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Polycistronic expression of a β-carotene biosynthetic pathway in *Saccharomyces cerevisiae* coupled to β-ionone production

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ABSTRACT

The flavour and fragrance compound β-ionone, which naturally occurs in raspberry and many other fruits and flowers, is currently produced by synthetic chemistry. This study describes a synthetic biology approach for β-ionone production from glucose by *Saccharomyces cerevisiae* that is partially based on polycistronic expression. Experiments with model proteins showed that the T2A sequence of the *Thosea asigna* virus mediated efficient production of individual proteins from a single transcript in *S. cerevisiae*. Subsequently, three β-carotene biosynthesis genes from the carotenoid-producing ascomycete *Xanthophyllomyces dendrorhous* (*crtI*, *crtE* and *crtYB*) were expressed in *S. cerevisiae* from a single polycistronic construct. In this construct, the individual *crt* proteins were separated by T2A sequences. Production of the individual proteins from the polycistronic construct was confirmed by Western blot analysis and by measuring the production of β-carotene. To enable β-ionone production, a carotenoid-cleavage dioxygenase from raspberry (*RiCCD1*) was co-expressed in the β-carotene producing strain. In glucose-grown cultures with a second phase of dodecane, β-ionone and geranylacetone accumulated in the organic phase. Thus, by introducing a polycistronic construct encoding a fungal carotenoid pathway and an expression cassette encoding a plant dioxygenase, a novel microbial production system has been established for a fruit flavour compound.

1. Introduction

β-Ionone (Fig. 1) is of interest to the flavour and fragrance industry. It has a warm, woody, berry, characteristic violet odour and is sensed at a very low threshold concentration, varying between 0.007 ppb in air and 1 ppb in water (Burdock, 2002). Annually, 4000–8000 tonnes of β-ionone are produced industrially. In addition to its direct application as a flavour or fragrance ingredient, ionone serves as an intermediate for synthesis of vitamins and aroma chemicals. The chemical production process of β-ionone involves base-catalyzed condensation from citral to linear pseudoionones, followed by acid-catalyzed cyclization (OECD, 2004). The resulting ionone cannot be marketed as a natural product, but rather must be labelled as being "nature identical". For application of β-ionone in food, a food-grade production process is essential. β-Ionone naturally occurs in raspberries (Beekwilder et al., 2008a), roses and many fruits, but mostly at low concentrations. Therefore it would be of interest to create a microbial production system for these compounds, based on the pathway as it has been unravelled in plants (Beekwilder et al., 2008a).

Naturally occurring β-ionone is an apo-carotenoid (*i.e.* a carotenoid-derived compound) resulting from the cleavage of its natural precursor β-carotene. The cleavage reaction is catalyzed by the *c*arotenoid *c*leavage *d*ioxygenase enzyme CCD1 which cleaves carotenoids in the presence of oxygen, at the 9–10 position and the 9′–10′ position (Fleischmann et al., 2003; Nacke et al., 2012) (Fig. 1). CCD1 enzymes and their encoding genes have now been identified in many plant species, including *Arabidopsis* (Schwartz et al., 2001), rose (Huang et al., 2009) and raspberry (Beekwilder

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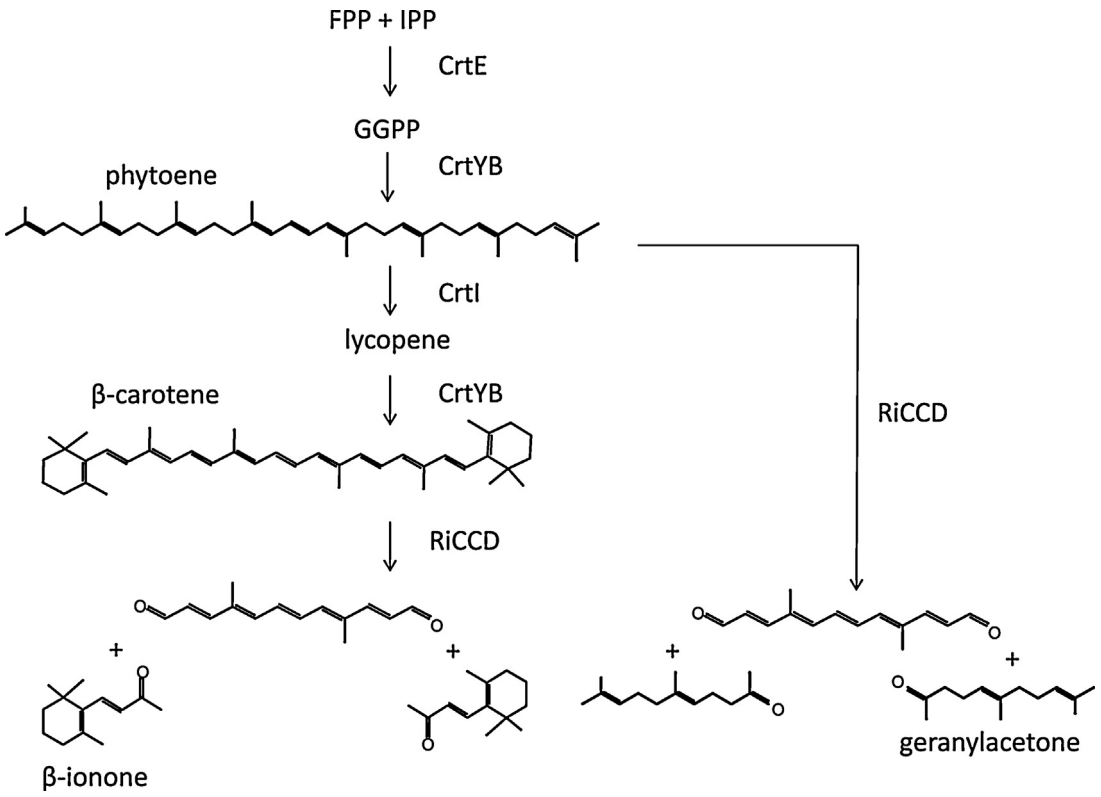

Fig. 1. Engineered β-ionone biosynthetic pathway in *Saccharomyces cerevisiae*. The engineered pathway includes the carotenoid biosynthetic proteins (crtI, crtE, crtYB) from *Xanthophyllomyces dendrohrous* and the *RiCCD1* gene from raspberry.

et al., 2008a). Co-expression of these CCD1s in *Escherichia coli* with a carotenoid operon from *Erwinia* has been shown to lead to production of β-ionone in the culture medium (Beekwilder et al., 2008a; Huang et al., 2009; Schwartz et al., 2001).

The yeast *Saccharomyces cerevisiae* could provide an interesting production platform for food-grade β-ionone. Strains for producing β-carotene have been constructed by introducing three genes from the carotenoid-producing fungus *Xanthophyllomyces dendrorhous* (Verwaal et al., 2007). *S. cerevisiae* strains expressing the *X. dendrorhous crtI*, *crtE* and *crtYB* genes under control of the constitutive *TDH3* promoter produced high levels of intracellular β-carotene, colouring the cultures orange. A transcriptional analysis showed that carotenoid production induces a pleiotropic drug resistance stress response in yeast (Verwaal et al., 2010). Product-induced stress is therefore likely to confer a selective advantage to non-producing strains. Indeed, cultures of strains where loss of *crt* expression cassettes can occur *via* homologous recombination display a rapid accumulation of non-producing mutants. In recent years, new technologies have been developed to express multiple genes from a single, polycistronic transcript, with the aim to reduce the number of promoters needed. One such technology comprises the expression of several proteins from a single open reading frame, in which each is separated by so-called 2A sequences (Ryan et al., 1991). The 2A sequences are small peptides (up to 20 amino-acids), mostly derived from viral polyproteins. When the ribosome translates the 2A sequence, it releases the nascent peptide and continues translation of the downstream sequence. As a result, several different, separate proteins can be formed from a single open reading frame. When applied for metabolic engineering in plants, co-ordinated and high level expression of several genes can be achieved simultaneously (Geu-Flores et al., 2009; van Herpen et al., 2010). However, for yeast, application of this system for metabolic engineering has hardly been explored (Park et al., 2008).

The aims of the present study were to achieve the production of β-ionone from yeast and to explore the use of 2A-based polycistronic expression for pathway engineering in *S. cerevisiae*. After engineering a stable 2A-based carotenoid production system in this yeast, β-ionone was produced by introduction of the raspberry gene encoding RiCCD1, which cleaves the carotenoid to release the volatile fruit odour component.

2. Materials and methods

2.1. Strains, media and DNA templates

The *S. cerevisiae* strains used in this study (Table 2) are derived from the CEN.PK lineage (Entian and Kotter, 2007; Nijkamp et al., 2012). Cultures for transformation were grown in complex YPD medium, containing 10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone and 20 g L^{-1} glucose. Synthetic medium (SM) was prepared according to Verduyn et al. (1990) and contained, per litre of demineralized water, 5 g $(NH_4)_2SO_4$, 3 g KH_2PO4, 0.5 g $MgSO_4 \cdot 7H_2O$, and trace elements. Filter-sterilized vitamins were added after heat sterilization of the medium at 120 °C for 20 min. Glucose was separately sterilized at 110 °C and added to a final concentration of 20 g L^{-1}. When required, the medium was supplemented with appropriate amounts of auxotrophic requirements (Pronk, 2002). Solid medium was prepared by adding 2% agar (w/v) to the media prior to heat sterilization. Plasmids pUG72 and pRS416 (Kuijpers et al., 2013) were maintained in *E. coli* DH5α and isolated with the GenElute™ Plasmid Miniprep Kit (Sigma–Aldrich, Zwijndrecht, The Netherlands).

2.2. Production of DNA fragments and transformation

Fragments for *in vivo* assembly were obtained from plasmid template DNA by extension PCR using Phusion® Hot Start II High

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Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). Primers were HPLC purified (Sigma) and are listed in Supplementary Table. To improve PCR efficiency, we slightly modified the conditions recommended by the supplier. The primer concentration was decreased from 500 nM to 200 nM and the polymerase concentration increased from 0.02 U μL^{-1} to 0.03 U μL^{-1}. Other conditions were according to the manufacturer's instructions. Primers were designed such that their annealing temperature was >65 °C to minimize non-specific product formation caused by false priming. Amplified fragments were concentrated by chromatography using Vivacon® 500 spin columns (Sartorius Stedim, Aubagne, France). Alternatively, fragments obtained from plasmid templates were purified by gel extraction, using Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, CA), to avoid contamination of the fragments by the linearized template plasmid and the ensuing formation of false positive clones. Restriction of pRS416 to obtain the linearized backbones was performed with FastDigest enzymes BamHI and SspI (Thermo Fischer Scientific) according to the manufacturer's instructions. For assembly of plasmids pUDC082 and pUDE269, six fragments were co-transformed into yeast (Fig. 3). The fragments CEN6/ARS4FG (primers Cen6 Ars4-r+G and Cen6 Ars4-f+F) and *E. coli*IA (primers Amprep-r+A and either AmpRep-f+I or G-fwd-coli) were amplified from pRS416. Fragment K.l.URA3AB was amplified from pUG72 using primers URA3Kl.-r+B and URA3Kl.-f+A. Fragments CrtYB, crtI and CrtE were amplified from plasmid YEPlac195-crtYB/crtI/crtE* (Verwaal et al., 2007). Fragment crtYB was amplified in two parts, using N-Fr-TDHfw and CrtYBIntRe, and CrtYBintFw and crtYB-re-t2a, as amplification of the full length ORF only occurred at low efficiency. Fragment crtI was amplified using primers crtI-re-t2a and t2a-crtI-fw. Fragment crtE was amplified using primers crtE-fw-t2a and Ttef-re-B. Plasmid pRiCCD was assembled in yeast from the same fragments, but also included a RiCCD1 fragment. Fragment RiCCD1 was amplified from plasmid pUC57-RiCCD using primers H-PPMA-fw and G-TPGK-re. pUC57-RiCCD contains a codon-optimized version of *RiCCD*1, 800 bp of the *PMA1* promoter region starting immediately at the start codon of *PMA1*, and 400 bp of the *PGK1* terminator. The protein sequence of RiCCD1 (CS363369) was reverse-translated to DNA optimized for expression in *S. cerevisiae* using the Java Codon Adaptation Tool (http://www.jcat.de/), and ordered from Genscript (Piscataway, NJ, USA). The 2μ fragment was obtained by SacII cleavage of plasmid pUD194, kindly provided by Barbara Kozak. DNA concentrations were measured by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and 200 fmol of each gene cassette and the *E. coli*IA fragment were pooled with 100 fmol of the K.l.URA3AB and CEN6/ARS4FG fragments in a final volume of 50 μL. A control pool lacking the *E.coli*IA cassette was generated in the same way. Both pools were transformed to *S. cerevisiae* strain CEN.PK113-5D using lithium acetate (Gietz and Schiestl, 2007). After transformation cells were selected on synthetic medium for 3–4 days at 30 °C.

For construction of the *S. cerevisiae* strains IME131, IME132 and IME134 with different plasmids bearing the GFP-2A-LEU2-HA constructs (pUDE155–pUDE157), three DNA fragments were transformed (Fig. 2). Strain IME131 with plasmid pUDE155 was constructed by transforming CEN.PK2-1C with the backbone obtained *via* PCR with pAG416-GPD as a template, using primers 1975 and 1976, the *GFP* fragment, amplified from template pAG416-EGFP with primers 2327 and 2315 and the *LEU2* fragment obtained from the template pAG415-GDP using primers 2319 and 2321. Overlapping regions as well as the 2A sequence were encoded within the primers. Similarly, IME132 with plasmid pUDE156 was obtained using the same templates for the PCR reactions but different primers for *GFP* (2327 and 2316) and *LEU2* (2320 and 2321). Likewise, the strain IME134 with plasmid pUDE157 was constructed with *GFP* amplified using primers 2327 and 2314 and *LEU2* with primers 2318 and 2321. Correct assembly was verified using primers 1153, 2322–2326 and restriction with BsrGI and EcoRV.

2.3. Western blotting

Two approaches were used to make cell extracts. For the experiments shown in Fig. 2, an equivalent of 1 mL with an OD_{660} of ~80 culture broth was centrifuged, followed by resuspension of the pellet in 200 μL Laemmli sample buffer (BioRad, Veenendaal, Netherlands) with subsequent heating for 10 min at 95 °C. After centrifuging, the supernatant was stored on ice and used as cell extract. For the experiments shown in Fig. 5 the procedure described by Kushnirov (2000) was followed.

The protein samples were loaded on a gel (12% Tris–HCl glycine; BioRad), together with a low range protein marker (BioRad) and/or a broad range protein marker (BioRad). The gel was run at 150 V for approximately 1 h using a Mini-PROTEAN gel system (BioRad), according to the manufacturer's instructions. After washing the gel in blot buffer (in demineralized water, 3.029 g L^{-1} Tris, 14.413 g L^{-1} glycine and methanol (20%, v/v)), the proteins were transferred to a PVDF membrane (Sigma) by blotting (Multiphor II NovaBlot, GE Healthcare Life Sciences, Piscataway, NJ, USA), according to the manufacturer's manual. The membranes were blocked with PBS-Tween (1X PBS (Sigma) with 0.05% Tween-20 (Acros, Geel, Belgium) containing 5% milk powder). The membranes were incubated for 1 h with the primary antibody (anti-GFP (Millipore), anti-HA (Sigma) or anti-2A (ABS31, Millipore)). After 4 washes with PBS-Tween, the membranes were incubated for 1 h with the secondary antibody (AP182P, Millipore). The membranes were then washed again 4 times with PBS-Tween, followed by detection of the signal using an enhanced chemiluminescence kit (Sigma).

2.4. DNA isolation and analysis

Analysis by multiplex PCR was done according to Kuijpers et al. (2013), using oligonucleotides A–I in Supplementary Table. One correctly assembled plasmid, as determined by multiplex PCR, was transformed to Electro Ten-Blue Electro-Competent *E. coli* cells (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. From a resulting clone, plasmid DNA was isolated and analysed by multiplex PCR. This isolated plasmid was named pUDC082. The resulting plasmid was further purified using the Zyppy Miniprep kit (Zymoresearch, Irvine, CA) and sequenced according to Kuijpers et al. (2013).

2.5. Growth for biochemical analysis

For biochemical analysis, single colonies were inoculated in 50 mL pre-cultures in SCD media without uracil (0.17% yeast nitrogen base without amino acids and ammonium sulphate, 0.5% ammonium sulfate, 4% glucose (all w/v) and 20 mg L^{-1} arginine, 10 mg L^{-1} adenine, 20 mg L^{-1} histidine, 20 mg L^{-1} methionine, 20 mg L^{-1} tryptophan, 30 mg L^{-1} isoleucine, 60 mg L^{-1} leucine, 30 mg L^{-1} threonine, 30 mg L^{-1} tyrosine, 30 mg L^{-1} valine and 50 mg L^{-1} phenylalanine). Glucose was sterilized separately. Cultures for analysis of product formation were grown in 300 mL shake flasks at 30 °C and 180 rpm in a horizontal shaking incubator, with a culture volume of 50 mL. For analysis of β-ionone, strains were grown with a second phase of dodecane (10%, v/v) in 300 mL baffled shake flasks for 22 h. All shake-flask cultures were inoculated from pre-cultures grown on the same medium, to an initial OD_{600} of 0.1.

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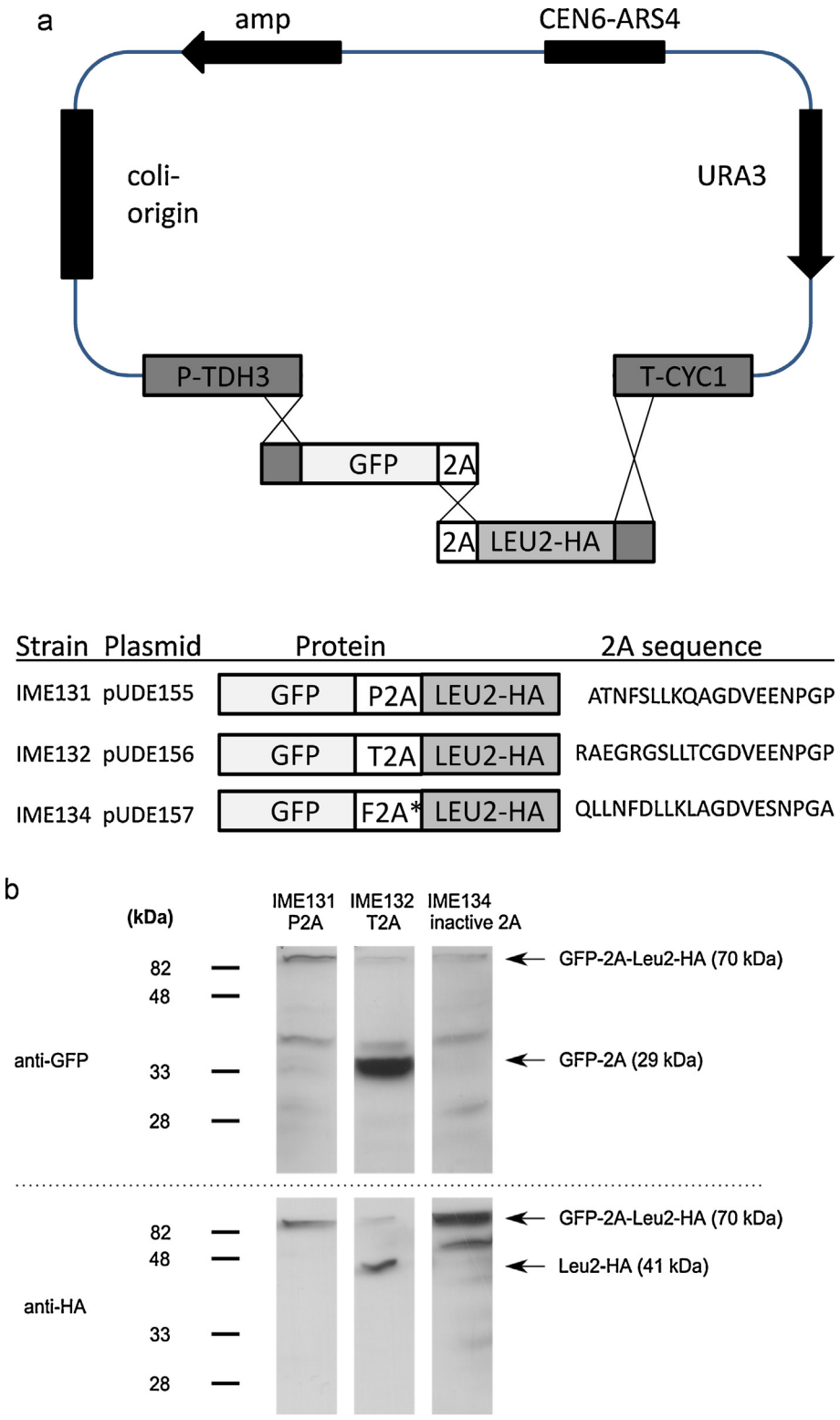

Fig. 2. Constructs for testing 2A activity in yeast. (A) Assembly strategy of *S. cerevisiae* strains IME131 (*GFP::P2A::LEU2::HA*), strain IME132 (*GFP::T2A::LEU2::HA*) and strain IME134 (*GFP::F2A$_{defect}$::LEU2::HA*). Protein coding regions (*GFP, 2A, LEU2-HA*) are represented in light grey, selection marker genes and origins of replication regions (amp, *URA3*, ColE1, *CEN6-ARS4*) in black and promoter/terminator regions (P-*TDH3*, T-*CYC1*) in dark grey. Recombination sites are indicated by crossing lines. (B) Representative Western blots of protein from strains IME131-133, using anti-GFP (top) and anti-HA (bottom). The predicted position of GFP-2A-Leu2-HA, GFP-2A (29.0 kDa) and Leu2-HA (40.5 kDa) is indicated.

2.6. Carotenoid analysis

Quantification of intracellular β-carotene was performed *via* HPLC analysis. For this purpose, 50 mL culture volume was centrifuged, washed with distilled H_2O and lyophilized overnight. Extraction of β-carotene was performed according to a published protocol (Visser and Verdoes, 2005). The vacuum dried carotenoids were suspended in 1 mL acetone and analysed on a

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Shimadzu SCL10-A system. A SPD20A UV/vis detector at 470 nm was used for analysis of β-carotene, lycopene and neurosporene and a SPDM10Avp photo diode array detector (PDA) at 286 nm and 292 nm for analysis of phytoene, respectively. An Altima C18-column (length: 250 mm, diameter: 4.5 mm, particle size: 10 μm; Alltech, Deerfield, USA) in combination with a binary gradient at a constant flow rate of 1 mL min^{-1} was used with an acetonitrile/methanol/2-propanol 85:10:5 mixture as eluent A and 100% 2-propanol as eluent B (all solvents HPLC grade). Eluent A was used for 31 min, followed by a 5 min elution with eluent B and again 10 min of eluent A. The temperature was kept constant at 32 °C. β-Carotene, neurosporene, lycopene and phytoene (retention times: 36.4, 29.7, 22, 37.4 min) were quantified *via* a calibration curve which was created by measuring known concentrations of the substances in acetone at the proper wavelengths.

2.7. GC–MS analysis

Analytes from 1 μL samples were separated using a gas chromatograph (5890 series II, Hewlett-Packard) equipped with a 30 m × 0.25 mm, 0.25 mm film thickness column (ZB-5, Phenomenex) using helium as carrier gas at flow rate of 1 mL min^{-1}. The injector was used in splitless mode with the inlet temperature set to 250 °C. The initial oven temperature of 45 °C was increased after 1 min to 300 °C at a rate of 10 °C/min and held for 5 min at 300 °C. The GC was coupled to a mass-selective detector (model 5972A, Hewlett-Packard). Compounds were identified by comparison of mass spectra and retention times with those of authentic standards of β-ionone and geranylacetone (Sigma). Quantification of apocarotenoids was conducted by determination of selective ion mode peak area of the sesquiterpene peaks from at least three independent fermentation experiments. Absolute concentrations were calculated from the peak area by comparison to a standard curve prepared by measuring a dilution series of authentic standards with known concentrations.

3. Results

3.1. Selection of a functional 2A sequence in yeast

To select a 2A sequence that functions well in *S. cerevisiae*, we evaluated the performance of two different 2A sequences: the P2A sequence from porcine teschovirus-1 and T2A from *Thosea asigna* virus (Szymczak et al., 2004). To test the functionality of these 2A sequences in *S. cerevisiae*, fusion genes were designed in which the open reading frames of *GFP* (encoding green fluorescent protein) and *LEU2* were separated by one of the 2A sequences (Fig. 2). In addition to these two, a negative control construct was made which included a defective F2A sequence, lacking a codon encoding proline, a residue that is essential for co-translational release (Donnelly et al., 2001).

Three plasmids carrying the bi-cistronic *LEU2-GFP* open reading frames were assembled by *in vivo* recombination (Gibson et al., 2008; Kuijpers et al., 2013) in *S. cerevisiae* CEN.PK2-1C (*ura3 his3 trp1 leu2*) (Fig. 2). In this way, we constructed three *S. cerevisiae* strains, each carrying one of the three 2A constructs: Strain IME131 (*GFP::P2A::LEU2::HA*), strain IME132 (*GFP::T2A::LEU2::HA*) and strain IME134 ($GFP::F2A_{defect}::LEU2::HA$) (Table 1). To determine whether the bi-cistronic genes were translated into two separate proteins, lysates of the three constructed strains were subjected to Western blot analysis using anti-GFP and anti-HA antisera. The predicted 29-kDa GFP::X2A and 40.5-kDa LEU2::HA band were only detected in the strain harbouring the T2A (IME132) sequence (Fig. 2B). The signal intensity of the two discrete bands was lower than the intensity of the larger band at 70 kDa (corresponding to the unprocessed polyprotein fusion). While the unprocessed polyprotein was expected in IME134 (strain harbouring a defective 2A), it was not anticipated for IME131 (P2A) which had been already successfully tested in rabbit reticulocyte lysates or wheat germ extracts (Donnelly et al., 2001) These results indicated that the T2A sequence mediates production of detached proteins in *S. cerevisiae*.

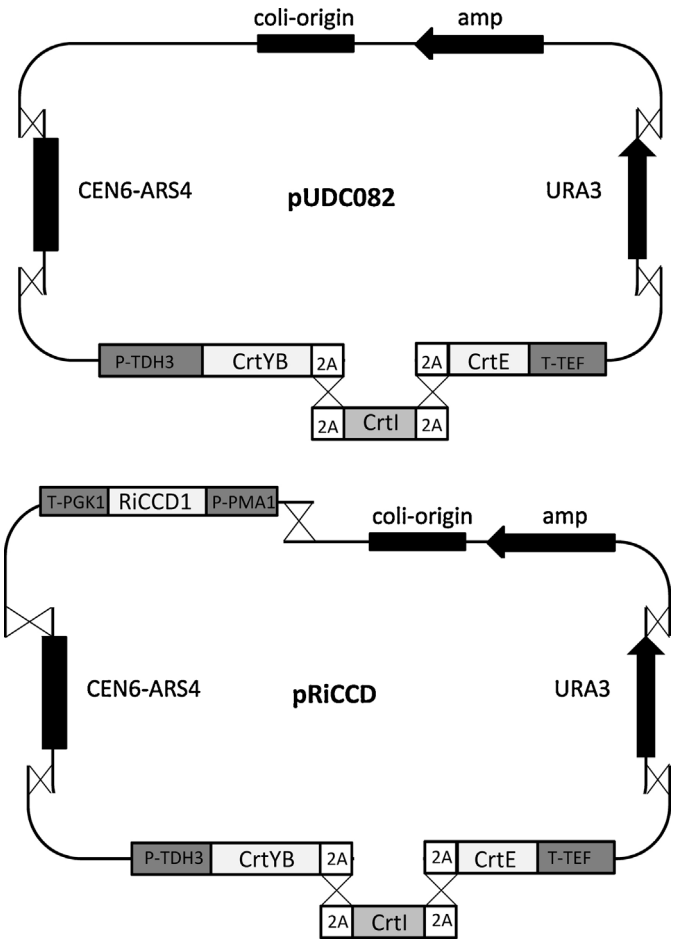

Fig. 3. Constructs and assembly strategies for carotenoid producing yeast strains. Top: pUDC082 (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE*); bottom: pRiCCD (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE RiCCD1*). Protein coding regions (*crtYB*, *crtI*, *crtE*, 2A, *RiCCD1*) are represented as light grey, marker and origin of replication regions (amp, *URA3*, coli-origin, *CEN6-ARS4*), in black and promoter/terminator regions (P-*TDH3*, T-*TEF*, P-*PMA1*, T-*PGK1*) in dark grey. Recombination sites are indicated by crossed lines.

3.2. Construction and expression of a polycistronic construct encoding a carotenoid pathway

To construct a pathway for β-carotene synthesis in *S. cerevisiae*, constructs were designed in which the coding regions *crtI*, *crtE* and *crtYB* genes from *X. dendrorhous* were fused in a single open reading frame, separated by T2A sequences (Fig. 3). To reduce the risk of loss of productivity due to recombination of the direct repeat caused by the two T2A-encoding sequences, the DNA sequences encoding the two T2A peptides were designed such that they differed in 18 out of 60 encoding nucleotides (Supplementary Figure 1). The expression of this polycistronic gene was controlled by the *TDH3* promoter. High-copy-number (pUDE269, 2μ) and low-copy-number (pUDC082, *ARS4 CEN6*) plasmid constructs carrying the polycistronic *crt* construct were generated by *in vivo* recombination in *S. cerevisiae* CENPK113-5D. In both centromeric and episomal recombinants, *ca.* 50% of the resulting colonies showed an orange

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Table 1
Strains and plasmids.

Strain	Relevant genotype	Origin
CEN.PK113-7D	*MATa MAL2-8c SUC2*	P. Kötter
CEN.PK2-1C	*MATa MAL2-8c SUC2 ura3-52 his3Δ1 trp1-289 leu2-3_112*	P. Kötter
IME131	*MATa MAL2-8c SUC2 ura3-52 his3Δ1 trp1-289 leu2-3_112* pUDE155	This study
IME132	*MATa MAL2-8c SUC2 ura3-52 his3Δ1 trp1-289 leu2-3_112* pUDE156	This study
IME134	*MATa MAL2-8c SUC2 ura3-52 his3Δ1 trp1-289 leu2-3_112* pUDE157	This study
IMC018	*MATa ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2* pUDC082	This study
IME167	*MATa ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2* pUDE269	This study
YB/I/E	*MATa ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2 YIplac211 YB/I/E**	Verwaal 2007
YEplac195 YB/I/E	*MATa ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2 YEplac195 YB/I/E*	Verwaal 2007
RiCCD1	*MATa MAL2-8c SUC2 ura3-52 his3Δ1 trp1-289 leu2-3_112* pRiCCD	This study

Plasmid	Description	Origin
pAG416-GDP	*CEN6-ARS4 URA3 pTDH3 tCYC1*	Alberti Lindquist 2007
YB/I/E*	YIplac211 *pTDH3-crtYB-tCYC1 pTDH3-crtI-tCYC1 pTDH3-crtE*-tCYC1*	Verwaal 2007
YEplac195 YB/I/E	YEplac195 *pTDH3-crtYB-tCYC1 pTDH3-crtI-tCYC1 pTDH3-crtE-tCYC1*	Verwaal 2007
pUDC082	*CEN6-ARS4 URA3 pTDH3-crtYB-T2A1-crtI-T2A2-crtE-tTEF*	This study
pUDE269	2 μ *URA3 pTDH3-crtYB-T2A1-crtI-T2A2-crtE-tTEF*	This study
pRiCCD	pRS426 *CEN6-ARS4 URA3 pTDH3-crtYB-T2A1-crtI-T2A2-crtE-tTEF pPMA1-RiCCD1-tPGK*	This study
pUDE155	pAG416-GDP *URA3 GFP-P2A-LEU2-HA*	This study
pUDE156	pAG416-GDP *URA3 GFP-T2A-LEU2-HA*	This study
pUDE157	pAG416-GDP *URA3 GFP-inactive 2A-LEU2-HA*	This study

* Indicates an A551G mutation in *crtE*.

colour, indicating carotenoid biosynthesis, while the remaining 50% were white. From the transformation plates, five orange colonies were selected from which plasmid DNA was isolated and amplified in *E. coli*. After confirming correct assembly by multiplex PCR, a full sequence analysis of plasmid pUDC082 was performed. No mutations were observed in the T2A sequences, and correct assembly was confirmed. The complete sequence of pUDC082 was deposited in GenBank under accession number KF680164. Subsequently, the characterized carotenogenic plasmids pUDC082 and pUDE269 were reintroduced in CEN.PK113-5D resulting in strains IMC018 (centromeric) and IME167 (episomal), respectively. The two strains displayed an orange colour indicating the functionality of the three heterologous genes.

To verify whether altered codon usage of the T2A sequences was sufficient to prevent loss of productivity by homologous recombination, we compared stability of β-carotene production in strains IMC018 (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE*) and IME167 (2μ *crtYB-T2A-crtI-T2A-crtE*) to that in *S. cerevisiae* strain YB/I/E (Verwaal et al., 2007). In the latter strain, the carotenogenic genes from *X. dendrorhous* (*crtI*, *crtE* and *crtYB*) are overexpressed from a single episomal vector (Yep195 (*URA3*)). Moreover, expression of each of the *crt* genes in this reference strain is controlled by the *TDH3* promoter and the *CYC1* terminator. Since all three genes are transcribed in the same direction, this leads to a high rate of loss of production due to recombination across the non-coding regions. When, after 24 h of cultivation in shake flasks on glucose

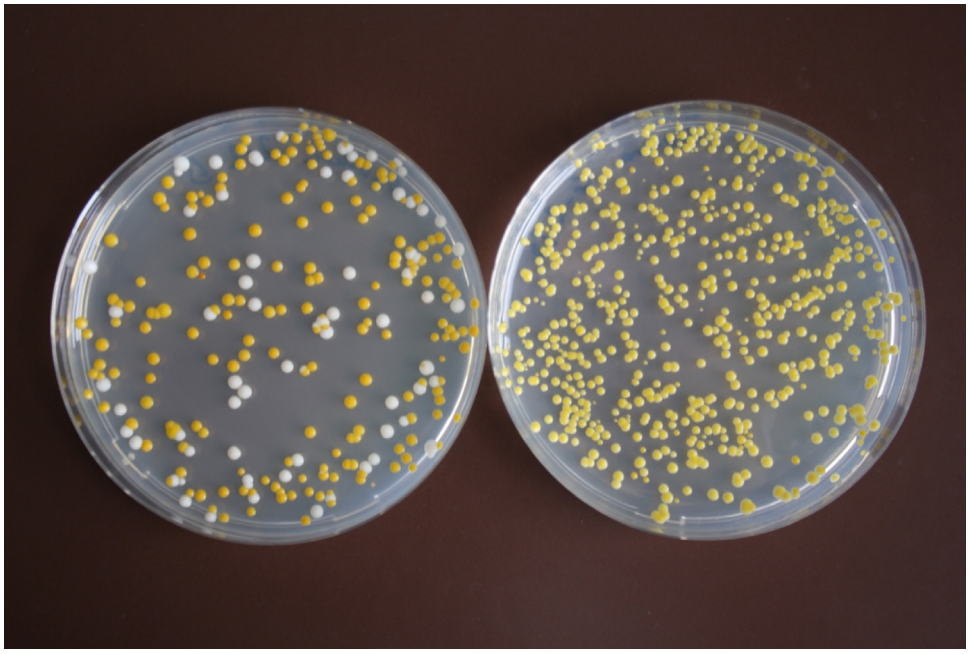

Fig. 4. Stability of carotenoid production in two *S. cerevisiae* strains. Strain YB/I/E (left) harbours the episomal plasmid *pTDH3-crtYB-tCYC1 pTDH3-crtI-tCYC1 pTDH3-crtE-tCYC1* in which each gene is expressed from its own TDH3 promoter; Strain IMC082 (right) harbours the centromeric plasmid (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE*). Shown are representative plates. Cells were plated after 24 h of growth on glucose synthetic medium in a shake flask, inoculated with an orange, β-carotene producing colony.

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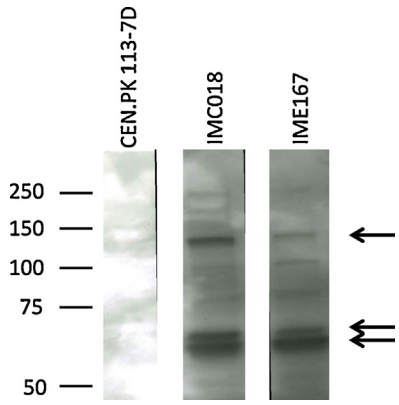

Fig. 5. Western blot analysis of yeast proteins. Shown is a representative chemoluminiscence detection of captured anti-2A antibodies by immunoblotted proteins from the yeast strains CENPK113-7D (empty), IMC018 (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE*) and IME167 (2μ *crtYB-T2A-crtI-T2A-crtE*). Arrows indicate the proteins corresponding to 65 kDa, 67 kDa and 144 kDa.

synthetic medium, dilutions were plated on agar plates, strain YB/I/E revealed a mixed population of white and orange colonies, and 43% of the plated colonies had lost colour (Fig. 4). PCR analysis results confirmed that the white colonies harboured a plasmid that had been reorganized through homologous recombination in between repeated promoter or terminator sequences (data not shown). In contrast, plates of strains IME167 and IMC018 exclusively contained orange colonies (Fig. 4).

3.3. Expression of Crt proteins in IMC018 and IME167

To assess the *in vivo* functionality of the 2A peptides in the carotenoid-producing constructs, cell lysates of strains IMC018 (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE*) and IME167 (2μ *crtYB-T2A-crtI-T2A-crtE*) were subjected to Western blot analysis using anti-2A peptide antibody, which specifically recognizes T2A sequence (Fig. 5). Based on the design of the constructs, probing with the 2A antibody should reveal bands corresponding to protein masses of 77 kDa and 67 kDa, corresponding to cleaved crtYB-T2A and crtI-T2A, respectively. Incomplete processing of the first T2A sequence should then lead to the formation of a 144 kDa product corresponding to the CrtYB-T2A-CrtI-T2A protein.

Western blotting of strains IMC018 (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE*) and IME167 (2μ *crtYB-T2A-crtI-T2A-crtE*) with 2A antibody indeed revealed bands with mobilities corresponding to 67 kDa (crtI-T2A) and 144 kDa (crtYB-T2A-crtI-T2A). The most intense band, which likely corresponds to crtYB-T2A (77 kDa) displayed an unexpectedly fast mobility, corresponding to a molecular weight of *ca.* 65 kDa (Fig. 5). The band around 144 kDa (corresponding to CrtYB-T2A-CrtI-T2A protein) was detected in both IMC018 and IME167, which indicated that polyprotein resulting from incomplete T2A functioning was also formed. Unexpectedly, the Western signal intensities were more intense in strain IMC018 than in strain IME167, although the latter carried a high-copy-number plasmid.

3.4. Analysis of produced carotenoid in S. cerevisiae strains

After confirming the stability of the synthetic T2A polycistronic *crt* construct and the processing of the individual crtYb, crtI and crtE proteins, we quantified the carotenoids produced by the engineered strains IMC018 (centromeric polycistronic construct), IME167 (episomal polycistronic construct), and the non-carotenoid-producing reference strain CEN.PK113-7D. These strains were further compared to strains expressing the three *crt* genes individually controlled by the constitutive *TDH3* promoter either expressed from an episomal plasmid (strain YB/I/E, 2μ) or from a chromosomal integration (strain YB/I/E, int) (Verwaal et al., 2007). The five strains were grown in shake flask cultures on synthetic medium. After 24 h, the cultures were sampled to measure β-carotene and the intermediates of its biosynthesis, neurosporene, phytoene and lycopene. All cultures reached a similar biomass density (Table 2).

The strain YB/I/E-int produced nearly fourfold higher β-carotene than its episomal counter YB/I/E-2μ despite its lower gene copy number, consistent with a previous study (Verwaal et al., 2007). This was also reflected in the phytoene levels, which were 3.5-fold higher in the strain harbouring the chromosomal integration construct (Table 2). In contrast, we observed a difference between IME167 and IMC018 that would agree with the plasmid copy number. However this difference remained lower than twofold. Furthermore, the comparison of the strains expressing the *crt* genes from an episomal plasmid (IME167 with the 2A plasmid and YB/I/E-2μ with promoter repeats) showed no significant differences in the production of β-carotene and phytoene (Table 2). Conversely, the strain IME167 that harboured the 2A construct did not accumulate intermediates neurosporene and lycopene while such intermediates were observed in YB/I/E-2μ (Table 2). These results indicated that the *crt* the 2A construct was fully functional in *S. cerevisiae*.

3.5. De novo production of β-ionone in S. cerevisiae

Having established stable β-carotene production in *S. cerevisiae*, we attempted to couple this pathway to a product that has not previously been made in this yeast, the apocarotenoid β-ionone. To this end, we combined the 2A *crt* construct with an expression cassette containing the CCD1 gene from raspberry (*Rubus ideaus*), in which *RiCCD1* was placed under control of the *PMA1* promoter (Fig. 3). The transformation and *in vivo* recombination of the *S. cerevisiae* strain CEN.PK113-5D resulted in a mix of white and orange colonies. All tested white colonies exhibited incorrect plasmid assembly, meaning that the loss of colour was not related to full conversion of orange β-carotene into colourless β-ionone. In contrast, the tested orange colonies ($n = 4$) showed a correct assembly, indicated the simultaneous presence of genes for production and dioxygenation of carotenoids. Carotenoid production by the four RiCCD1 strains was compared to that of strain IMC018. To this end, the strains were grown in shake flasks containing a second phase (10%, v/v) of dodecane to trap any hydrophobic metabolites released from the yeast cultures. The second phase of dodecane did not impair growth of the *S. cerevisiae* strains. The RiCCD1 strains reached a lower density than the IMC018 after 24 h (Table 3). Analysis of carotenoids from the cell material showed that carotenoid production was reduced in the RiCCD1 strains. Phytoene production was reduced by 50%, and β-carotene production by 65% (Table 3).

To quantify β-ionone and other RiCCD1-mediated breakdown products of carotenoids, the dodecane layers of the IMC018 and the four RiCCD1 strain cultures were analysed by GC-MS. In *RiCCD1* expressing strains, β-ionone and geranylacetone could be observed (Fig. 6), to a level of 0.22 mg L^{-1}. Geranylacetone is expected to result from dioxygenase activity on phytoene (Fig. 1). Neither β-ionone nor geranylacetone were detectable in cultures of the IMC018 reference strain. Quantification showed that the reduced production of carotenoids by the *RiCCD1* expressing strains was mostly compensated by the produced apocarotenoids (Table 3).

4. Discussion

4.1. Polycistronic expression of a heterologous pathway in yeast

Synchronized and coordinate expression of genes can be crucial for efficient production of secondary metabolites. For example,

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Table 2
Carotenoid content of yeast cultures after 24 h of growth. The average values of three cultures and the standard deviation are shown.

Strain	OD600	Phytoene [mg g^{-1} cdw]	Neurosporene [mg g^{-1} cdw]	Lycopene [mg g^{-1} cdw]	β-Carotene [mg g^{-1} cdw]
CEN.PK (WT)	8.5 ± 0.3	0	0	0	0
IMC018 (2a centromeric)	7.9 ± 0.1	1.6 ± 0.02	0	0	0.35 ± 0.01
IME167 (2a episomal)	8.9 ± 0.3	2.5 ± 0.4	0	0	0.55 ± 0.01
YB/I/E (genomic)	7.9 ± 0.2	6.5 ± 2.0	0	0.03 ± 0.01	1.94 ± 0.66
YEplac195 (episomal)	8.1 ± 0.1	1.8 ± 0.03	0.27 ± 0.02	0.08 ± 0.02	0.55 ± 0.02

Carotenoid content of cultures after 24 h of growth.

Table 3
Carotenoid and apocarotenoid content of yeast cultures overlaid with dodecane. Cultures were grown for 24 h and 10% (v/v/) dodecane was included in the medium. Shown are the average values of three (IMC018) or four (RiCCD1) cultures and the standard deviation.

Strain	OD600	Phytoene [mg g^{-1} cdw)	β-Carotene [mg g^{-1} cdw]	Geranylacetone [mg g^{-1} cdw]	β-Ionone [mg g^{-1} cdw]
IMC018	8.4 ± 0.1	1.8 ± 0.2	0.51 ± 0.08	0	0
RiCCD1	5.3 ± 0.7	1.0 ± 0.2	0.19 ± 0.04	0.86 ± 0.03	0.22 ± 0.06

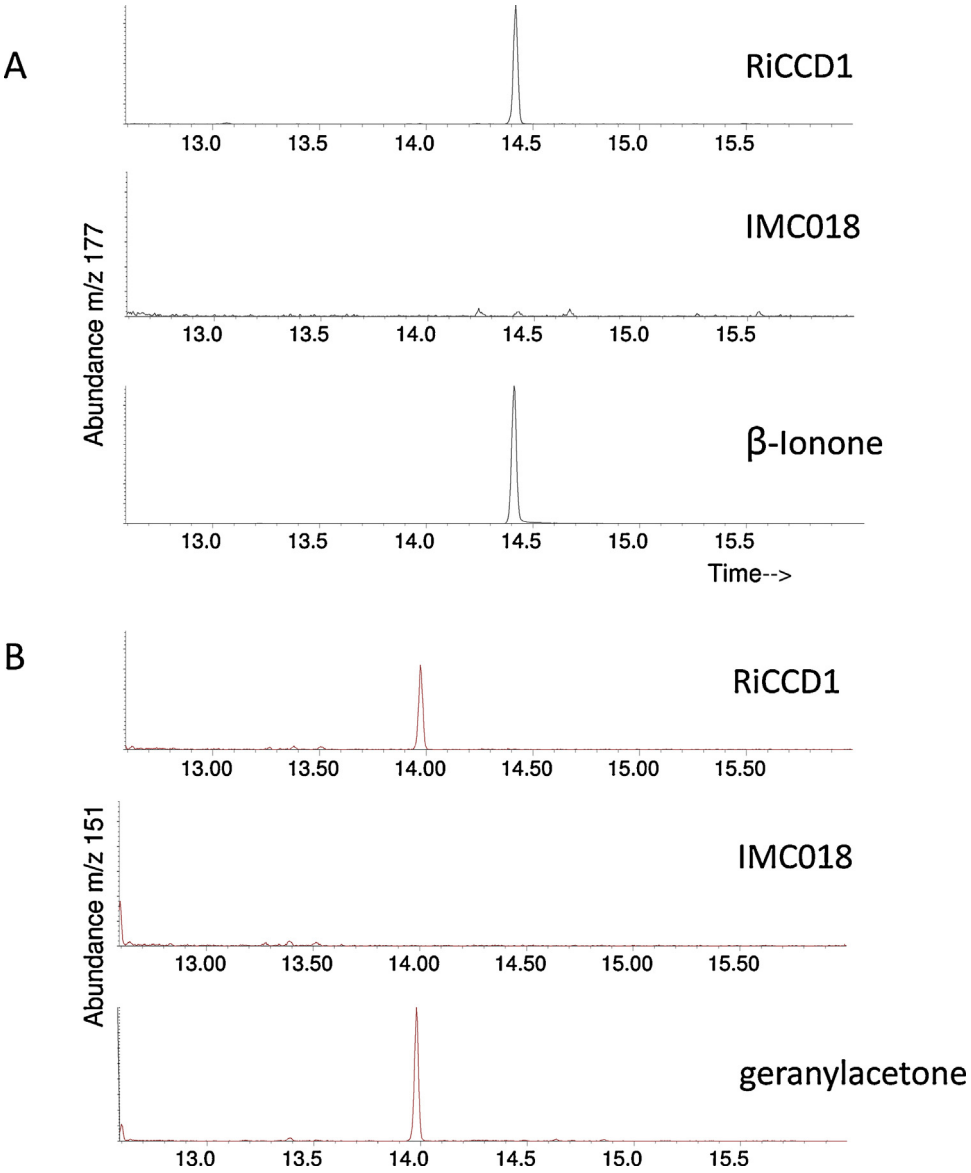

Fig. 6. GC MS analysis of the dodecane phase from cultures of the RiCCD1 (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE RiCCD1*) and IMC018 (*CEN6-ARS4 crtYB-T2A-crtI-T2A-crtE*) strains. Shown are representative chromatograms. (A) Detection of ion $m/z = 177$ representative of β-ionone in strains RiCCD and IMC018, and in a 5 μg mL^{-1} standard solution. (B) Detection of ion $m/z = 151$ representative of geranylacetone in strains RiCCD1 and IMC018, and in a 5 μg mL^{-1} standard solution.

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in plants, metabolic pathways leading to complex molecules such as flavonoids, glucosinolates and steroidal glycoalkaloids are encoded by 10–20 genes whose expression is tightly co-regulated (Beekwilder et al., 2008b; Itkin et al., 2013; Koopman et al., 2012). In such pathways, individual genes are transcribed from their own promoters, which are coordinated by transcription factors. When pathways for the synthesis of complex secondary plant metabolites are introduced in yeast, achieving synchronized expression may represent a challenge. Repeated use of identical promoters may lead to instability of constructs, since yeast maintains active homologous recombination machinery (Siddiqui et al., 2012). However, very few yeast promoters have been extensively characterized and most synthetic biology concepts repeatedly use a single highly expressed promoter, *e.g.* from the *GAL1* or *TDH3* gene (Paddon et al., 2013; Verwaal et al., 2007).

In the current study, an alternative approach for synchronized expression of heterologous pathway genes was explored. Only few previous studies report on the activity of 2A sequences which, in principle, allow the generation of multigene transcripts, in *S. cerevisiae* (de Felipe et al., 2003; Doronina et al., 2008; Ito-Harashima et al., 2007; Park et al., 2008). Most of these studies focused on the mechanism of ribosomal release of the nascent peptide. The efficiency of co-translational release for 2A sequences, as tested in reticulocyte lysates and wheat germ extracts, is known to vary from 18% to 99% (Donnelly et al., 2001). In this study we tested two 2A sequences that had not been previously tested in yeast: the T2A and the P2A sequences, which both show high ribosomal release efficiencies in higher eukaryotic systems. In *S. cerevisiae*, the P2A sequence did not allow a detectable level of ribosomal release, while T2A displayed a well detectable formation of release products, and hardly any detectable fusion protein (Fig. 3). The lack of activity of the P2A sequence, which is active in other eukaryotic expression systems (Donnelly et al., 2001), may relate to the interaction between the 2A peptide and species-specific sequences or conformations of the ribosome. Co-translational release is known to depend on pausing of the ribosome and on the concentration of release factors (Doronina et al., 2008). The 2A peptide is hypothesized to induce a conformation change in the ribosome, which delays incorporation of the last proline residue in the peptide chain, but instead promotes binding of release factors. Interestingly, all nine C-terminal amino acid residues are identical in P2A and T2A. It is known that residues upstream those nine residues may strongly affect the ribosomal release at 2A sequences (Donnelly et al., 2001). Possibly different interaction with the ribosome, which, in case of P2A in yeast, fails to induce the conformational change in the ribosome, but the effect of mutations on such conformational changes needs to be explored more fundamentally to explain the failure of P2A to act as a ribosomal release sequence in yeast.

An alternative system for expressing multiple proteins from a single promoter uses Internal Ribosomal Entry Sites (IRES), which allows *de novo* translation initiation for multiple genes in one transcript (Zhou et al., 2003). However, such IRES technologies have not been extensively deployed in metabolic engineering, likely since they result in substantially reduced expression levels relative to normal cap-dependent translation mechanisms (de Felipe, 2002; Siddiqui et al., 2012). A more successful way to achieve coordinated, high expression of a set of genes seems to be by the use of a set of promoters inducible by a single gene switch (Liang et al., 2013).

The combination of the 2A system with the *in vivo* recombination approach should allow a rapid and powerful method to generate multiple constructs for expressing pathway modules in yeast. Many applications may be found in the production of secondary metabolites, such as shown here for (apo-)carotenoids, but which may also be expanded to *e.g.* flavonoids, glucosinolates and sesquiterpenoids (Koopman et al., 2012; Mikkelsen et al., 2012; Paddon et al., 2013).

4.2. Apocarotenoid production in yeast

The *de novo* production of apocarotenoids in yeast has so far not been reported in the scientific literature. This study provides a first proof of concept that these compounds can be produced in *S. cerevisiae*. There are clear opportunities to build on these experiments and expand the repertoire of yeast-produced aroma compounds, since many apocarotenoids exist which have important flavour and/or fragrance characteristics. Well known examples include safranal, α-ionone, and β-damascenone, which are important for the aroma of fruits such as grapes, passion fruit, quince and starfruit, but also spices such as saffron and red pepper and fermented products such as wine, tea and tobacco (Winterhalter and Rouseff, 2002). These apocarotenoids often require a dioxygenase that cleaves at a different position in the carotenoid, and are derived from modified carotenoids other than β-carotene. Thus, different, and possibly more genes may need to be incorporated for diversification of yeast-produced apocarotenoids. Clearly the production of β-ionone in yeast as reported here is a valuable proof of concept which has a great potential for expanding towards other interesting aroma compounds.

In addition to potential for diversification, there are still opportunities to increase the production levels of β-ionone in *S. cerevisiae*. For example, the efficiency of conversion of the carotenoid may be enhanced. Strains expressing the *RiCCD1* still accumulate β-carotene, albeit at lower levels than in an isogenic reference strain that did not express *RiCCD1* (Table 3). Increased expression of RiCCD1 seems a logical strategy to achieve a more complete conversion of β-carotene. The *PMA1* promoter, which was used here, normally drives expression of the major plasma membrane H^+-ATPase, which is an abundant protein that functions in controlling intracellular pH. Expression of *PMA1* is generally high, but is not optimal in cultures growing on high concentrations of glucose, in particular during the later stages of the growth (Fernandes and Sa-Correia, 2003). Possibly, transcription of *RiCCD1* driven by alternative promoters or expression of carotenoid cleavage dioxygenase genes from other organisms might increase the yield of β-ionone. A second improvement could be in the increased production of isoprenoid precursors. In *S. cerevisiae*, carotenoids are produced from the isoprenoid precursor farnesyl diphosphate FPP (Verwaal et al., 2007). Extensive and successful optimizations of the biosynthetic pathway towards FPP in this yeast have been described and applied to enhance production of the antimalarial compound artemisinin (Paddon et al., 2013). Similar approaches could be used to enhance carotenoid synthesis (Lange and Steinbuchel, 2011). Thirdly, optimization of FPP conversion towards GGPP may form a bottleneck in carotenoid formation. Optimization of crtE, which limits the entry of carbon into the carotenoid biosynthetic pathway (Fig. 1), may improve carotenoid production (Verwaal et al., 2007). In the construct introduced in strain IMC018, *CrtE* is the last of the three cistrons. It has been reported that efficiency of translation decreases after every 2A sequence (de Felipe et al., 2006), which could affect the expression of crtE protein. Limitations in crtE protein expression, and consequently in GGPP production in IMC018 could explain a lower carotenoid yield relative to three promoter strains such as crtYB/I/E.

Notably, the ratio between phytoene and carotene as reported in Table 2 is similar for the 3-promoter YB/I/E strains and IMC018. This indicates that the *crtI* activity, which should take phytoene as a substrate, is in both strains limiting the completion of the pathway to β-carotene. Improvements in the β-carotene to phytoene ratios can be achieved by overexpressing additional copies of *crtI* (Verwaal et al., 2007).

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Clearly, tuning of production of crtYB, crtI and crtE proteins will help to achieve an optimal functioning pathway. One way to achieve this could be to altering the order of the reading frames in the 2A constructs and testing 2A sequences with different activities, and in this way try to optimize the ratio between the produced proteins. This, together with improved precursor supply and expression of the CCD1 gene in yeast, should enhance β-ionone production to economic levels.

5. Conclusion

For the first time, a yeast strain was constructed which produces β-ionone from glucose. This was achieved by combining genes from raspberry and a carotenoid producing *X. dendrorhous* in *S. cerevisiae*, leading to production of an industrially relevant fruit flavour compound.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.12.016.

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