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Canthaxanthin production with modified *Mucor circinelloides* strains

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Abstract Canthaxanthin is a natural diketo derivative of β-carotene primarily used by the food and feed industries. *Mucor circinelloides* is a β-carotene-accumulating zygomycete fungus and one of the model organisms to study the carotenoid biosynthesis in fungi. In this study, the β-carotene ketolase gene (*crtW*) of the marine bacterium *Paracoccus* sp. N81106 fused with fungal promoter and terminator regions was integrated into the *M. circinelloides* genome to construct stable canthaxanthin-producing strains. Different transformation methods including polyethylene glycol-mediated transformation with linear DNA fragments, restriction enzyme-mediated integration and *Agrobacterium tumefaciens*-mediated transformation were tested to integrate the *crtW* gene into the *Mucor* genome. Mitotic stability, site of integration and copy number of the transferred genes were analysed in the transformants, and several stable strains containing the *crtW* gene in high copy number were isolated. Carotenoid composition of selected transformants and effect of culturing conditions, such as temperature, carbon sources and application of certain additives in the culturing media, on their carotenoid content were analysed. Canthaxanthin-producing transformants were able to survive at higher growth temperature than the untransformed strain, maybe due to the effect of canthaxanthin on the membrane fluidity and integrity. With the application of glucose, trehalose, dihydroxyacetone and L-aspartic acid as sole carbon sources in minimal medium, the *crtW*-expressing *M. circinelloides* strain, MS12+pCA8lf/1, produced more than 200 μg/g (dry mass) of canthaxanthin.

Keywords β-carotene ketolase · Canthaxanthin · Echinenone · Heterologous gene expression · *Paracoccus* sp. N81106 · Filamentous fungi

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Introduction

Canthaxanthin is a dark red, natural diketo derivative of β-carotene (β,β-carotene-4,4′dione; Fig. 1). It is used as food colorant and feed additive, primarily in aquacultures and poultry industries (Bhosale and Bernstein 2005; Dufossé 2006). This carotenoid has stronger antioxidant activity than β-carotene (Palozza and Krinsky 1992), and several beneficial effects have been attributed to it. Canthaxanthin induced apoptosis in human cancer cell lines (Kumaresan et al. 2008; Palozza et al. 1998), reduced the risk of several

types of cancer in animal models (Mayne and Parker 1989) and effectively stimulated immune defences compared to other carotenoid species (Jyonouchi et al. 1996; Okai and Higashi-Okai 1996). Although several bacteria, such as *Corynebacterium michiganense* (Saperstein and Star 1954), *Micrococcus roseus* (Cooney et al. 1966), *Brevibacterium* sp. strain KY 4313 (Nelis and De Leenheer 1989) and *Gordonia jacobaea* MV-1 (De Miguel et al. 2001; Veiga-Crespo et al. 2005), and some microalgae, such as *Chlorella pyrenoidosa* (Czygan 1964) and *Chlorella zofingiensis* (Pelah et al. 2004), have been reported as canthaxanthin producers, compounds for the food and feed industries are primarily produced by chemical synthesis (Ernst 2002). The canthaxanthin market is growing but hampered by the lack of appropriate microbial sources (Bhosale and Bernstein 2005).

Mucor circinelloides is a β-carotene-producing filamentous fungus. Besides *Phycomyces blakesleeanus* and *Blakeslea trispora*, this species has been involved in the study of the molecular background of the carotene biosynthesis in zygomycetes. Consequently, the biosynthetic process is well resolved, and several structural and regulatory genes participating in or related with the pigment production have been isolated and characterized (Navarro et al. 2001; Papp et al. 2006; Velayos et al. 2000a, b; 2003). Although the main carotenoid product in *M. circinelloides* is β-carotene, it also has a poor β-carotene hydroxylase activity; thus, the fungus is able to produce β-cryptoxanthin and zeaxanthin in small amounts (Álvarez et al. 2006; Papp et al. 2006). We previously reported the heterologous expression of a β-carotene ketolase (*crtW*) from the marine bacterium, *Paracoccus* sp. N81106 (formerly *Agrobacterium aurantiacum*) in *M. circinelloides* (Csernetics et al. 2011; Papp et al. 2006). In those studies, *crtW* was introduced into the fungus in autonomously replicating plasmids, and the resulting transformants were able to produce astaxanthin and canthaxanthin. However, the copy number of the plasmids (0.07–1 per host genome) and the amount of the new carotenoid products remained low. Therefore, the aim of the present study was the construction of *M. circinelloides* strains, which harbour the bacterial *crtW* gene integrated in the genome and able to effectively express it to produce canthaxanthin in significant amount.

It is fairly difficult to obtain stable transformants in zygomycetes. The transferred DNA rarely integrates into the genome but often form autonomously replicating, occasionally rearranged and/or concatenated structures (Michielse et al. 2004; Ibrahim and Skory 2007; Papp et al. 2010). Such transformants generally display mitotic instability losing their extrachromosomal elements under nonselective conditions. Integration can be forced by transformation with linear fragments holding extensive homologous regions at their termini to direct homologous recombination and gene replacement. For this purpose, polyethylene glycol (PEG)-mediated transformation is traditionally used (Navarro et al. 2001; Silva et al. 2006), but the integration frequency is generally low in this system. *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been established for some zygomycetes. However, stability of the transformants remained problematic in case of *Mucor* (Monfort et al. 2003; Nyilasi et al. 2005). Modification of the integrated DNA resulting in rearrangements, excision and recircularization may also occur in the integrative transformation systems of zygomycetes (Michielse et al. 2004; Ibrahim and Skory 2007).

In this study, different methods, such as transformation with linear DNA fragments, restriction enzyme-mediated integration (REMI) (Maier and Schäfer 1999) and ATMT, were tested to integrate the bacterial gene into the *Mucor* genome. Carotenoid composition and effect of some

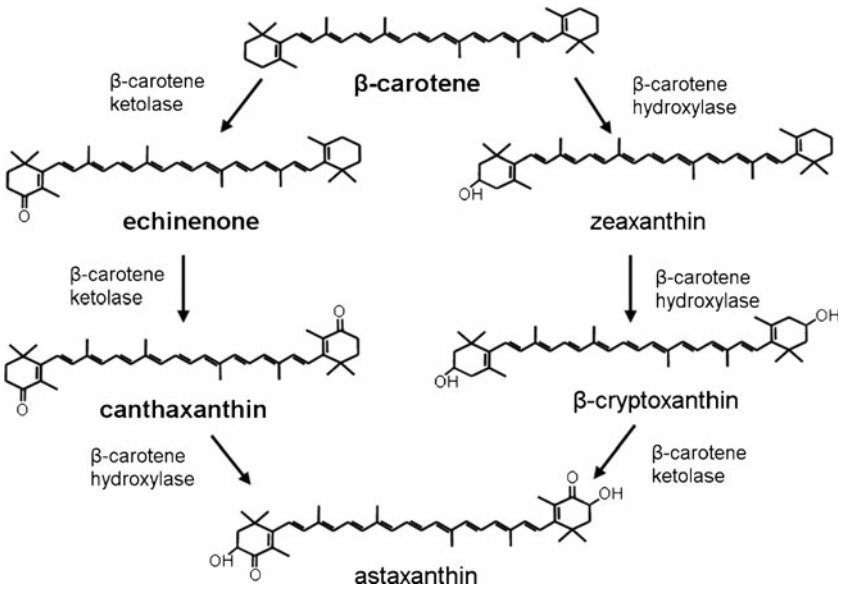

Fig. 1 Conversion of β-carotene to its keto derivatives and astaxanthin and the main intermediate compounds of the process

culturing conditions, such as temperature, carbon sources and application of certain additives, on the carotenoid content were analysed in the resulting transformants.

Materials and methods

Strains, media and growth conditions

MS12, a *leuA*$^-$ and *pyrG*$^-$ mutant (Benito et al. 1992) derived from the wild-type *M. circinelloides* strain CBS277.49, was used in the transformation experiments; this strain is auxotrophic for leucine and uracil but wild type for the carotene biosynthesis. The *A. tumefaciens* strain GV3101 containing the pMP90 helper plasmid was used in the ATMT experiments; this strain harbours rifampicin and gentamicin resistance markers in the bacterial genome and the helper plasmid, respectively. GV3101 was grown on LB medium (Sambrook et al. 1989) containing 25 $\mu g mL^{-1}$ gentamicin and 100 $\mu g mL^{-1}$ rifampicin at 28 °C. Induction medium (IM) for ATMT was prepared as described by Bundock and Hooykaas (1996). *Escherichia coli* strain DH5α was used in all cloning experiments and plasmid amplifications; it was cultivated on LB medium containing 50 $\mu g mL^{-1}$ ampicillin at 37 °C. For both nucleic acid and carotenoid extraction, *M. circinelloides* strains were cultured on solid minimal medium (YNB, 10 g glucose, 0.5 g yeast nitrogen base without amino acids, 1.5 g $(NH_4)_2SO_4$, 1.5 g sodium glutamate and 20 g agar/L) supplemented with leucine or uracil (0.5 $mg mL^{-1}$) as required. To test the mitotic stability of the transformants, malt extract agar (MEA, 10 g glucose, 5 g yeast extract, 10 g malt extract and 20 g agar/L) was used as a complete medium. Fungal cultures were grown for 4 days under continuous light at 25 °C. Temperature dependence of the carotenoid production was tested cultivating the fungal strains on YNB at 20, 25, 30 35 and 37 °C. To examine the effect of different carbon sources on the carotenoid production, glucose was replaced with mannose, trehalose, fructose, galactose, cellobiose, maltose, ethanol, glycerol, glycerol-L-monoacetate, dihydroxyacetone and L-aspartic acid in a final concentration of 1 % in YNB. When the effect of some chemical additives was analysed, 20 mM H_2O_2, 1 % palm oil or 1 mM $FeSO_4$, $CuSO_4$ or $CoCl_2$ were added to YNB containing 1 % glucose.

Molecular techniques

General procedures for plasmid DNA preparation, cloning, transformation of *E. coli* and Southern blotting were performed by following standard methods (Sambrook et al. 1989). Genomic DNA was prepared from mycelia disrupted with a pestle and mortar in liquid nitrogen. DNA was isolated using a method described earlier (Iturriaga et al. 1992) or with the DNeasy Plant Mini Kit (Qiagen). DNA sequencing was performed by LGC Genomics (Berlin, Germany). For Southern hybridizations, probes were labelled with the digoxigenin-based PCR DIG Probe Synthesis Kit (Roche), and the DIG Nucleic Acid Detection Kit (Roche) was used for immunological detection of the nucleic acid blots, following the instructions of the manufacturer.

Construction of plasmids

Plasmids used in the present study are summarized in Table 1. In all constructions, either the *leuA* gene encoding the α-isopropylmalate isomerase or the *pyrG* gene encoding the orotidine-5′-monophosphate decarboxylase was used as a selection marker; these genes complement the leucine and the uracil auxotrophy of the MS12 strain, respectively. The *crtW* cassette containing the β-carotene ketolase gene (*crtW*) of *Paracoccus* sp. N81106 fused with the promoter (*gpd1*P) and terminator (*gpd1*T) regions of the glyceraldehyde-3-phosphate dehydrogenase 1 gene (*gpd1*) of *M. circinelloides* was derived from the pPT51 plasmid (Papp et al. 2006). Map of the plasmids constructed in this study is presented in Online Resource 1.

The plasmid pCA8 was constructed by placing the *crtW* cassette between the *leuA* gene and its 5′ adjacent region, as follows: *leuA* cut from the pAVB107 plasmid (Velayos 2000) with *Xba*I and *Pst*I enzymes was placed between the corresponding sites of pUC18 (Fermentas) giving rise pCA7; then, the 4.01-kb *Sma*I-*Sca*I fragment of pCA7 and the 8.67-kb *Nae*I-*Sca*I fragment derived from pPT51 were ligated to form pCA8.

To construct pCA9, the *crtW* casette and *pyrG* were built between the fragments of the 18S and 28S ribosomal RNA genes (rDNA) of *M. circinelloides*. The 18S and 28S rDNA fragments were amplified from the genomic DNA of *M. circinelloides* using the RIB1–RIB2 and RIB3–RIB4 primer pairs (Table 2), respectively, and they were cloned into pUC18 (Fermentas) giving rise the p18S and p28S plasmids, respectively. These plasmids were digested with the enzymes *Sal*I and *Bam*HI, and the 1.41-kb fragment of p28S was ligated with the 4.5-kb fragment of p18S resulting in p18S-28S. At the same time, pCA2 was constructed by ligating the 5.01- and 1.32-kb fragments obtained by the *Xba*I and *Pst*I digestion of pPT51 and pEPM901 (Benito et al. 1992), respectively. Finally, p18S-28S and pCA2 were digested with *Cla*I and *Kpn*I, and the arising 5.65-kb fragment of p18S-28S and the 3.45-kb fragment of pCA2 were used to create pCA9. The pCA15 plasmid used in the ATMT experiments was derived from the pPK2 *Agrobacterium* binary vector (Covert et al. 2001) by the replacement of

Table 1 Plasmids used in the study

Plasmid	Genotype or description[a]	Source or reference
pUC18	General cloning vector for *E. coli* (Amp)	Fermentas
pAVB107	*leuA* gene of *M. circinelloides* in pBluescript II SK (Amp)	Velayos 2000
pEPM901	*pyrG* gene of *M. circinelloides* (Amp)	Benito et al. 1992
pPT51	Expression cassette *gpd1*P *crtW gpd1*T (Amp; *leuA*)	Papp et al. 2006
pPK2	Expression cassette *Aspergillus nidulans gpd*P *hph A nidulans trpC*T (Kan)	Covert et al. 2001
p18S	A 1,826-kb fragment of the 18S rRNA gene in pUC18	This study
p28S	A 1,497-kb fragment of the 28S rRNA gene in pUC18	This study
p18S-28S	The 1,826- and the 1,405-kb fragments of the 18S and 28S rRNA genes, respectively, in pUC18	This study
pCA2	Expression cassette *gpd1*P *crtW gpd1*T (Amp; *pyrG*)	This study
pCA7	*leuA* gene of *M circinelloides* in pUC18 (Amp; *leuA*)	This study
pCA8	Expression cassette *gpd1*P *crtW gpd1*T bordered by the *leuA* gene and it's 5′ adjacent region (Amp; *leuA*)	This study
pCA9	Expression cassette *gpd1*P *crtW gpd1*T bordered by the fragments of the 18S and 28S rRNA genes (*Amp*; *pyrG*)	This study
pCA15	Expression cassette *gpd1*P *crtW gpd1*T in pPK2 (*Kan*; *pyrG*)	This study

Amp ampicillin resistance, *hph* hygromycin B resistance gene of *E. coli*, *Kan* kanamycin resistance

[a] Selection markers (e.g. genes for antibiotic resistance and genes complementing the auxotrophy of the recipient strain) are shown in parentheses

the original *hph* cassette with the modified *crtW* and the *pyrG* genes (Papp et al. 2012).

Transformation

Protoplasts of *M. circinelloides* were prepared as described earlier (Papp et al. 2006). In all transformation experiments, transformants were selected on the basis of auxotrophy complementation and colour change cultivating them on YNB, supplemented with leucine or uracil if required. Transformation with linear fragments and REMI was carried out via PEG-mediated transformation of protoplasts, which was performed according to van Heeswijck and Roncero (1984). Map of DNA fragments and molecules for transformation are shown in Online Resource 1 and 2. To force gene replacement, the linear DNA molecules pCA8lf and pCA9lf obtained by the *Cla*I-*Aat*II and *Aat*II-*Nhe*I digestion of pCA8 and pCA9, respectively, were used to transform MS12. For REMI, the *Sal*I-*Sac*I fragment of pCA8 (pCA8′R), the *Cla*I-*Kpn*I and *Pst*I-*Kpn*I fragments of pCA9 (pCA9′R1 and pCA9′R2, respectively) and the *Nae*I-*Nde*I, *Nae*I-*Cla*I and *Sal*I-*Sca*I fragments of pPT51 (pPT51′R1, pPT51′R2 and pPT51′R3, respectively) were introduced into the recipient MS12 strain. REMI experiments were performed according to the PEG-mediated method except that the restriction enzymes used previously to digest the plasmid were added to the protoplasts together with the linear DNA, and the solution was incubated for 0.5 h on ice and 1.5 h at 33 °C.

For ATMT, pCA15 was introduced into the *A. tumefaciens* strain GV3101 by electroporation at 2.5 kV and 25 μF; LB medium supplemented with 25 $\mu g mL^{-1}$ gentamicin, 100 $\mu g mL^{-1}$ rifampicin and 40 $\mu g mL^{-1}$ kanamycin was used to select the cells that contain pPCA15 and pMP90. ATMT was performed according to Papp et al. (2012). Co-cultivation of *A. tumefaciens* cells and *M. circinelloides* protoplasts was performed on cellophane sheets placed on the surface of IM supplemented with 0.8 M sorbitol and 200 μM acetosyringone at 28 °C for 3 days. Following the incubation, the cellophane sheets were transferred to selection medium (YNB) and incubated at room temperature for 4–10 days. Putative transformants were then transferred to fresh YNB plates to obtain monosporangial (i.e. colonies from single spores) cultures.

Molecular analysis of the transformants

Real-time quantitative PCR (qPCR) was used to determine the copy number of the transferred gene in the total DNA samples. The qPCR experiments were performed in iQ5 and CFX96 real-time PCR detection systems (Bio-Rad), using the iQ™ SYBR Green Supermix (Bio-Rad). The primers used in the qPCR amplifications are shown in Table 2. The amplification conditions were as follows: an initial denaturing step of 3 min at 95 °C was followed by 40 cycles of 15-s denaturation (95 °C) and 30-s annealing and extension (69 °C). The relative quantification of the copy number was performed using the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001). Copy number of the *crtW* was referred to those of the *carG* and *isoA* genes in a given DNA sample; the latter genes exist in one copy in the *M. circinelloides* genome (Csernetics et al. 2011).

Table 2 Primers used in the study

Primer	Sequence 5′-3′	Amplified DNA or analysed transformant
RIB1	CTCGAGCTCCTGGTTGATCCTGCTTGTAGTCA	18S rRNA gene
RIB2	CTCGGTACCAATGATCCTTCCGCAGGTTCACCT	
RIB3	CTCGGATCCTAATCAATAATTTTGGCTTGTCCA	28S rRNA gene
RIB4	AGAGTTTCCTCTGGCTTCACCCTATTCAGG	
crtW1	ATGAGCGCACATGCCCTGC	crtW
crtW2	TCATGCGGTGTCCCCCTTGGTG	
Primers used in the qPCR experiments		
crtWreal-time1	CTTCATCGGCACCTATTTCGGCT	crtW
crtWreal-time2	CGAACACGAACAGCTGGATCGAC	
isoAreal-time1	ATCTCGACTGTTACGGTGCTCCT	isoA
isoAreal-time2	CTTGCGTTGTTCGGGATTAGCCA	
carGreal-time1	CAACATCATCAGCCAGAAGCCCA	carG
carGreal-time2	ACCACCCAAACGCTTGATTTCCT	
Primers used in the IPCR experiments		
leuAv8i1	CTGGTCATGAAGTGCCCTTTGAGGT	MS12+pCA8lf
leuAe8i2	CTCGCTCGTCTACTAGCAGGTTGT	
gpdPe8i3	GGCTGCGCGTGGTTCACACTAAT	
arsMe8i4	GTTTGGCTGTGCTCATCGCACT	
18S9i1	GTAATTTGCACGCCTGCTGCCT	MS12+pCA9lf
28S9i2	CCGTCTTGAAACACGGACCAAGGA	
pPT51i1	CAACAGCGGTAAGATCCTTGAGAGT	MS12+pPT51′R
pPT51i2	GGGCTAGACTGCTCTCCAAATGCA	
pCA8remiI2	CACTTTATGCTTCCGGCTCGTATGT	MS12+pCA8′R
gpdTv8remiI1	GCTGTTACTGTTACTATCGCCCT	
Primers used in the plasmid rescue experiments		
amprev1	GGCGACACGGAAATGTTGAATAC	-
amprev2	CGAAATAGACAGATCGCTGAG	

The inverse PCR (IPCR) method (Ochman et al. 1988) was used to analyse the integration site of the transferred DNA. About 1 μg genomic DNA of the tested transformants was digested with one of the restriction enzymes *Sal*I, *Sca*I, *BstX*I, *Cla*I, *Sma*I or *Nhe*I overnight and precipitated with two volumes of ethanol at −20 °C. Then, the samples were washed with 70 % ethanol, dried under vacuum and resuspended in 10 μl distilled water. The digested samples were ligated at 8 °C overnight and incubated at 65 °C for 5 min to inactivate the ligase. The ligation samples were precipitated and washed again and dissolved in 20 μl distilled water. These samples (20–50 ng) served as templates in the IPCR experiments using different combinations of the primers shown in Table 2. Amplifications were performed with the *Pfu* polymerase (ZenonBio), and the PCR products were cloned using the CloneJET PCR Cloning Kit (Thermo Scientific) and sequenced. To determine the sites of integration, the resulting sequences were tested in BLAST searches against the *M. circinelloides* genome database (DoE Joint Genome Institute; *M. circinelloides* CBS277.49 v2.0; http://genome.jgi-psf.org/Mucci2/Mucci2.home.html). The IPCR strategy is shown in Online Resource 2.

Plasmid rescue experiments were performed as follows. *E. coli* DH5α cells were transformed with 3 μg genomic DNA of the MS12+pCA8′R transformants. The bacterium transformation protocol followed general procedures (Sambrook et al. 1989). As pCA8′R fragments contain the appropriate bacterial gene, bacterial transformants were selected on the basis of their resistance to ampicillin. The plasmids were purified from the transformants using the Mini Plus Ultrapure Plasmid DNA kit (Viogene) and then sequenced with the primers designed to the ampicillin resistance gene (Table 2). Location of these primers on the pCA8′R fragment is shown in Online Resource 2.

Carotenoid extraction and analysis

Carotenoid extraction was performed as described earlier (Papp et al. 2006). For high-performance liquid chromatography (HPLC), samples were analysed by using a modular

Shimadzu low-pressure gradient HPLC system. The dried samples were re-dissolved in 100 μL tetrahydrofuran supplemented with butylated hydroxytoluene (100 $\mu g mL^{-1}$), and 2 μL was subjected to HPLC analysis on a Prodigy ODS-3 (4.6×150, ODS 3 μm) column (Phenomenex). The separation was performed with a gradient (min/volume of solvent A%/volume of solvent B% was 0/99/1; 8/60/40; 13/46/54; 15/0/100; 18/0/100; 21/99/1; and 25/99/1), 4 % water-96 % methanol being used as solvent A and 100 % methyl-*tert*-butyl ether as solvent B, at a flow rate of 1 mL min^{-1}. The detection wavelength was 450 nm. The following standards were used to identify the carotenoids: astaxanthin, lycopene and β-carotene (Sigma), β-cryptoxanthin, zeaxanthin and canthaxanthin (Carl Roth) and echinenone (DHI Water and Environment); γ-carotene was purified by HPLC from *Mucor azygosporus*.

Results

Transformation, transformation frequency and mitotic stability

Transformation with linear fragments and REMI was performed via PEG-mediated protoplast transformation. All these experiments resulted in transformant colonies except REMI using the two pCA9′R fragments; the transformation frequencies were relatively low, one to four colonies per 10^5 protoplasts using 10–15 μg linear DNA. During the optimization of the transformation protocol, application of the DNA for transformation in higher amounts did not lead to further increase in the number of the transformants. In case of the REMI, application of 30 U of the restriction enzymes and incubation of transformation mixture containing the protoplasts, the DNA fragment and the restriction enzymes for 30 min on ice and subsequently for 1.5 h at 33 °C proved to be suitable.

ATMT experiments had a similar transformation frequency resulting in one to eight transformant colonies per experiment. All stable transformants obtained in this study were deposited in the culture collection of the University of Szeged (Szeged Microbiology Collection, Szeged, Hungary). After the second cultivation, the presence of the *crtW* gene could be detected clearly in the transformants by PCR (data not shown). Southern hybridization analysis verified the integration in the majority of the transformants (Fig. 2).

To analyse their mitotic stability, transformants were passed several times onto selective and non-selective media. Strains transformed with the linear fragments, pCA8lf or pCA9lf, were stable after more than 15 consecutive cultivation steps under non-selective conditions (i.e. on MEA plates). Out of the REMI constructed strains, the strains

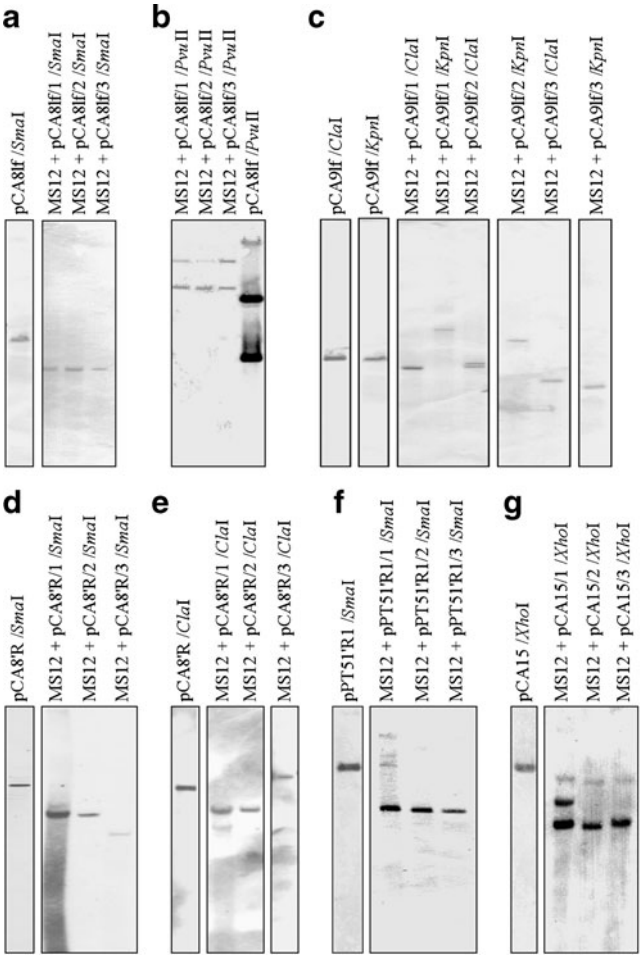

Fig. 2 Examples of Southern analyses performed to prove the presence of the *crtW* gene and its integration into the genome of the *M. circinelloides* transformants using the digoxigenin-labelled *crtW* gene as a hybridization probe. Panels: the fragment pCA8lf and the DNAs of three MS12+pCA8lf strains digested with *Sma*I (**a**) and *Pvu*II (**b**), the fragment pCA9lf and the DNAs of three MS12+pCA9lf strains digested with *Cla*I and *Kpn*I (**c**), the fragment pCA8′R and the DNAs of three MS12+pCA8′R strains digested with *Sma*I (**d**) and *Cla*I (**e**), the fragment pPT51′R and the DNAs of three MS12+pPT51′R strains digested with *Sma*I (**f**) and the plasmid pCA15 and the DNAs of three MS12+pCA15 strains digested with *Xho*I (**g**)

transformed with pCA8′R and pPT51′R3 were found to be stable. ATMT transformants proved to be unstable losing the transferred DNA during the first three cultivation cycles.

Molecular analysis of the mitotically stable transformants

The site of integration was analysed only in the stable transformants by using the IPCR technique. In all tested MS12 +pCA8lf and MS12+pCA8′R transformants, integration occurred by gene replacement in the homologous site and was directed by the *leuA* gene and its 5 adjacent region. IPCR analysis detected ectopic integration in the tested MS12 +pCA9lf and MS12+pPT51′R3 transformants in different

genomic regions (the genomic regions revealed by the IPCR experiments are presented in Online Resource 3). In several REMI transformants, IPCR analysis suggested multiple integrations and/or rearrangements in the transferred DNA and the adjacent host regions. Sequences of the fragments amplified by PCR from the genomic DNA of these transformants using the inverse primers also suggested the existence of such multicopy structures.

Copy number of the *crtW* gene in the transformants was analysed by using real-time qPCR (Table 3). *Mucor* species generally produce multinucleate protoplasts and spores and form coenocytic mycelia. As a consequence, primary integrative transformants are heterokaryotic to the transferred DNA. In order to obtain homokaryotic strains, it is necessary to isolate monosporangial colonies and to perform some consecutive cultivation cycles on the selection medium with them. Copy number increased in all tested transformants in the consecutive generations. In several transformants, such as in MS12+pCA8lf/1-2, MS12+pCA9lf/1 and MS12+pPT51′R3/2, the copy number detected after the 13th cultivation cycle was very high (Table 3). In the cases of the MS12+pCA8lf/1 and MS12+pCA9lf/1, IPCR also suggested the presence of multiple copies of the introduced DNA. In line with the increasing copy number, the primary yellowish colony colour of these transformants changed to orange-red indicating the accumulation of keto-carotenoids in consequence of the expression of the transferred *crtW* gene (see Online Resource 4). In some transformants, the copy number of the *crtW* gene did not reached one copy per genome even after more than ten generations showing that these strains remained heterokaryotic to the transferred DNA (Table 3).

To reveal the potential existence of autonomously replicating plasmids came from the excision and recircularization of the integrated DNA fragments, plasmid rescue experiments were performed with the MS12+pCA8′R transformants. Circular plasmids with sizes significantly lower than the originally transferred DNA (2,000–5,500 bp) could be recovered. Sequencing of these plasmids revealed rearranged and truncated fragments of the transferred DNA, but most of them did not contain the *crtW* gene or its parts.

Carotenoid production of the transformants

Expression of the bacterial *crtW* gene led to the production of keto derivatives of β-carotene, mainly canthaxanthin and echinenone and very small amounts of astaxanthin in the transformants. Detailed carotenoid composition of some transformants is shown in Table 3. During the first three cultivation cycles, concentration of the keto carotenoids was low, except in some MS12+pPT51′R3 transformants, which had red-orange colour directly after the transformation. After several cultivation cycles (>10), the amount of canthaxanthin and echinenone increased significantly in most of the transformants corresponding to the elevated copy number of the *crtW* gene. At the same time, the REMI constructed MS12+pCA8′R transformants produced only small amounts of the keto derivatives independently from the number of the cultivation cycles. Out of the good canthaxanthin-producing strains, MS12+pCA8lf/1 and MS12+pCA9lf/1 were selected to examine the effect of the culturing temperature, the carbon source and certain chemical additives on the carotenoid content and composition.

Effect of the culturing temperature on the carotenoid production

Carotenoid content of the two selected transformants in comparison with that of the MS12 strain was examined after cultivating the fungi on YNB at 20, 25, 30, 35 and 37 °C. Figure 3 shows the average β-carotene and canthaxanthin content of the three strains measured at the different temperatures. The canthaxanthin and echinenone production was the highest at 20 °C and decreased at the higher cultivation temperatures. At the same time, the highest total carotenoid and β-carotene levels were detected at 35 °C. Interestingly, transformants produced significantly higher amounts of β-carotene at this temperature than MS12. The maximum growth temperature of *M. circinelloides* is 36 °C. In accordance with this, the original MS12 strain did not grow at 37 °C. However, the tested transformants were able to survive and produce carotenoids at this temperature.

Effect of different carbon sources and chemical additives on the carotenoid production

This experiment was performed with strains passed more than 20 times after the transformation. MS12, MS12+pCA8lf/1 and MS12+pCA9lf/1 were cultured on solid YNB, where glucose was replaced to different compounds as the sole carbon sources (Table 4). Some chemical additives given to the glucose containing minimal medium were also tested. Canthaxanthin content of the two transformants significantly differed on glucose: MS12+pCA8lf/1 contained 33 % canthaxanthin and 7 % β-carotene compared to the total carotenoid content; in contrast, these proportions were 13 and 36 %, respectively, in the case of MS12+pCA9lf/1. Other carbon sources and the tested additives also exerted different effects on their carotenoid composition. Although application of fructose, trehalose and mannose enhanced the total carotenoid level in both transformants, the canthaxanthin–β-carotene ratio of the strains changed only on fructose. L-aspartic acid significantly stimulated the canthaxanthin production of the MS12+pCA8lf/1 strain, while glycerine had a similarly positive effect on that of MS12+pCA9lf/1. Dihydroxyacetone increased the canthaxanthin content in both transformants. Although the other tested carbon sources and additives did

Table 3 Copy number of the transferred gene and carotenoid composition of the different *crtW*-expressing transformants after different cultivation cycles

Strain	Cultivation step	Copy number	Lycopene	β-Carotene	γ-Carotene	β-Cryptoxanthin	Zeaxanthin	Echinenone	Canthaxanthin	Astaxanthin	Total carotenoid
MS12	–	–	12±2 (3)	249±23 (61)	22±4 (5)	26±7 (6)	8±1 (2)	–	–	–	412±31
MS12+pCA8lf/1	3	3	9±1 (2)	296±12 (49)	29±4 (5)	18±3 (3)	8±2 (1)	31±13 (5)	16±7 (3)	1±0.8 (0.2)	604±41
	13	250	12±2 (2)	43±5 (8)	32±8 (6)	26±4 (5)	4±1 (1)	**117**±5 (22)	**148**±21 (28)	3±1 (0.6)	529±29
MS12+pCA8lf/2	3	12	10±1 (1)	295±31 (43)	38±7 (6)	14±3 (2)	6±2 (1)	60±8 (9)	65±9 (9)	–	692±63
	13	55	15±3 (2)	78±7 (13)	41±6 (7)	15±3 (3)	6±1 (1)	**135**±19 (22)	**123**±18 (20)	2±0.5 (0.4)	614±52
MS12+pCA8lf/3	3	0.03	12±2 (2)	373±55 (49)	32±4 (4)	21±3 (3)	9±2 (1)	27±5 (4)	16±2 (2)	1±0.1 (0.1)	757±71
	13	0.07	10±2 (2)	295±33 (46)	30±4 (5)	18±4 (3)	9±1 (1)	43±7 (7)	20±3 (3)	1±0.2 (0.1)	644±52
MS12+pCA9lf/1	3	0.5	11±1 (2)	377±34 (55)	30±7 (4)	24±3 (4)	7±1 (1)	9±1 (1)	6±1 (1)	–	688±47
	13	140	12±1 (2)	190±31 (30)	32±4 (5)	16±2 (3)	8±1 (1)	57±9 (9)	71±8 (11)	2±0.5 (0.2)	636±43
MS12+pCA9lf/2	3	0.5	21±2 (3)	397±78 (51)	31±3 (4)	32±2 (4)	10±2 (1)	11±1 (1)	9±1 (1)	–	785±72
	13	62	15±2 (2)	281±21 (40)	35±4 (5)	20±2 (3)	5±1 (1)	45±5 (7)	50±5 (7)	2±0.2 (0.3)	697±36
MS12+pCA8′R/2	3	0.9	10±2 (2)	358±42 (67)	30±5 (6)	29±4 (5)	11±2 (2)	5±1 (1)	3±0.5 (0.5)	0.3±0 (0.1)	533±75
	13	1.12	14±1 (3)	360±37 (67)	30±4 (5)	25±4 (5)	7±1 (1)	4±1 (1)	8±1 (2)	0.7±0.1 (0.1)	540±63
MS12+pCA8′R/4	3	45	12±1 (3)	209±35 (52)	25±3 (6)	18±3 (4)	6±1 (2)	31±3 (8)	37±4 (9)	–	405±74
	13	50	12±1 (3)	196±39 (48)	31±6 (8)	16±2 (4)	6±1 (2)	29±3 (7)	44±7 (11)	–	407±70
MS12+pCA8′R/5	3	1.22	8±2 (2)	325±49 (66)	28±5 (6)	29±5 (6)	14±2 (3)	6±1 (1)	3±0.5 (1)	–	495±91
	13	1.41	13±1 (3)	337±53 (66)	28±3 (6)	25±5 (5)	6±1 (1)	4±1 (1)	8±1 (2)	0.4±0 (0.1)	508±54
MS12+pPT51′R3/2	3	3	23±4 (5)	58±17 (13)	51±5 (11)	17±2 (4)	4±1 (1)	**120**±27 (26)	**109**±12 (24)	0.4±0 (0.1)	460±46
	13	75	11±2 (2)	40±12 (8)	28±3 (6)	21±2 (4)	8±1 (2)	97±21 (19)	**119**±13 (24)	0.1±0 (0.02)	501±35
MS12+pPT51′R3/4	3	0.001	12±1 (2)	359±61 (61)	25±3 (4)	20±4 (3)	9±1 (2)	7±2 (1)	6±1 (1)	0.5±0.1 (0.1)	583±71
	13	0.01	–	390±69 (54)	21±4 (3)	38±4 (5)	10±2 (1)	6±2 (1)	10±2 (1)	1±0.2 (0.2)	729±75

The indicated amounts are average values calculated from the data of three independent cultures. Copy number means relative copy number per host genome. Amounts of the different carotenoids are given in micrograms per gram (dry mass)±standard deviations; the percentage of a carotenoid species within the total carotenoid content is presented in parenthesis. Echinenone and canthaxanthin amounts higher than 100 $\mu g g^{-1}$ (dry mass) are indicated with bold characters. DNA and carotenoid extraction were carried out after cultivation of the strains on YNB for 4 days at 25 °C under continuous light

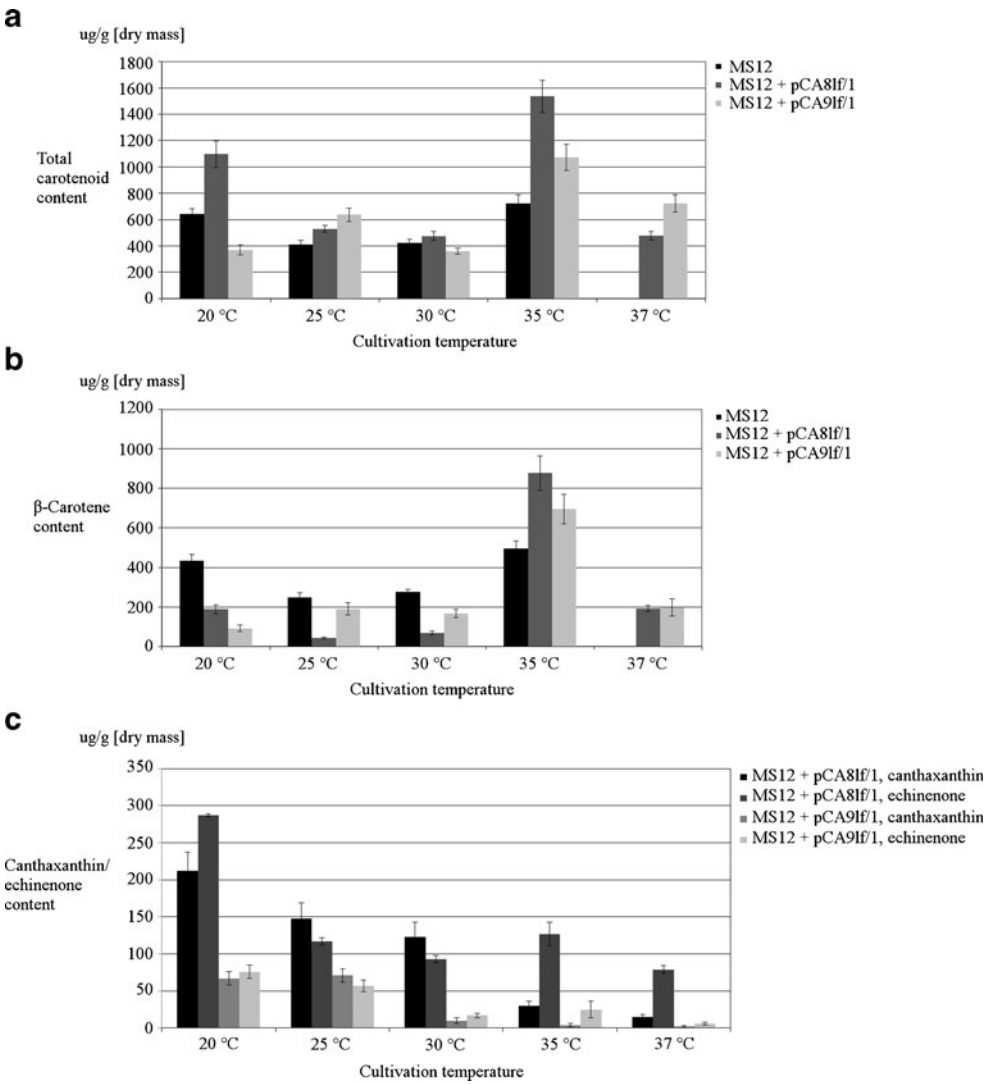

Fig. 3 Effect of the cultivation temperature on the total carotenoid (**a**), the β-carotene (**b**) and the canthaxanthin and echinenone (**c**) levels in two transformants and the original MS12 strain. The presented amounts are averages of three independent experiments; the *error bars* indicate standard deviations

not change or even decreased the total carotenoid level, several compounds increased the proportion of the keto derivatives of β-carotene, such as H_2O_2 and $CuSO_4$ in the case of MS12+pCA8lf/1 or glycerol and ethanol in the case of MS12 +pCA9lf/1. Online Resource 4 shows the colony colours of the MS12+pCA8lf/1 strain cultured on the different carbon sources.

Discussion

In the present study, attempts to integrate the *crtW* gene of *Paracoccus* sp. N81106 encoding a β-carotene ketolase into the *M. circinelloides* genome were carried out using different techniques. Transformation systems that allow stable integration of the transferred DNA into the host genome are essential for the genetic modification of the organisms as well as for the functional analysis of genes. It is well known that transformation of zygomycetes with circular plasmids generally results in strains that maintain the introduced plasmids episomally without any integration event (Ibrahim and Skory 2007; Papp et al. 2010). Such transformants generally prove to be mitotically unstable because of the poor segregation of their plasmids into the spores (Appel et al. 2004). However, at least in the case of *M. circinelloides*, integration has been achieved by using linear DNA fragments for transformation, which harbour homologous sequences at their termini to direct the double-crossing over gene replacement (Navarro et al. 2001; Silva et al. 2006), and several zygomycetes have been successfully transformed with the ATMT method (Michielse et al. 2004; Monfort et al. 2003; Nyilasi et al. 2005; 2008), which generally led to single-copy integration. Successful REMI transformation has not yet been reported in zygomycetes. Despite the fact that several transformation methods are established for zygomycetes, construction of stable transformants, which maintain and express a heterologous (especially a bacterial) gene integrated into the genome, has remained a great challenge (Ibrahim and Skory

Table 4 Effect of different carbon sources and other additives in the culture media on the carotenoid production of two *crtW*-expressing transformants and the original *M circinelloides* strain (MS12)

Strain	Carbon source/additive	Total carotenoid	β-Carotene	Canthaxanthin	Echinenone
MS12	Glucose	412±31	245±12 (60)	–	–
MS12+pCA8lf/1		511±24	34±8 (7)	169±15 (33)	103±7 (20)
MS12+pCA9lf/1		596±51	215±30 (36)	80±17 (13)	65±14 (11)
MS12	Mannose	524±41	344±26 (66)	–	–
MS12+pCA8lf/1		749±68	74±12 (10)	198±17 (26)	165±18 (22)
MS12+pCA9lf/1		799±85	271±40 (34)	139±23 (17)	84±16 (10)
MS12	Trehalose	310±32	186±18 (60)	–	–
MS12+pCA8lf/1		684±57	61±16 (9)	**209**±12 (31)	149±10 (22)
MS12+pCA9lf/1		781±61	268±31 (34)	122±19 (16)	88±7 (11)
MS12	Fructose	582±79	318±12 (64)	–	–
MS12+pCA8lf/1		595±59	88±23 (15)	109±18 (18)	136±35 (23)
MS12+pCA9lf/1		746±91	199±50 (27)	139±45 (19)	86±11 (12)
MS12	Galactose	472±48	246±17 (58)	–	–
MS12+pCA8lf/1		584±81	68±9 (12)	153±26 (26)	124±19 (21)
MS12+pCA9lf/1		631±75	166±45 (26)	108±17 (17)	108±23 (17)
MS12	Cellobiose	211±21	127±11 (60)	–	–
MS12+pCA8lf/1		314±18	19±3 (6)	114±8 (36)	58±5 (19)
MS12+pCA9lf/1		203±19	56±8 (28)	43±7 (21)	18±3 (9)
MS12	Maltose	315±32	172±10 (54)	–	–
MS12+pCA8lf/1		428±78	27±7 (6)	139±29 (33)	82±15 (19)
MS12+pCA9lf/1		385±53	95±12 (25)	49±15 (13)	27±12 (7)
MS12	Ethanol	360±41	129±14 (34)	–	–
MS12+pCA8lf/1		341±44	40±9 (12)	117±17 (34)	28±8 (8)
MS12+pCA9lf/1		208±27	14±5 (7)	85±11 (41)	35±7 (17)
MS12	Glycerol	236±30	98±14 (41)	–	–
MS12+pCA8lf/1		411±47	53±14 (13)	79±11 (19)	35±7 (8)
MS12+pCA9lf/1		437±32	50±7 (11)	163±21 (37)	34±5 (8)
MS12	Glycerol-L-monoacetate	172±22	99±4 (58)	–	–
MS12+pCA8lf/1		430±70	40±17 (9)	169±15 (39)	69±11 (16)
MS12+pCA9lf/1		402±54	126±20 (38)	71±13 (18)	42±6 (11)
MS12	Dihydroxyacetone	325±45	184±9 (57)	–	–
MS12+pCA8lf/1		426±61	15±2 (4)	**232**±21 (55)	35±10 (8)
MS12+pCA9lf/1		590±41	77±10 (13)	144±21 (24)	142±23 (24)
MS12	L-aspartic acid	334±27	194±4 (58)	–	–
MS12+pCA8lf/1		494±64	21±4 (4)	**222**±28 (45)	78±13 (16)
MS12+pCA9lf/1		609±58	204±24 (38)	50±6 (8)	87±14 (14)
MS12	Palm oil	122±12	53±5 (43)	–	–
MS12+pCA8lf/1		209±15	17±3 (8)	54±2 (26)	41±6 (20)
MS12+pCA9lf/1		205±10	48±6 (23)	23±5 (11)	16±3 (8)
MS12	H_2O_2	467±37	263±13 (53)	–	–
MS12+pCA8lf/1		192±21	11±1 (6)	77±5 (40)	27±4 (14)
MS12+pCA9lf/1		325±27	52±13 (16)	78±18 (24)	43±5 (13)
MS12	$FeSO_4$	398±40	167±8 (42)	–	–
MS12+pCA8lf/1		312±24	24±3 (8)	110±12 (35)	47±6 (15)
MS12+pCA9lf/1		385±27	104±7 (27)	67±7 (17)	27±5 (7)
MS12	$CuSO_4$	130±5	18±3 (14)	–	–
MS12+pCA8lf/1		98±9	6±1 (6)	42±2 (43)	10±2 (10)
MS12+pCA9lf/1		246±12	62±8 (25)	44±5 (18)	26±3 (10)

Table 4 (continued)

Strain	Carbon source/additive	Total carotenoid	β-Carotene	Canthaxanthin	Echinenone
MS12	$CoCl_2$	273±12	96±8 (35)	–	–
MS12+pCA8lf/1		362±21	62±7 (17)	26±4 (7)	70±6 (19)
MS12+pCA9lf/1		229±20	30±3 (13)	– (–)	39±5 (17)

The indicated amounts are average values calculated from the data of three independent cultures and are given in micrograms per gram (dry mass)± standard deviations; the percentage of a carotenoid species within the total carotenoid content is presented in parenthesis. Canthaxanthin amounts higher than 200 $\mu g g^{-1}$ (dry mass) are indicated with bold characters. Carotenoid extraction was carried out after cultivation of the strains for 4 days at 25 °C under continuous light

2007; Michielse et al. 2004; Obraztsova et al. 2004; Papp et al. 2010).

In our study, all tested methods, including PEG-mediated protoplast transformation with linear DNA fragments, REMI and ATMT, resulted in transformants. However, ATMT and certain REMI transformants proved to be unstable losing the transferred DNA during the first few cultivation cycles. In a previous experiment, ATMT of *M. circinelloides* with a bacterial gene (hygromycin B phosphotransferase) also led to the formation of unstable transformants (Nyilasi et al. 2005), and a similar instability was found in the ATMT of other Mucorales, such as *Rhizomucor miehei* and *Backusella lamprospora* (Monfort et al. 2003; Nyilasi et al. 2008). Stable transformants were obtained by ATMT from *Rhizopus oryzae* but only if the integrated gene was endogenous or originated from a closely related organism (Michielse et al. 2004; Ibrahim and Skory 2007). REMI method generally leads to random integration where the applied enzymes and the site of integration are important factors of the stability of the integrated DNA.

Although majority of the transformants produced by PEG-mediated protoplast transformation with linear DNA fragments and REMI retained the transferred DNA even under non-selective cultivation conditions, rearrangements to large concatemers and/or excision and recircularization of the integrated linear fragments can be suggested in several strains based on the results of the IPCR and the plasmid rescue experiments. Besides the multiple integration of the transformed DNA, such processes also may explicate the extremely high copy number of the transferred DNA detected in certain transformants (Table 3). Rearrangements of the transferred DNA have often observed in zygomycetes (Yanai et al. 1990; Takaya et al. 1996; Papp et al. 2010). Several authors proposed the existence of a genome defence mechanism that eliminates the heterologous DNA via these DNA rearrangements and deletions (Ibrahim and Skory 2007; Michielse et al. 2004; Nyilasi et al. 2005; Obraztsova et al. 2004; Papp et al. 2010). However, this was not the case in our transformants, where rearrangements did not eliminate the bacterial gene. In contrast, copy number of the *crtW* gene increased, sometimes dramatically, during the consecutive cultivation cycles. Higher copy number of *crtW* generally led to increased canthaxanthin and echinenone production, but this relation was not directly proportional in the different transformants (Table 3) indicating that the site of integration, possible DNA rearranges and/or other factors, such as gene regulation or silencing, may also affect the efficacy of the heterologous gene expression as reported in other organisms (Verdoes et al. 1995; Liang et al. 1996; Lubertozzi and Keasling 2009). Over a certain expression level of the exogenous gene, the amount of the available precursor, i.e. β-carotene, could also limit the synthesis of canthaxanthin.

As linear DNA molecules used in the PEG-mediated transformations held homologous sequences at their termini to direct gene replacement, integration was expected in the corresponding homologous sites. This could be proven only in the transformants produced by using the fragment pCA8lf and REMI with pCA8′R. In the other cases, ectopic integration occurred. As restriction enzymes digest the genomic DNA at several sites, it is well known that REMI frequently causes random integration (Turgeon et al. 2010). Although we expected gene replacement with the pCA9lf fragment, it also integrated ectopically. Maybe the extensions of the homologous regions were not sufficient to direct the double-crossing over or integration into the ribosomal cluster caused defects in the host genome.

After the 13th cultivation step, some transformants produced more than 100 $\mu g g^{-1}$ (dry mass) canthaxanthin on glucose-containing minimal medium (Table 3), which is a similar amount to that measured previously in *Paracoccus* sp. N81106 by Yokoyama and Miki (1995). Interestingly, total carotenoid content of the *crtW*-harbouring transformants also proved to be higher than that of the untransformed MS12 strain suggesting that formation and/or presence of the keto derivatives stimulate the β-carotene biosynthesis (Table 3). It is known that β-carotene itself and its derivatives produced by the fungus have feedback effect on the β-carotene biosynthesis (Lampila et al. 1985; Fraser et al. 1996; Bhosale 2004). It is possible that the β-carotene level, which decreased radically in consequence of its conversion to canthaxanthin, affected the activity of the carotenoid biosynthesis genes. On the other hand, canthaxanthin may

also be able to stimulate the carotenogenic pathway since it holds keto groups on the β-ionone rings, which was previously found to be essential in the positive feedback effect of several chemical regulators of the pathway, such as trisporic acids (Bhosale 2004). Previously, *crtW* was introduced into the same *M. circinelloides* strain using a circular plasmid (Csernetics et al. 2011; Papp et al. 2006). Under the same culturing conditions, the canthaxanthin content of those transformants was much lower [6–13 $\mu g g^{-1}$ (dry mass)], maybe in consequence of the low copy number and the unequal distribution of the plasmids in the mycelium and the spores. Similarly to previous studies (Papp et al. 2006; Csernetics et al. 2011), the astaxanthin level remained low in consequence of the low activity of the endogenous β-carotene hydroxylase.

As an important environmental factor, temperature affects several biosynthetic pathways including carotenoid biosynthesis (Bhosale 2004). In our experiments, both the transformed and the original *M. circinelloides* strains produced the highest amounts of β-carotene at 35 °C (Fig 3). A similar effect of the temperature to the carotenoid production was previously observed in other *Mucor* species, such as in *Mucor rouxii* (Mosqueda-Cano and Gutierez-Corona 1995). Contrarily, canthaxanthin and echinenone formation of the transformants was higher at lower cultivation temperature, maybe due to the temperature requirements of the heterologous β-carotene ketolase. Interestingly, *crtW* containing transformants were able to survive at higher temperature than the untransformed fungus. Canthaxanthin has the ability to interact with and incorporate in the plasma membrane, where it exerts significant influence on the structural and dynamic properties of the membrane even in very small concentration (Sujak et al. 2007). Recently, Kumaresan et al. (2008) also reported thermotolerance of a mutant *Aspergillus carbonarius* strain connected with its canthaxanthin content. The effect of canthaxanthin on membrane fluidity and structure may contribute in the higher thermotolerance of the transformants. Moreover, at higher cultivation temperatures, canthaxanthin may protect against the increased endogenous generation of reactive oxygen species. Indeed, canthaxanthin proved to be more effective against free radicals than β-carotene in membrane models (Palozza and Krinsky 1992).

Effects of different carbon sources and chemical additives on the carotenoid production of two selected transformants were also tested. Glucose, mannose, trehalose and fructose had a positive effect on the whole carotenoid content of both transformants tested. Previously, glucose, cellobiose and maltose enhanced the carotenogenesis of *M. rouxii* in the highest degree compared to other carbon sources (Mosqueda-Cano and Gutierez-Corona 1995). In the case of MS12+pCA8lf/1, application of dihydroxyacetone and L-aspartic acid resulted in that β-carotene almost completely converted to its keto derivatives, mainly to canthaxanthin. We suppose that both compounds affected the keto-carotenoid level through the induction of the *gpd1* promoter, which drives the expression of the transferred *crtW* gene. With the application of glucose (at 20 °C, see Fig. 3), trehalose, dihydroxyacetone and L-aspartic acid (Table 4), MS12+pCA8lf/1 produced more than 200 $\mu g g^{-1}$ (dry mass) of canthaxanthin. This amount is comparable with the canthaxanthin content of the wild-type *G. jacobaea* [200 $\mu g g^{-1}$ (dry mass)] (Veiga-Crespo et al. 2005), but lower than those of *Brevibacterium* KY-4313 [600 $\mu g g^{-1}$ (dry mass)] (Nelis and De Leenheer 1989) and *Haloferax alexandrinus* [700 $\mu g g^{-1}$ (dry mass)] (Asker and Ohta 2002).

The aim of this study was to examine the biological requirements of the application of *M. circinelloides* as a xanthophyll producer and to develop strains and methods, which could be used in the further applied studies. Stable strains expressing a bacterial β-carotene ketolase and producing canthaxanthin in considerable amounts were constructed. Taking into account that the canthaxanthin level reported in this study for MS12+pCA8lf/1 was achieved in minimal medium and the echinenone level remained relatively high in the majority of experiments, we expect that optimization of the growth conditions and improvement of the culturing medium will allow much higher carotenoid production and more complete β-carotene–canthaxanthin conversion. Stable canthaxanthin-producing mutants also can be used as a host for heterologous genes to construct astaxanthin-producing strains as well as for model organisms to study the physiological effect of the canthaxanthin accumulation. Although further studies are needed to increase the canthaxanthin productivity of the modified *M. circinelloides* strains, our results indicate that they may be promising candidates for further canthaxanthin-producing strain improvement studies.

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