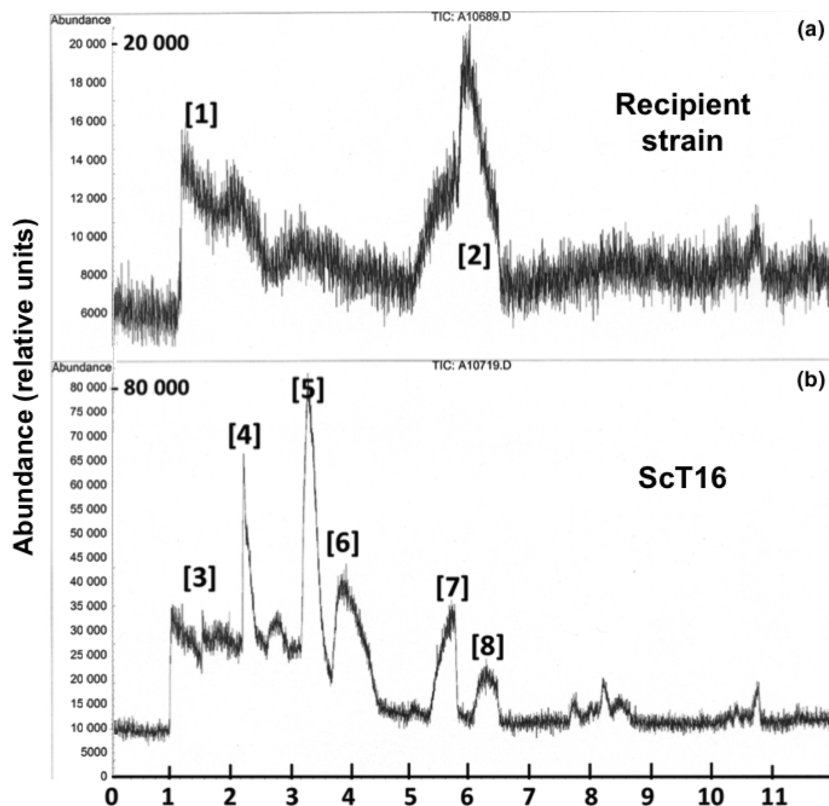

Figure 6 *Saccharomyces cerevisiae* culture headspace vapour analysis by Agilent Technologies 6890 GC/5973 MSD equipped with a DB-XLB column. GC-MS analysis of headspace gases produced by cultures of *S. cerevisiae* recipient and ScT16 transformant strains. Yeast cells were grown in YPG liquid media for 48 h at 30°C with shaking at 200 rev min^{-1}. The cultures were then sealed and incubated under the same conditions for an additional 48 h. Subsequently, 1 ml of gas was withdrawn from the headspace of the sealed flasks and analysed. (a) Recipient strain (untransformed). Peak [1] with retention time of 1·55 min was unspecific, as multiple low-amplitude GC-MS lines could not be attributed to any distinctive chemical species (see Fig. S2A). Peak [2] with retention time of 6·02-6·15 min displayed a single GC-MS line '57' attributed to 1,6-Hexanediol. (b) *S. cerevisiae* isoprene synthase (*IspS* gene) transformant ScT16. Peak [3] with retention time of 1·55 min displayed dominant 53, 67 and 68 lines attributed to isoprene. Peaks [4] through [6] with retention times of 2·2–3·8 min were dominated by lines (55, 56, 57 and 70) indicative of 5-carbon branched alcohols (2-methyl-3-buten-1-ol, 3-methyl-1-butanol), probably derived from the nonspecific hydroxylation of isoprene. Peak [7] with retention time of 5·71–5·74 min displayed a single GC-MS line (96) attributed to 5-hydroxypyrimidine, whereas peak [8] with retention times of 6·15–6·26 min displayed a single GC-MS line (57) attributed 1,6-hexanediol.

retention times of 6·15–6·26 min displayed a single GC-MS line (57) attributed 1,6-hexanediol (also present in the GC-MS profile of the recipient strain). Table 1 provides a detailed summary of the GC-MS analysis results, correlating GC retention time, corresponding MS lines, and the chemical species that corresponds to each of the GC peaks shown in Fig. 6.

In addition to the GC-MS analysis, we conducted a GC-FID measurement of headspace gases from control (recipient strain) and the *IspS* ScT16 transformant strain of *S. cerevisiae.* Fig. S2d shows that the headspace of the recipient strain cultures contained CO_2 and ethanol, the latter appearing as a dominant GC peak with a 5·4 min retention time (Fig. S2d, left panel). The headspace *of SckIspS* and *kIspS* transformants contained CO_2, ethanol and isoprene (Fig. S2d, middle panel). All seven *IspS* transformant lines examined, but only the transformants produced isoprene, which appeared as a separate and distinct GC peak with a 6·8 min retention time (Fig. S2d, middle and right panels).

These results clearly show that the expressed IspS transgenic protein is active in the yeast transformants. It is also evident that chemical modification of the isoprene molecule takes place in the yeast growth medium, with various hydroxylated isoprene derivatives being the dominant product. The ratio of secondary product to isoprene differed among different yeast cultures and preparations, suggesting a variable and time-dependent process for the secondary modification of isoprene (see also below). Quantitative analysis of the SckIspS-specific products

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Table 1 Summary of GC-MS analysis results from Fig. 6

Peak number	GC retention, time, min	MS lines	Chemical species
[1]	1·55	Multiple lines, noise level	Unspecific
[2]	6·02	57	1,6-Hexanediol
	6·15	57	
[3]	1·55	53,67,68; 72*	Isoprene
[4]	2·27	56	2-methyl-3-buten-1-ol
[5]	3·32	55,56,57,70,(77)	3-methyl-1-butanol
	3·43	55,56,57,70,(77)	
[6]	3·77	55,56,57,70,(77)	3-methyl-1-butanol
	3·83	55,56,57,70,(77)	
[7]	5·71	96	5-Hydroxypyrimidine
	5·74	96	
[8]	6·15	57	1,6-Hexanediol
	6·26	57	

Peak numbers were assigned on the basis of retention time in the GC, and chemical species identification was enabled upon comparative analysis of the resultant lines with known standards. Peaks [1] and [2] are from Fig. 6a. Peaks [3] through [8] are from Fig. 6b.
*Line 72 was putatively identified as one of several different molecules, including (i) 3-phenyl-2-propenoic acid, 2-methyl-2-propenyl ester, (ii) N,N′-diisopropylethylenediamine, (iii) 2-fluoro-1,3-butadiene, or (iv) 3-methoxy-1-propanamine. MS lines in parentheses indicate low relative abundance.

showed a yield of over 500 μg of isoprene and isoprene derivatives accumulated in the headspace per l culture in the course of 72 h of anaerobic incubation. This was a rather conservative estimate of product accumulation in the headspace. Moreover, it did not take into account hydroxylated isoprene derivatives that remained in the liquid medium of the culture.

Similar kinetics of isoprene accumulation were noted in both of the *SckIspS* and *kIspS* transformants, reaching a maximum at about 72 h after sealing of the culture flasks (Fig. 7a and b). It is of interest to observe that *SckIspS* transformants (Fig. 7a) accumulated about twice as much isoprene as their *kIspS* counterparts (Fig. 7b). It was reproducibly observed that, among all transformants, strain ScT16 was more efficient in isoprene production. These quantitative isoprene production results (Fig. 7a) are consistent with the Western blot analyses (Fig. 5a), showing that codon use optimization is an important tool in efforts to improve the performance of transgenes.

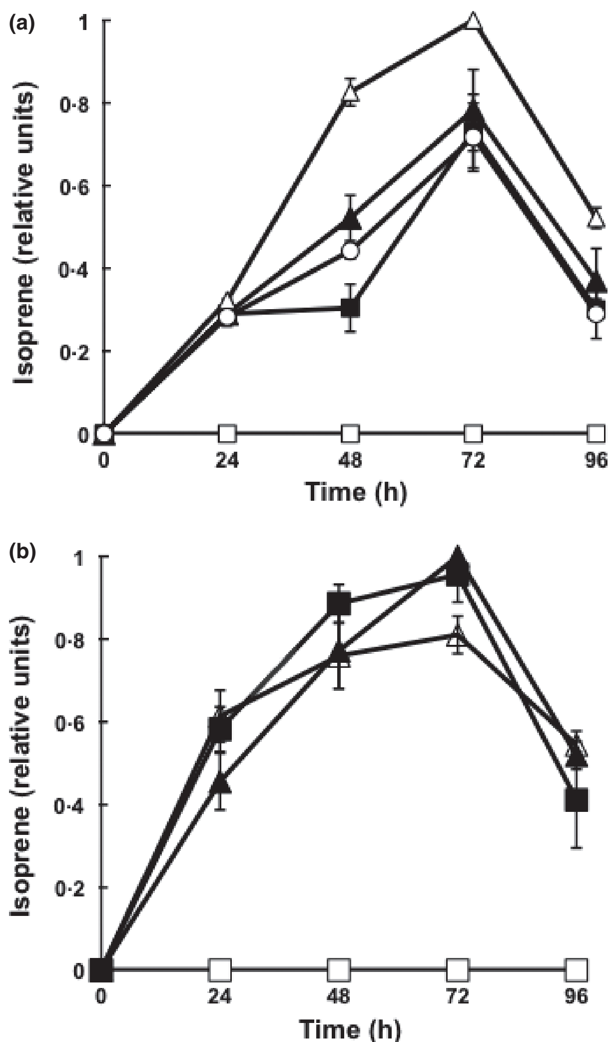

Figure 7 Time course of isoprene accumulation in the culture headspace of *Saccharomyces cerevisiae* recipient strain and transformants carrying the *SckIspS* (a) or *kIspS* (b) transgene. Yeast cells were cultured in YPG liquid medium under aerobic conditions at 30°C upon shaking at 200 rev min^{-1}. The culture flasks were sealed at 48 h after inoculation and further incubated under the same conditions for the indicated periods of time. Gas samples from the headspace of the sealed flasks were used for quantitative GC isoprene analysis.

Stability of multi-copy integrated IspS in transformants

To investigate whether the multi-copy integration of the *IspS* transgenes was stably maintained in the yeast transformants, we tested for tryptophan prototrophy, copy number of *IspS* and IspS activity as a function of time over many generations of yeast cell growth. For tryptophan prototrophy, 100 colonies were tested after the ScT16 strain was cultured in YPD as a nonselective rich medium until 100 generations of growth. All ScT16 colonies showed prototrophy to tryptophan after growth for 100 generations (not shown). Copy number of *IspS* was determined by Southern blot analysis using genomic DNA isolated from the ScT16 strain before and after 100 generations of growth. The intensity of the transgenic band at 4·1 kb was approximately 1/3 of that of the endogenous rDNA band at 2·06 kb in the samples from

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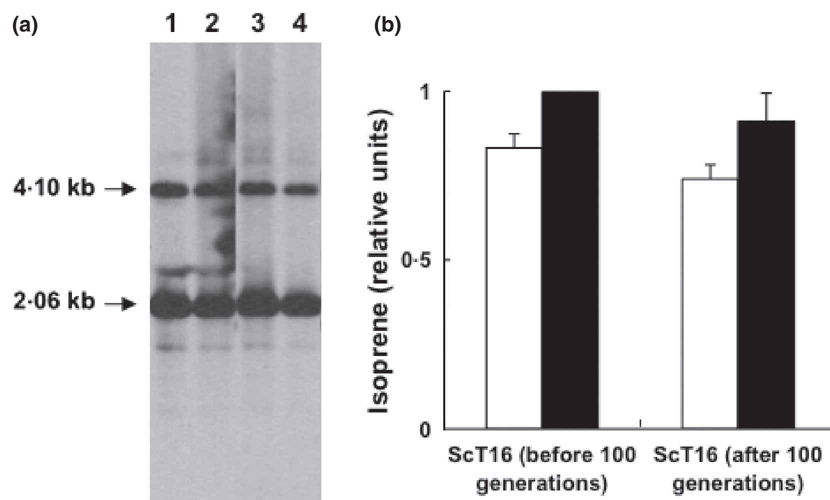

Figure 8 Southern blot analysis and isoprene production using the ScT16 *SckIspS* transformant before and after propagation for 100 generations in YPD nonselective rich medium. (a) Genomic DNA was isolated from the ScT16 before (lanes 1 and 2) or after strain propagation for 100 generations (lanes 3 and 4). The DNA was digested with *Pvu*II, and hybridized with an rDNA fragment (NTS2 region) as a probe. (b) Yield of isoprene production from the ScT16 transformant strain harbouring the *SckIspS* construct before and after propagation for 100 generations.

both before (Fig. 8a, lanes 1 and 2) and after propagation for 100 generations (Fig. 8a, lanes 3 and 4). Importantly in this respect, the ratio of transgenic/endogenous bands intensity (4·1 kb/2·06 kb) remained the same before and after strain propagation for 100 generations (Fig. 8a).

IspS enzymatic activity of the transformants was also compared, based on isoprene production, which was measured using the ScT16 cultures before and after propagation for 100 generations. No statistically significant differences could be observed between ScT16 cultures before and after 100 generations (Fig. 8b). These results support the notion of stability of the *IspS* transgene in the *S. cerevisiae* transformants. They are also consistent with the earlier finding that a high proportion of yeast transformants (> 97% of the colonies) retain their prototrophy characteristics and are stable for over 200 generations under nonselective media conditions (Lopes *et al.* 1989; Nieto *et al.* 1999).

Discussion

Yeast and fungi are commercially employed in large-scale fermentations to generate industrially valuable products. However, it was unclear whether the eukaryotic metabolism of Ascomycota can express the isoprene synthase (*IspS*) transgene, and whether isoprene might be toxic to the cells. Evidence was presented in this work to show stable heterologous transformation of *Saccharomyces cerevisiae* with a plant isoprene synthase gene (*IspS*), conferring to the yeast cells constitutive volatile isoprene (C_5H_8) hydrocarbons production. Two specific criteria for expression of the *IspS* gene in *S. cerevisiae* were applied, aiming to enhance prospects of isoprene hydrocarbons production. One criterion entailed multiple independent integrations of the *IspS* gene into the rDNA locus in the yeast genome so as to express as high amounts of the IspS protein as possible and thus to generate a genetically stable yeast strain with sustained isoprene production properties. The other criterion was optimization of codon usage of the *IspS* gene for specific application in *S. cerevisiae*.

It was previously reported that multi-copy integration can be obtained in *S. cerevisiae* if a defective selectable marker is used (Lopes *et al.* 1989; Nieto *et al.* 1999). In this work, we used *TRP1d* (conferring tryptophan auxotrophy) as a defective selectable marker to target multiple integrations of the *IspS* gene into the rDNA region on the yeast chromosome. Conferring tryptophan auxotrophy is an attractive selectable marker for industrial strain construction, as undefined media do not contain this amino acid after sterilization (Lopes *et al.* 1991). *Saccharomyces cerevisiae* transformants carrying multi-copy integrations (25–50 copies) of the *IspS* construct were generated in which the *IspS* gene was expressed under control of the *GPD* promoter, a yeast promoter designed to confer constitutive expression of the *IspS* gene (Fig. 2). Analysis of the transformants showed that they constitutively produced isoprene and showed high mitotic stability, which are desirable features for long-term culturing of yeast in industrial fermentations.

An alternative approach to express the *IspS* gene would be to employ a standard episomal yeast expression vector

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for isoprene production. However, episomal expression vectors could easily be lost from the cells in the course of continuous long-term cultivation. An additional disadvantage of episomal yeast expression vectors is the required presence of selectable markers (antibiotics) for the maintenance of the vector. The latter is not desirable for isoprene commercialization conditions, as large-scale use of antibiotics substantially increases cost and also raises environmental issues.

Our experimental approach entailed sealing the cultures to prevent escape of the volatile isoprene gas, followed by a subsequent sampling of the gas in the headspace of the cultures. In the context of this experimental approach, we noted that substantial amounts of isoprene converted into primarily hydroxylated products, that is, 2-methyl-3-buten-1-ol and 3-methyl-1-butanol. Quantitative analysis of the headspace of one of the *IspS* transformants showed a hydroxylated isoprene-to-isoprene ratio of about 100 : 1. This ratio is probably >100 : 1, as hydroxylated isoprene molecules have greater than isoprene solubility in the aqueous medium; hence, they were not quantitatively present in the reactor headspace. Accordingly, total productivity of isoprene and its hydroxylated products in the IspS transformants is much greater than the 500 $\mu g\ l^{-1}$ estimated in this work. Thus, the average rate of isoprene accumulation in this experiment was >7 μg per l culture per h.

Rates of isoprene accumulation in different transformants are available from the literature. The cyanobacterium *Synechocystis* photoproduced 4 μg isoprene per l culture per h (Lindberg *et al.* 2010; Bentley and Melis 2012). A study of isoprene release by *Bacillus subtilis* indicated rates of 50 μg isoprene per l culture per h (Julsing *et al.* 2007). Biosynthesis of isoprene in *E. coli* via the methylerythritol phosphate (MEP) pathway reportedly generated 2·8 mg isoprene per l culture per h (Zhao *et al.* 2011). In contrast, isoprene production in *E. coli* batch cultures via the MVA pathway showed rates of 18 mg isoprene per l culture per h (Zurbriggen *et al.* 2012). These measurements are preliminary and their significance should be moderated by their respective commercialization value. A techno-economic analysis for commercial feasibility of the above-mentioned isoprene production processes is outside the scope of this work. Nevertheless, it is clearly understood that improvements in endogenous substrate partitioning towards isoprene rather than biomass need be achieved with all of the above-mentioned systems before anyone could contemplate commercialization. Approaches to increase the isoprene yield and to prevent or regulate the chemical modification of isoprene in the yeast cell and/or growth media are currently under investigation in this lab. For example, alcohol dehydrogenase gene disruption would prevent endogenous substrate flux towards ethanol and, thus, improve the isoprene-to-ethanol and isoprene-to-biomass carbon-partitioning ratio of the cultures.

Commercially, isoprene is currently generated as a by-product of ethylene production from a petroleum feedstock. However, this work showed that isoprene can be generated from *S. cerevisiae* through heterologous transformation of the cell with a codon-optimized *IspS* construct, and by driving isoprene biosynthesis through the MVA pathway. Isoprene is a volatile and highly hydrophobic small hydrocarbon that can apparently pass from the cytosol, where it is generated, and through the yeast cell membranes without exerting any toxic effects, ending up in the headspace of the culture. The spontaneous separation from the cells is a property that simplifies product isolation. Spontaneous and efficient product separation from the biomass also alleviates potential feedback inhibition or product toxicity to the cells, which applies when products accumulate in microbial cells (Fischer *et al.* 2008). Our results demonstrate the potential of *S. cerevisiae* serving as a platform for the production of renewable isoprene hydrocarbons through further optimization of the MVA pathway.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Diagram of the 9.1 kb rDNA repeat unit in *S. cerevisiae*. The yeast rDNA consists of 100-200 tandemly repeated copies of a 9.1 kb unit on the right arm of chromosome XII. 5S, 5.8S, 18S, and 25S rRNAs are transcribed from the rDNA by RNA polymerase. Each rRNA form is present in a single copy in an 80S yeast ribosome. Abbreviations used: NTS = non-transcribed spacer; ITS = internal transcribed spacer; ETS = external transcribed spacer.

Figure S2 GC-MS analysis of the culture headspace from *S. cerevisiae* recipient strain and ScT16 transformant. (a) GC and mass spectra of headspace gases generated by the recipient strain *S. cerevisiae* culture. Upper panel: GC profile of headspace gases from the culture. Lower panel: Mass profile of headspace gases from the same culture. Note the low level (noise level) multiple unspecific lines. (b) GC and mass spectra of headspace vapor generated by *S. cerevisiae* ScT16 transformant cultures. Upper panel: GC profile of headspace gases from the culture of *S. cerevisiae* ScT16 transformant. Note the specific peaks not present in the corresponding analysis of the recipient strain. Lower panel: Mass profile of gases from the culture of *S. cerevisiae* ScT16 transformant. Note the presence of MS lines 53, 67, and 68 from isoprene. Also note the presence of MS line 72 attributed to a variety of products (see Table 1). (c) Mass spectrum lines 53, 67, and 68 from an isoprene standard. (d) 1 ml of gas was withdrawn from the headspace of the sealed flasks and analyzed by GC-FID. Ethanol peaks are shown with a retention time of 5.4 min. Isoprene peaks are shown with a retention time of 6.8 min. Left panel: GC-FID profile of gases from the culture of *S. cerevisiae* recipient strain. Middle panel: GC-FID profile of gases from the culture of *S. cerevisiae* ScT16 transformant carrying the *SckIspS* transgene. (Note: hydroxylated isoprene products overlap with the ethanol peak in this GC analysis.) Right panel: GC-FID profile of an isoprene standard.

Table S1 Primer sequences used in the *IspS*-containing plasmid construction.

Table S2 Primer sequences used in the yeast colony PCR, Southern hybridization, genomic PCR, and RT-PCR.

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