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ORIGINAL PAPER

Expression of *Xanthophyllomyces dendrorhous* cytochrome-P450 hydroxylase and reductase in *Mucor circinelloides*

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Abstract Carotenoids are natural pigments that act as powerful antioxidants and have various beneficial effects on human and animal health. *Mucor circinelloides* (Mucoromycotina) is a carotenoid producing zygomycetes fungus, which accumulates β-carotene as the main carotenoid but also able to produce the hydroxylated derivatives of β-carotene (i.e. zeaxanthin and β-cryptoxanthin) in low amount. These xanthophylls, together with the ketolated derivatives of β-carotene (such as canthaxanthin, echinenone and astaxanthin) have better antioxidant activity than β-carotene. In this study our aim was to modify and enhance the xanthophyll production of the *M. circinelloides* by expression of heterologous genes responsible for the astaxanthin biosynthesis. The *crtS* and *crtR* genes, encoding the cytochrome-P450 hydroxylase and reductase, respectively, of wild-type and astaxanthin overproducing mutant *Xanthophyllomyces dendrorhous* strains were amplified from cDNA and the nucleotide and the deduced amino acid sequences were compared to each other. Introduction of the *crtS* on autonomously replicating plasmid in the wild-type *M. circinelloides* resulted enhanced zeaxanthin and β-cryptoxanthin accumulation and the presence of canthaxanthin, echinenone and astaxanthin in low amount; the β-carotene hydroxylase and ketolase activity of the *X. dendrorhous* cytochrome-P450 hydroxylase in *M. circinelloides* was verified. Increased canthaxanthin and echinenone production was observed by expression of the gene in a canthaxanthin producing mutant *M. circinelloides*. Co-expression of the *crtR* and *crtS* genes led to increase in the total carotenoid and slight change in xanthophyll accumulation in comparison with transformants harbouring the single *crtS* gene.

Keywords Cytochrome-P450 hydroxylase · Cytochrome-P450 reductase · β-Carotene hydroxylase and ketolase · Heterologous expression · Xanthophylls

Introduction

Carotenoids are widely used natural pigments mostly because of their antioxidant properties (Bhosale and Bernstein 2005). Carotenoids protect cells against photooxidation by quenching singlet oxygen, free radicals (e.g. prevention of lipid peroxidation) and reactive oxygen species (Edge et al. 1997; Bhosale and Bernstein 2005). Xanthophylls are substituted oxygen-containing carotene derivatives; commercially the most important compounds are derived from β-carotene with 3,3′-hydroxylation and/or 4,4′-ketolation. β-cryptoxanthin (3-hydroxy-β,β-carotene), zeaxanthin (3,3′-dihydroxy-β,β-carotene), canthaxanthin (β,β-carotene-4,4′dione) and astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) are more powerful antioxidants than β-carotene, so they are frequently used as ingredients of various food, pharmaceutical, cosmetic and feed products (Palozza and Krinsky 1992; Bhosale and Bernstein 2005). Beside and due to their antioxidant properties, xanthophylls have several beneficial effects on human and animal health, e.g. xanthophylls effectively stimulate the immune defences (Jyonouchi et al. 1996; Okai and

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Higashi-Okai 1996), canthaxanthin induced apoptosis in human cancer cell lines (Palozza et al. 1998; Kumaresan et al. 2008), β-cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin reduced the risk of several types of cancer in animal models (Mayne and Parker 1989; Mayne 1996; Chew et al. 1999; Nishino et al. 2002), zeaxanthin prevents of age-related macular degeneration (AMD; Mares-Perlman et al. 2002; Beatty et al. 2004), astaxanthin is used as a medical ingredient against heart disease (Guerin et al. 2003) and β-cryptoxanthin has a preventive effect against rheumatoid arthritis (Pattison et al. 2004) and bone diseases (Yamaguchi 2004). Currently, major part of the commercially available carotenoids is synthesized chemically but an increasing interest can be observed in microbial sources (Bhosale and Bernstein 2005).

Mucor circinelloides (Mucoromycotina) is a carotenoid producing fungus and together with the related *Phycomyces blakesleeanus* and *Blakeslea trispora* is among the most studied model organisms for microbial carotene biosynthesis (Velayos et al. 2000a, b, 2003, 2004; Navarro et al. 2001; Papp et al. 2006, 2013; Csernetics et al. 2011). Today, *B. trispora* is used for industrial production of carotenoids, but the lack of an effective transformation system makes it less applicable for genetic engineering (Iturriaga et al. 2001, 2005; Dufossé 2006). At the same time, a well-developed transformation system for the expression of exogenous genes is available for *M. circinelloides* (Papp et al. 2010). *M. circinelloides* is a β-carotene producing fungus, but it is also able to synthesise hydroxylated derivatives of β-carotene in low amount (zeaxanthin and β-cryptoxanthin); that is, the fungus has a poor β-carotene hydroxylase activity, while it does not show any ketolase activity (Álvarez et al. 2006; Papp et al. 2006; Csernetics et al. 2011). The genetic background of this hydroxylase activity is still unknown.

The basidiomycetes yeast *Xanthophyllomyces dendrorhous* produces astaxanthin as the major carotenoid compound (Johnson 2003; Álvarez et al. 2006). The enzymatic background of carotene biosynthesis in *X. dendrorhous* has been studied and the genes involved in the formation of astaxanthin have been cloned and characterized (Verdoes et al. 1999a, b, 2003; Visser et al. 2003; Lodato et al. 2004; Álvarez et al. 2006; Lodato et al. 2007; Alcaíno et al. 2008; Niklitschek et al. 2008; Fig. 1). Gene named as *ast*, *asy* or *crtS* by the different authors, encoding a cytochrome-P450-type enzyme may be responsible for the formation of astaxanthin from β-carotene, i.e. addition of two hydroxyl and two keto groups to the β-ionone rings of β-carotene (Hoshino et al. 2000; Verdoes et al. 2003; Álvarez et al. 2006; Ojima et al. 2006; Lodato et al. 2007; Contreras et al. 2013). Overexpression of the *crtS* gene in *X. dendrorhous* resulted higher level of astaxanthin production (Contreras et al. 2013), and complementation of the astaxanthin non-producing and β-carotene accumulating *X. dendrorhous* ATCC 96815 mutant with *crtS* restored the astaxanthin biosynthesis (Álvarez et al. 2006). Although the β-carotene hydroxylase and ketolase activity of the enzyme was verified, Álvarez et al. (2006) found that CrtS had only β-carotene hydroxylase activity when it was expressed in *M. circinelloides*. The CrtS (Ast) protein seems to have near resemblance with cytochrome-P450 hydroxylases; the oxygen and heme binding motifs as well as a domain involved in the maintenance of the three-dimensional structure of the enzyme have been identified (Álvarez et al. 2006). In its active state, the protein is reduced by a cytochrome-P450 reductase (McLean et al. 2005): the electron donor is required for the addition of oxygen-bearing functional group to the substrate (Alcaíno et al. 2008). The corresponding reductase is encoded by the *crtR* gene in *X. dendrorhous*. Deletion of the *crtR* resulted astaxanthin non-producing mutants, indicating that the gene is also necessary for the formation of astaxanthin (Alcaíno et al. 2008).

The aim of this study was to express the *crtS* and *crtR* genes in *M. circinelloides* to achieve the conversion of β-carotene to xanthophylls. The *crtS* and *crtR* of different *X. dendrorhous* strains, including wild-type and astaxanthin overproducing mutant, were amplified and the sequences were compared to each other. The genes were introduced on autonomously replicating plasmids into wild-type and canthaxanthin producing mutant *M. circinelloides*. The carotenoid composition of the transformants, copy number of the transferred plasmids and relative transcript levels of the exogenous genes were analysed.

Materials and methods

Strains and growth conditions

MS12, a *leuA*$^-$, *pyrG*$^-$ mutant strain (Benito et al. 1992) derived from the wild-type *M. circinelloides* CBS 277.49 and MS12 + pCA8lf/1, a *leuA*$^+$, *pyrG*$^-$, *crtW*$^+$ mutant derived from the MS12 strain (Papp et al. 2013) were used in the transformation experiments. MS12 is auxotrophic for leucine and uracil but wild type for the carotenoid biosynthesis, while MS12 + pCA8lf/1 is auxotrophic for uracil and harbours the *crtW* gene encoding the β-carotene ketolase of *Paracoccus* sp. N81106 (formerly *Agrobacterium aurantiacum*) integrated into the genome and able to synthesise canthaxanthin, echinenone and small amount of astaxanthin (Papp et al. 2013). The *crtS* and *crtR* genes were isolated from the following *X. dendrorhous* strains: ATCC 24229 (wild-type), SZMC 9073 (astaxanthin

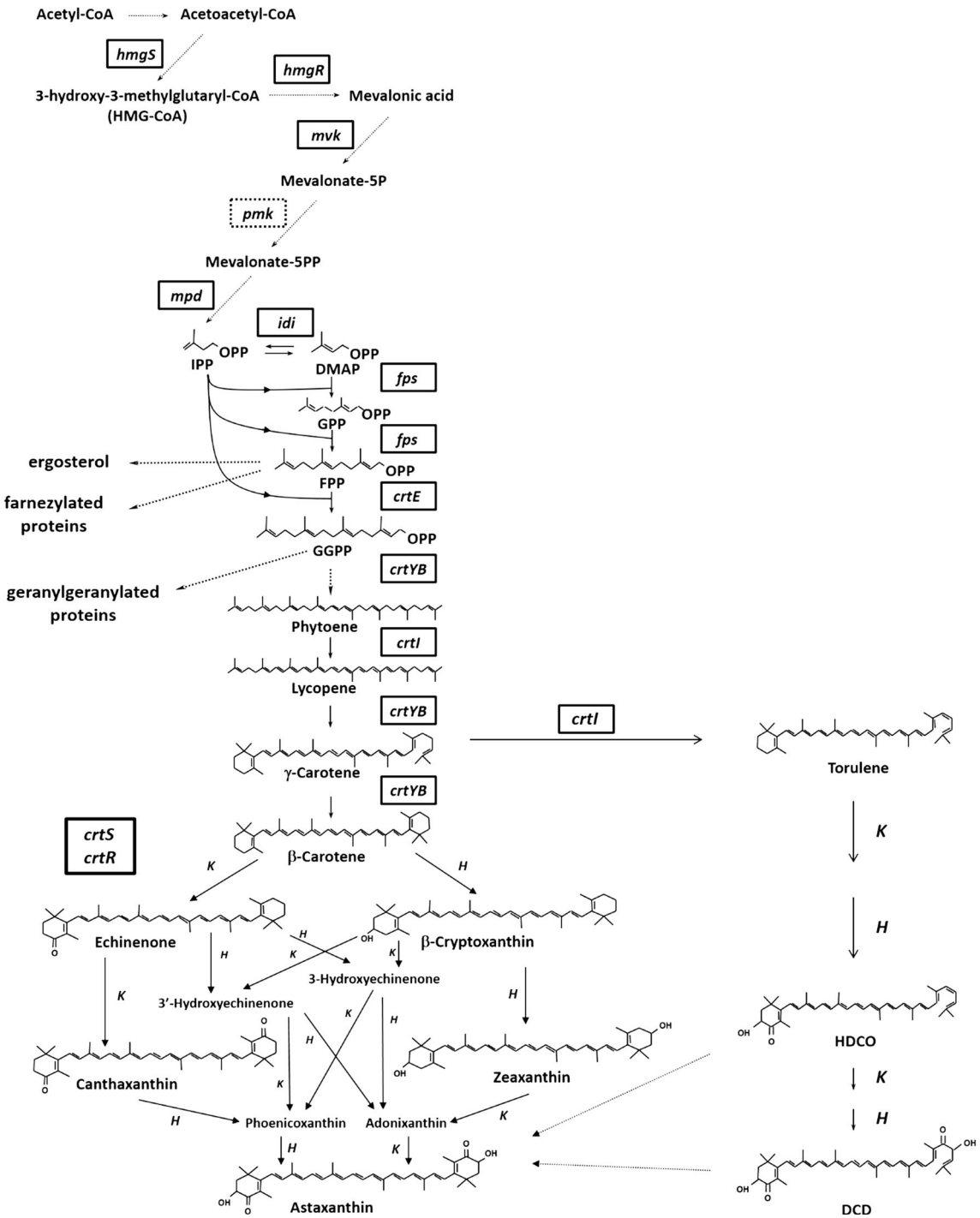

Fig. 1 Carotene and xanthophyll biosynthesis pathway in *X. dendrorhous* based on Visser et al. (2003) and Álvarez et al. (2006). The genes are indicated in *boxes*, the presumed gene with *dotted frame*. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl pyrophosphate; DMAP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HDCO, 3-hydroxy-3′,4′-didehydro-β,ψ-carotene-4-one; DCD, 3,3′-dihydroxy-β,ψ-carotene-4,4′-dione. Genes and the encoded proteins: *hmgS*, HMG-CoA synthase; *hmgR*, HMG-CoA reductase; *mvk*, mevalonate kinase; *pmk*, phosphomevalonate kinase; *mpd*, pyrophosphomevalonate decarboxylase; *idi*, IPP isomerase; *fps*, FPP synthase; *crtE*, GGPP synthase; *crtYB*, phytoene synthase/lycopene cyclase (phytoene–β-carotene synthase); *crtI*, phytoene desaturase; *crtS*, cytochrome-P450 hydroxylase (astaxanthin synthase); *crtR*, cytochrome-P450 reductase. H–β-carotene hydroxylase activity of cytochrome-P450 hydroxylase; K–β-carotene ketolase activity of cytochrome-P450 hydroxylase

Table 1 Fungal strains and plasmids used in this study

Strain/plasmid	Genotype or description	References
MS12	Leucine and uracil auxotrophic, wild-type for carotenoid production (*leuA*$^-$, *pyrG*$^-$)	Benito et al. (1992)
MS12 + pCA8lf/1	Uracil auxotrophic, canthaxanthin and echinenone producing mutant, expressing the β-carotene ketolase of *Paracoccus* sp. N81106 (*pyrG*$^-$, *crtW*)	Papp et al. (2013)
X. dendrorhous ATCC 24229	Prototrophic, wild-type for carotenoid production	American Type Culture Collection
X. dendrorhous SZMC 9073	Prototrophic, astaxanthin overproducing mutant, derivative of ATCC 24229	Szeged Microbiological Collection
X. dendrorhous CBS 6938	Prototrophic, wild-type for carotenoid production	Centraalbureau voor Schimmelcultures
MS12 + pPT81-crtS/1–10	Leucine auxotrophic, expressing the cytochrome-P450 hydroxylase of *X. dendrorhous* CBS 6938 (*leuA*$^-$, *crtS*)	This study
MS12 + pPT86-crtR/1–10	Uracil auxotrophic, expressing the cytochrome-P450 reductase of *X. dendrorhous* CBS 6938 (*pyrG*$^-$, *crtS*)	This study
MS12 + pPT81-crtS + pPT86-crtR/1–10	Prototrophic, expressing the cytochrome-P450 hydroxylase and reductase of *X. dendrorhous* CBS 6938 (*crtS*, *crtR*)	This study
MS12 + pCA8lf/1 + pPT81-crtS/1–10	Prototrophic, expressing the β-carotene ketolase of *Paracoccus* sp. N81106 and the cytochrome-P450 hydroxylase of *X. dendrorhous* CBS 6938 (*crtW*, *crtS*)	This study
pJET1.2/blunt	General cloning vector for *E. coli* (*amp*)	Thermo Scientific
pBluescript II KS	General cloning vector for *E. coli* (*amp*)	Stratagene
pPT81	*M. circinelloides gpd1* promoter and terminator regions (*gpd1*P–*gpd1*T, *pyrG*, *amp*), same as pPT43pyr	Csernetics et al. (2011)
pPT86	Expression casette *gpd1*P–*isoA* of *M. circinelloides*–*gpd1*T (*leuA*, *amp*)	Csernetics et al. (2011)
pJET-*crtS*_ATCC_24229	*crtS* of *X. dendrorhous* ATCC 24229 into pJET1.2/blunt (*amp*)	This study
pKS-*crtS*_SZMC_9073	*crtS* of *X. dendrorhous* SZMC 9073 into pBluescript II KS (*amp*)	This study
pKS-*crtS*_CBS_6938	*crtS* of *X. dendrorhous* CBS 6938 into pBluescript II KS (*amp*)	This study
pJET-*crtR*_ATCC_24229	*crtR* of *X. dendrorhous* ATCC 24229 into pJET1.2/blunt (*amp*)	This study
pJET-*crtR*_SZMC_9073	*crtR* of *X. dendrorhous* SZMC 9073 into pJET1.2/blunt (*amp*)	This study
pJET-*crtR*_CBS_6938	*crtR* of *X. dendrorhous* CBS 6938 into pJET1.2/blunt (*amp*)	This study
pPT81-crtS	Expression casette *gpd1*P–*crtS* of *X. dendrorhous* CBS 6938–*gpd1*T (*pyrG*, *amp*)	This study
pPT86-crtR	Expression casette *gpd1*P–*crtR* of *X. dendrorhous* CBS 6938–*gpd1*T (*leuA*, *amp*)	This study

Encoded proteins: *leuA*, α-isopropylmalate isomerase; *pyrG*, orotidine-5′-monophosphate decarboxylase; *crtW*, β-carotene ketolase of *Paracoccus* sp. N81106; *crtS*, cytochrome-P450 hydroxylase of *X. dendrorhous*; *crtR*, cytochrome-P450 reductase of *X. dendrorhous*; *isoA*, farnesyl pyrophosphate synthase of *M. circinelloides*; *amp*, ampicillin resistance

overproducing mutant of ATCC 24229 described by Palágyi et al. (2006) as ATCC 24229/S119) and CBS 6938 (wild-type; Table 1).

Escherichia coli strain TOP10F-was used in all cloning experiments and plasmid amplifications. *E. coli* was cultivated on Luria–Bertani (LB) medium (Sambrook et al. 1989) containing 100 μg mL^{-1} ampicillin at 37 °C.

For growth tests, nucleic acid and carotenoid extraction, *M. circinelloides* strains were cultivated on solid minimal medium (YNB, 10 g glucose; 0.5 g yeast nitrogen base

Table 2 Primers used in this study and the size of the amplified fragments

Primer	Sequence 5′–3′	Amplicon size (bp)
XantcrtS1	GGC ATCGAT ATG TTC ATC TTG GTC TTG CTC	1,674
XantcrtS2	CTT GCGGCCGC TCA TTC GAC CGG CTT GAC CT	
XantcrtR1	GA CTCGAG ATG GCC ACA CTC TCC GAT CTT GTC	2,241
XantcrtR2	AAT GCGGCCGC CTA CGA CCA GAC GTC CAT CAA CAA	
crtSreal-time1	CCG ATC CGA AAG TCT TCA ACC	110
crtSreal-time2	CGC CGT AAC AAC ACC ATC TC	
crtRreal-time1	TCT TCT CCG AAA CTT CAC CC	177
crtRreal-time2	CTG TCC GTC GCT AAT CAT TG	
isoAreal-time1	ATC TCG ACT GTT ACG GTG CTC CT	119
isoAreal-time2	CTT GCG TTG TTC GGG ATT AGC CA	
actAreal-time1	CAC TCC TTC ACT ACC ACC GCT GA	117
actAreal-time2	GAG AGC AGA GGA TTG AGC AGC AG	
T3	ATT AAC CCT CAC TAA AGG GA	Sequence determination
T7	TAA TAC GAC TCA CTA TAG GG	
pJET1.2 forward sequencing	CGA CTC ACT ATA GGG AGA GCG GC	Sequence determination
pJET1.2 reverse sequencing	AAG AAC ATC GAT TTT CCA TGG CAG	

without amino acids; 1.5 g $(NH_4)_2SO_4$, 1.5 g sodium glutamate and 20 g agar per litre) supplemented with leucine and/or uracil (0.5 mg mL^{-1}) as required. Strains were grown at 25 °C for 4 days under continuous light. For growth test 10^5 spores were inoculated onto the centre of the YNB plates. For RNA extraction, *X. dendrorhous* strains were cultivated for 4 days in liquid malt extract broth (MEB: 10 g glucose; 10 g malt extract; 5 g yeast extract per litre) with shaking at 150 rpm at 20 °C. For analysis of the mitotic stability of the transformants and to maintain the *X. dendrorhous* strains, solid malt extract medium (MEA: 10 g glucose; 10 g malt extract; 5 g yeast extract; 20 g agar per litre) was used.

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). Plasmid DNA was purified with the E.Z.N.A. Plasmid Mini Kit II (Omega Bio-Tek) or with the Viogene Mini Plus and Midi Plus Plasmid DNA Extraction Systems (Viogene). Genomic DNA and total RNA samples were prepared from *M. circinelloides* mycelia disrupted with a pestle and mortar in liquid nitrogen. DNA was isolated according to Iturriaga et al. (1992) or with the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) and RNA was purified by the E.Z.N.A. Total RNA Kit II (Omega Bio-Tek).

The coding regions of *crtS* and *crtR* genes were amplified from *X. dendrorhous* cDNA with *Pfu* Polymerase (Zenon) or Phusion High Fidelity DNA Polymerase (Thermo Scientific). After cultivation, yeast cells were collected by centrifugation (3,200 rpm, 10 min, 25 °C) and incubated for 2 h in protoplast-forming solution (0.7 M potassium-chloride; 1.5 % home-made snail enzyme; 1 % Novozym234). Total RNA was purified from protoplasts/spheroplasts with E.Z.N.A. Total RNA Kit II (Omega Bio-Tek). After DNase treatment performed with DNaseI (Thermo Scientific), cDNA was synthesized with Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) using oligo$(dT)_{18}$ primers, following the instructions of the manufacturers. XantcrtS1–XantcrtS2 and XantcrtR1–XantcrtR2 primer pairs were designed for PCR amplification of *X. dendrorhous crtS* and *crtR* genes, respectively (Table 2). The reaction mixtures were subjected to the following programs: (1) an initial denaturing step of 3 min at 94 °C was followed by 35 cycles of 60 s denaturation (94 °C), 60 s annealing (58 °C), 2 min extension (72 °C), followed by 10 min final extension (72 °C) with *Pfu* Polymerase (Zenon) or (2) an initial denaturing step of 30 s at 98 °C was followed by 35 cycles of 10 s denaturation (98 °C), 50 s annealing and extension (72 °C), followed by 10 min final extension (72 °C) with Phusion High Fidelity DNA Polymerase (Thermo Scientific). Amplified fragments were purified from agarose gel using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific). DNA sequencing was performed by LGC Genomics (Berlin, Germany). For Southern hybridization, 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 expression vectors and transformation

Amplified *X. dendrorhous crtS* and *crtR* genes were cloned into *EcoRV* digested pBluescript II KS plasmid (Stratagene) (pKS-*crtS*_SZMC_9073 and pKS-*crtS*_CBS_6938 plasmids) or into pJET1.2 Blunt Cloning Vector (Thermo Scientific) (pJET-*crtS*_ATCC_24229, pJET-*crtR*_ATCC_24229, pJET-*crtR*_SZMC_9073 and pJET-*crtR*_CBS_6938 plasmids; Table 1). The pKS-*crtS*_CBS_6938 was digested with *Cla*I and *Not*I restriction endonucleases and the *crtS* gene was ligated at the corresponding sites between the promoter and terminator regions of the *Mucor* glyceraldehyde-3-phosphate dehydrogenase (*gpd1*) gene into the pPT81 plasmid (same as pPT43pyr in Csernetics et al. 2011). The final pPT81-crtS plasmid also holds the *Mucor pyrG* as selection marker, which can complement the uracil auxotrophy of MS12 (Table 1; Fig. 2). The pJET-*crtR*_CBS_6938 plasmid was digested with *Xho*I and *Not*I restriction endonucleases and the *crtR* gene was cloned in the corresponding sites of the plasmid pPT86 replacing the *isoA* gene between the promoter and terminator regions of the *gpd1* gene (Csernetics et al. 2011). The *Xho*I recognition site can be found in the *crtR*, therefore partial digestion was used: the *Xho*I was added to the reaction mixture in 30 % of the suggested concentration and digestion was stopped after 0.5 h. The constructed pPT86-crtR plasmid holds the *Mucor leuA* gene, which can complement the leucine auxotrophy of the MS12 strain (Table 1; Fig. 2).

For protoplast formation *Mucor* spores harvested from cultures grown for 4 days, were inoculated in small drops onto cellophane sheets placed on YNB media supplemented with uracil and leucine, as required, and grown at 25 °C for 16 h. Young colonies were transferred into protoplast-forming solution (10 mM sodium-phosphate buffer, pH 6.4; 0.8 M sorbitol; 1.5 % home-made snail enzyme) and incubated at 25 °C for 3 h with continuous gentle shaking. Protoplasts were separated from mycelia with filtration through three sheets of gauze, collected with centrifugation (3,200 rpm, 15 min, 4 °C) and washed once with SMC buffer (0.8 M sorbitol; 50 mM $CaCl_2$; 10 mM 3-(*N*-morpholino)propanesulfonic acid). The PEG/$CaCl_2$-mediated transformation of protoplasts was performed according to van Heeswijck and Roncero (1984). Transformants were selected on the basis of auxotrophy complementation on YNB solid media supplemented with leucine or uracil, as required. Introduction of the pPT81-crtS and/or pPT86-crtR plasmids into MS12 or MS12 + pCA8lf/1 resulted in the MS12 + pPT81-crtS, MS12 + pPT86-crtR, MS12 + pPT81-crtS + pPT86-crtR and MS12 + pCA8lf/1 + pPT81-crtS transformants (Table 1).

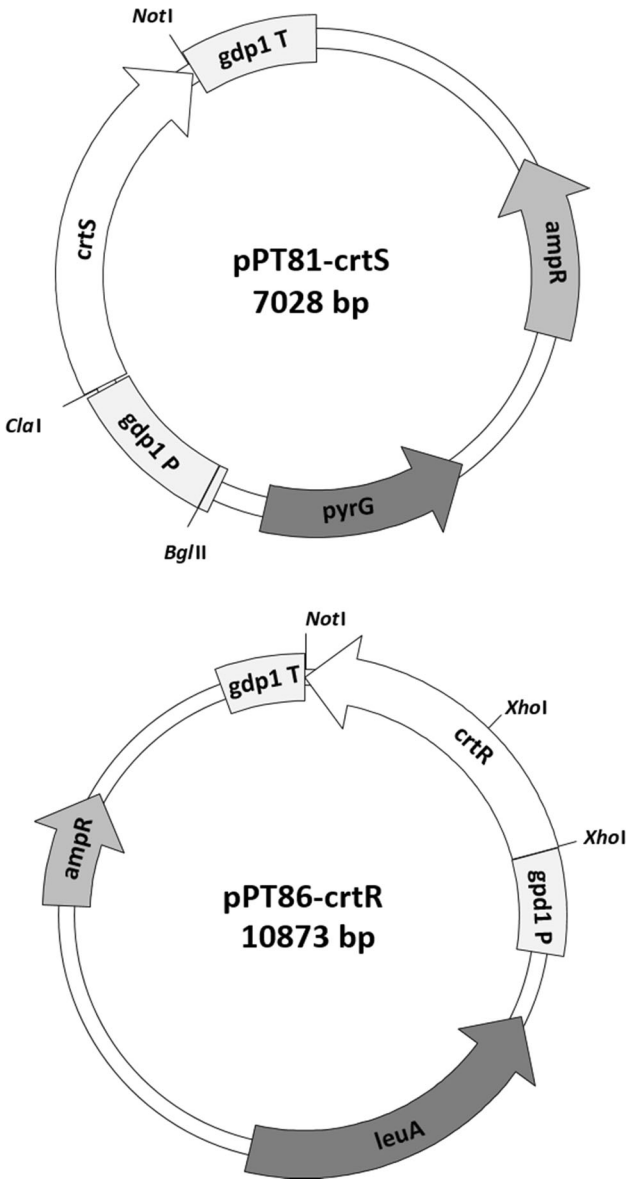

Fig. 2 Plasmid constructions used for transformation of MS12 and MS12 + pCA8lf/1 strains. The plasmids carries the coding regions of *crtS* or the *crtR* genes amplified from the cDNA of *X. dendrorhous* CBS 6938 inside the *Mucor gpd1* promoter and terminator regions. The *pyrG* and *leuA* selection marker genes are responsible for the complementation of the uracil and leucine auxotrophy, respectively; *ampR* (gene for ampicillin resistance) is responsible for the bacterial selection

Molecular analysis of the transformants

Real-time quantitative PCR (qPCR) was used to determine the copy number of the plasmids and the relative transcript levels of the exogenous genes. Total DNA and RNA of the

Table 3 Origin of the compared *crtS* and *crtR* genes with GenBank accession numbers

GenBank accession number	Gene	References
HG939452	*crtS* of *X. dendrorhous* ATCC 24229	This study
HG939453	*crtS* of *X. dendrorhous* SZMC 9073	This study
HG939455	*crtS* of *X. dendrorhous* CBS 6938	This study
DQ201828	*crtS* of *X. dendrorhous* VKPM Y2410	Álvarez et al. (2006)
DQ202402	*crtS* of *X. dendrorhous* ATCC 24203	Álvarez et al. (2006)
DQ002007	*ast* of *X. dendrorhous* ATCC 24230	Niklitschek et al. (2008)
EU713462	*crtS* of *X. dendrorhous* atx5	Niklitschek et al. (2008)
JQ342968	*crtS* of *X. dendrorhous* WtA	Barbachano-Torres et al. (unpublished)
JQ342969	*crtS* of *X. dendrorhous* WtA.1	Barbachano-Torres et al. (unpublished)
JQ342970	*crtS* of *X. dendrorhous* P26	Barbachano-Torres et al. (unpublished)
JQ342971	*crtS* of *X. dendrorhous* R5	Barbachano-Torres et al. (unpublished)
JQ342972	*crtS* of *X. dendrorhous* R17	Barbachano-Torres et al. (unpublished)
JQ342973	*crtS* of *X. dendrorhous* Y13	Barbachano-Torres et al. (unpublished)
KJ783313	*ast* of *X. dendrorhous*	Chen and Li (unpublished)
EU884134	*crtR* of *X. dendrorhous* ATCC 24230	Álcaino et al. (2008)
LN554258	*crtR* of *X. dendrorhous* ATCC 24229	This study
LN554259	*crtR* of *X. dendrorhous* SZMC 9073	This study
LN554260	*crtR* of *X. dendrorhous* CBS 6938	This study

transformants was purified as described above. For analysis of the relative transcript levels, after DNase treatment performed with DNaseI (Thermo Scientific), cDNA was synthesized with Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) using random hexamer and oligo(dT)$_{18}$ primers, following the instructions of the manufacturer. qPCR experiments were performed in a CFX96 real-time PCR detection system (Bio-Rad) using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and the primers presented 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), 30 s annealing (60 °C) and 30 s extension (72 °C) with detection. The relative quantification of plasmid copy number and gene transcript levels were achieved with the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), respectively, using the actin gene (*actA*) of *M. circinelloides* as a reference. In all experiments, qPCR was performed from the same RNA extract for each gene. Experiments were performed in biological and technical triplicates. For Southern hybridization XantcrtS1–XantcrtS2 and XantcrtR1–XantcrtR2 primers were used for the synthesis of gene probe from pPT81-crtS and pPT86-crtR plasmid DNA (Table 1; Fig. 2). The total DNA of transformants and plasmids were digested with *Sma*I, *Pst*I and *Sal*I restriction endonucleases.

Carotenoid extraction and analysis

Carotenoids were extracted from 500 mg mycelial powder as described earlier (Papp et al. 2006). For high-performance liquid chromatography (HPLC), samples and standards 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 µg mL^{-1}) directly before the analysis and 2 µL was subjected to HPLC analysis on a Phenomenex Prodigy column (4.6 × 250, ODS 3 µm). The separation was performed with a gradient (where min/solvent A %/solvent B % was 0/99/1; 8/60/40; 13/46/54; 15/0/100; 18/0/100; 21/99/1; 25/99/1) using 4 % water-96 % methanol 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 and the column thermostat temperature was 35 °C. For identification of carotenoids the following standards were used: astaxanthin, lycopene, β-carotene (Sigma-Aldrich), β-cryptoxanthin, canthaxanthin, zeaxanthin (Carl Roth) and echinenone (DHI Water and Environment), while for identification of γ-carotene a standard was purified with HPLC from *Mucor azygosporus*.

Results

Cloning and comparison of the nucleotide and predicted amino acid sequences of *crtS* and *crtR* genes from different *X. dendrorhous* strains

The *crtS* and *crtR* genes, encoding the cytochrome-P450 hydroxylase and reductase, respectively, were amplified from the cDNA of wild-type (ATCC 24229 and CBS 6938)

and astaxanthin overproducing mutant (SZMC 9073) strains of *X. dendrorhous* in biological replicates. The fragments were cloned into pBluescript II KS or pJET 1.2 Blunt Cloning Vector and sequences of five clones were determined in each case. The gene sequences were deposited in EMBL–EBI (see Table 3 for the accession numbers). Nucleotide sequences of the *crtS* and *crtR* genes and the deduced amino acid sequences were compared with those available in GenBank (Figs. 3, 4, S1 and S2).

The nucleotide sequences of *crtS* of ATCC 24229 and SZMC 9073 shows 100 % identity to each other, while five nucleotide differences was observed in comparison of the *crtS* of CBS 6938 with those of ATCC 24229 and SZMC 9073, but the genes encode the same protein (Figs. 3, S1). Predicted amino acid sequences of the CrtS of ATCC 24229, SZMC 9073 and CBS 6938 compared to CrtS/Ast sequences found in GenBank with the accession numbers DQ201828, DQ202402, DQ002007, JQ342969 and KJ783313 did not differ to each other; the incidental nucleotide polymorphism did not result in amino acid changes (Table 3; Figs. 3, S1). At the same time, differences in one or two amino acid positions were detected comparing the aforementioned and other GenBank sequences (accession numbers: EU713462, JQ342968, JQ342970, JQ342971, JQ342972 and JQ342973; Table 3; Fig. 3). Since the encoded proteins did not differed to each other, plasmid was constructed using the amplified *crtS* of CBS 6938 to examine the effect of the encoded protein to the carotenoid production of untransformed and canthaxanthin producing mutant *Mucor* strains (Fig. 2).

The *crtR* of ATCC 24229, SZMC 9073 and the nucleotide sequence determined by Alcaíno et al. (2008) showed 100 % identity to each other, while *crtR* of CBS 6938 differed from those in six nucleotides resulted in two amino acid changes (Table 3; Figs. 4, S2). Astaxanthin accumulation of wild-type *X. dendrorhous* CBS 6938 is significant, non-functional CrtR must be lead to astaxanthin non-accumulating and β-carotene producing mutant (Alcaíno et al. 2008). Moreover, *X. dendrorhous* CBS 6938 accumulates more astaxanthin than ATCC 24229 (Palágyi et al. 2006), therefore the gene *crtR* of CBS 6938 was selected for plasmid construction and transformation experiments.

Heterologous expression of the *X. dendrorhous crtS* and *crtR* genes in *M. circinelloides*

MS12 protoplasts were transformed with pPT81-crtS and pPT86-crtR circular plasmids (Fig. 2). Vectors were constructed to contain either the *pyrG* or the *leuA*, as selection markers to allow the co-transformation. Protoplasts of MS12 + pCA8lf/1 were transformed with the pPT81-crtS plasmid (Fig. 2; Table 1). The transformation frequency was 15–30 colony per 10^5 protoplasts. No differences in the growth curve, but slight colony colour change was observed when transformants were compared with *Mucor* strains not harbouring the *crtS* and *crtR* genes (Figs. S3, S4). Ten isolates per transformation experiments were selected for further analysis.

Fig. 3 Comparison of the predicted CrtS (Ast) amino acid sequences of different *X. dendrorhous* strains. Different positions are *underlined* and given in *bold*. GenBank accession numbers, HG939452, HG939453, HG939455, corresponding to the sequences of CrtS of ATCC 24229, SZMC 9073 and CBS 6938, respectively are also *underlined* and in *bold face*. Only entire amino acid sequences available in GenBank are represented ▶

PCR with XantcrtS1–XantcrtS2 and XantcrtR1–XantcrtR2 primers verified the presence of the plasmid DNA in the transformants (results not shown). The plasmid copy number and transcript levels were determined by qPCR. Originally, the *crtS* and *crtR* genes are not presented in the *Mucor* genome. Therefore, serial dilutions of the pPT81-crtS, pPT86-crtR and pPT86 plasmids were used in the control experiments. The amplification signals of the *crtS*, *crtR*, *isoA* and *actA* reached the threshold line at the same Ct value, which presume that the used qPCR primers operate with equal efficiency and can be used to determine the relative copy number and transcript level of the analysed genes. The relative copy number of pPT81-crtS and pPT86-crtR varied between 1 and 10 copies per genome in the transformants and the number of the two plasmids was generally different in the co-transformants (Table 4). The relative transcript level of the *crtS* and *crtR* genes were relatively low compared to the *actA* (varied between 0.002 and 1.32) in all transformants, even if the copy number of the plasmid was high. Interestingly, the relative transcript level of *crtR* remained low (relative transcript level was 0.05–0.1 referred to *actA*), but that of the *crtS* increased significantly in the transformants MS12 + pPT81-crtS + pPT86-crtR. Similarly, 3–50 times higher relative transcript level of *crtS* was observed in the transformants MS12 + pCA8lf/1 + pPT81-crtS in comparison with MS12 + pPT81-crtS. Copy number and relative transcript level of the *crtW* gene did not change significantly in the transformants MS12 + pCA8lf/1 + pPT81-crtS in comparison with the recipient MS12 + pCA8lf/1. Southern hybridization patterns verified that transformants harbour the introduced foreign DNA as autonomously replicating plasmids, but plasmid rearranges could also be suggested (Fig. 5).

Carotenoid content of the transformants (10 isolates per each transformation experiment) was analysed after the 10th cultivation cycle by HPLC technique in independent biological replicates. MS12 and MS12 + pCA8lf/1 strains were used as controls and the average carotenoid contents are shown in Table 4. In transformants carrying only the *crtR* gene, a slight increase was observed in the total carotenoid content, but the carotenoid composition did not changed significantly in comparison with MS12. In

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Ast_DQ002007        MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_DQ201828       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_HG939455       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
Ast_KJ783313        MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_HG939453       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_HG939452       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_DQ202402       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_JQ342969       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_JQ342970       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_JQ342968       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTG 60
CrtS_EU713462       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTD 60
CrtS_JQ342973       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPSHTNYFTGNFLDILSARTG 60
CrtS_JQ342972       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPDHTNYFTGNFLDILSARTG 60
CrtS_JQ342971       MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPDHTNYFTGNFLDILSARTG 60
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Ast_DQ002007        EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_DQ201828       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_HG939455       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
Ast_KJ783313        EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_HG939453       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_HG939452       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_DQ202402       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_JQ342969       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_JQ342970       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_JQ342968       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_EU713462       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_JQ342973       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_JQ342972       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPEVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
CrtS_JQ342971       EEHAKYREKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDG 120
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Ast_DQ002007        VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_DQ201828       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_HG939455       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
Ast_KJ783313        VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_HG939453       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_HG939452       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_DQ202402       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_JQ342969       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_JQ342970       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_JQ342968       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_EU713462       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_JQ342973       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_JQ342972       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
CrtS_JQ342971       VVTAEGEAHKRHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGE 180
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Ast_DQ002007        KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
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CrtS_HG939455       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
Ast_KJ783313        KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_HG939453       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_HG939452       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
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CrtS_JQ342969       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_JQ342970       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_JQ342968       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_EU713462       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_JQ342973       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_JQ342972       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
CrtS_JQ342971       KKATRLETEGVDVKDWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLD 240
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Ast_DQ002007        SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
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CrtS_HG939455       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
Ast_KJ783313        SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_HG939453       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_HG939452       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_DQ202402       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_JQ342969       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_JQ342970       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_JQ342968       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIGLMEQKKRAVLGSASDQAVDKKDV 300
CrtS_EU713462       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_JQ342973       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_JQ342972       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
CrtS_JQ342971       SFKAIMWDFVPYFRTMKRRHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDV 300
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CrtS_DQ201828       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_HG939455       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
Ast_KJ783313        QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_HG939453       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_HG939452       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
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CrtS_JQ342969       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_JQ342970       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_JQ342968       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_EU713462       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_JQ342973       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
CrtS_JQ342972       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSAVLTWMFHRLSEDKA 360
CrtS_JQ342971       QGRDILSLLVRANIAANLPESQKLSDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKA 360
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Ast_DQ002007        VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
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CrtS_HG939455       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
Ast_KJ783313        VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_HG939453       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_HG939452       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_DQ202402       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_JQ342969       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_JQ342970       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_JQ342968       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_EU713462       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_JQ342973       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_JQ342972       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
CrtS_JQ342971       VQDKLREEICQIDTDMPTLDELNALPYLEAFVKESLRLDPPSPYANRECLKDEDFIPLAE 420
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Ast_DQ002007        PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
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CrtS_HG939455       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
Ast_KJ783313        PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
CrtS_HG939453       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
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CrtS_JQ342969       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
CrtS_JQ342970       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
CrtS_JQ342968       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
CrtS_EU713462       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
CrtS_JQ342973       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
CrtS_JQ342972       PVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGEDAEEFRPERWLEDVTDSLNSIEA 480
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Ast_DQ002007        PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
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CrtS_HG939455       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
Ast_KJ783313        PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_HG939453       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_HG939452       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_DQ202402       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_JQ342969       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_JQ342970       PYGHQASFISGPRACFGWRSAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_JQ342968       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_EU713462       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_JQ342973       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_JQ342972       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
CrtS_JQ342971       PYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPIISHPEYEHITLIISRPRIV 540
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Ast_DQ002007        GREKEGYQMRLQVKPVE 557
CrtS_DQ201828       GREKEGYQMRLQVKPVE 557
CrtS_HG939455       GREKEGYQMRLQVKPVE 557
Ast_KJ783313        GREKEGYQMRLQVKPVE 557
CrtS_HG939453       GREKEGYQMRLQVKPVE 557
CrtS_HG939452       GREKEGYQMRLQVKPVE 557
CrtS_DQ202402       GREKEGYQMRLQVKPVE 557
CrtS_JQ342969       GREKEGYQMRLQVKPVE 557
CrtS_JQ342970       GREKEGYQMRLQVKPVE 557
CrtS_JQ342968       GREKEGYQMRLQVKPVE 557
CrtS_EU713462       GREKEGYQMRLQVKPVE 557
CrtS_JQ342973       GREKEGYQMRLQVKPVE 557
CrtS_JQ342972       GREKEGYQMRLQVKPVE 557
CrtS_JQ342971       GREKEGYQMRLQVKPVE 557
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Fig. 3 continued

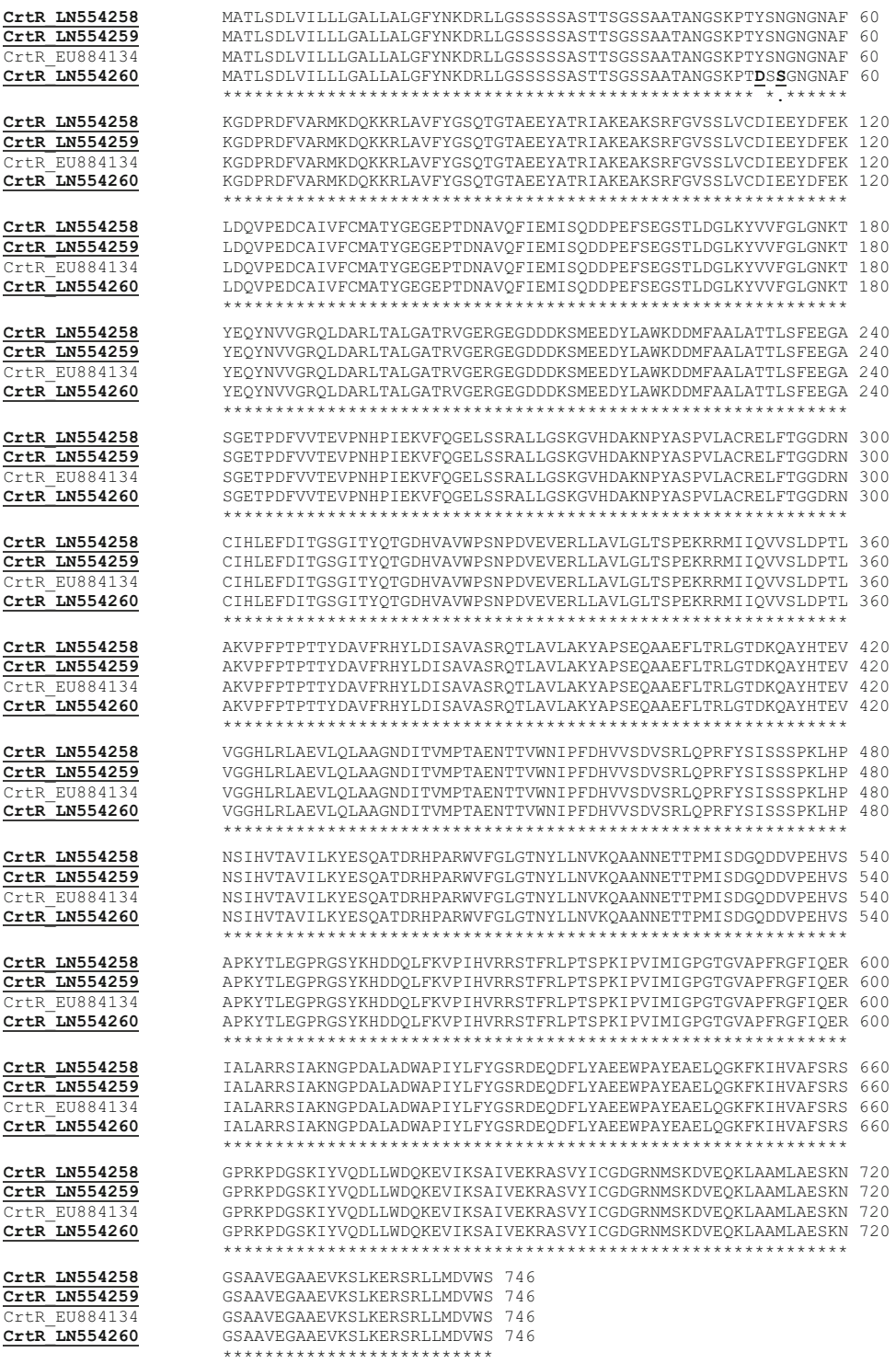

Fig. 4 Comparison of the predicted CrtR amino acid sequences of different *X. dendrorhous* strains. Different positions are *underlined* and given in *bold*. GenBank accession numbers, LN554258, LN554259, LN554260, corresponding to the sequences of CrtR of ATCC 24229, SZMC 9073 and CBS 6938, respectively are also *underlined* and in *bold face*

transformants harbouring only the *crtS* as exogenous gene, the β-carotene remained the main carotenoid component, although, enhanced β-cryptoxanthin and zeaxanthin accumulation was observed in comparison with the untransformed MS12. Canthaxanthin, echinenone and astaxanthin production was also detected in low amount in most of these transformants; the β-carotene hydroxylase and ketolase activity of the CrtS in transformants was verified. Co-expression of the *crtS* and *crtR* genes increased the total carotenoid content, and only slightly changed the carotenoid composition in comparison with expression only the *crtS* gene. In transformants MS12 + pCA8lf/1 + pPT81-

Table 4 Total carotenoid content and composition of transformants, MS12 and MS12 + pCA8lf/1 strains

Strain	Relative copy number of *crtS* or *crtR* (copy/genome)	Total carotenoid	β-Carotene	β-Cryptoxanthin	Zeaxanthin	Echinenone	Canthaxanthin	Lycopene	γ-Carotene	Astaxanthin
MS12	–	424 ± 34	280 ± 12	24 ± 4	8 ± 3	–	–	11 ± 3	23 ± 3	–
MS12 + pPT81-crtS/1–10	*crtS*: 1–10	469 ± 35	315 ± 19	43 ± 6	18 ± 3	3 ± 1	3 ± 1	11 ± 2	33 ± 4	2 ± 1
MS12 + pPT86-crtR/1–10	*crtR*: 2–4	462 ± 42	321 ± 17	29 ± 4	10 ± 3	–	–	11 ± 2	31 ± 3	–
MS12 + pPT81-crtS + pPT86-crtR/1–10	*crtS*: 2–5 *crtR*: 1–3	554 ± 49	371 ± 27	55 ± 7	19 ± 3	3 ± 2	4 ± 2	15 ± 3	36 ± 3	4 ± 2
MS12 + pCA8lf/1	–	541 ± 48	29 ± 5	25 ± 5	5 ± 2	115 ± 18	145 ± 14	28 ± 2	41 ± 4	3 ± 2
MS12 + pCA8lf/1 + pPT81-crtS/1–10	*crtS*: 1–9	508 ± 47	44 ± 7	39 ± 7	11 ± 4	132 ± 15	170 ± 20	25 ± 5	45 ± 7	3 ± 1

The presented values [μg g^{-1} (dry mass)] are averages of ten transformants from three independent carotenoid extraction and measurements. Fluctuations in plasmid copy number in transformants are also represented

crtS/1–10, the amount of echinenone, canthaxanthin and β-cryptoxanthin increased significantly; the average canthaxanthin content in some of these strains was around 190 μg g^{-1} (dry mass). The γ-carotene accumulation increased in most of the transformants in comparison with MS12 (Table 4). Fluctuation in the total carotenoid content and plasmid copy number was also observed during the consecutive cultivation cycles in the transformants.

Discussion

In this study, the *crtS* and *crtR* genes were amplified from cDNA of three *X. dendrorhous* strains; two of them were wild-type for the carotenoid production (CBS 6938 and ATCC 24229) and one of them was an astaxanthin over-producing mutant strain (SZMC 9073). Nucleotide sequence polymorphisms, observed among *crtS* of ATCC 24229, SZMC 9073, CBS 6938 and several GenBank sequences did not affected the amino acid sequences, while few amino acid differences were found in the case of EU713462, JQ342968, JQ342970, JQ342971, JQ342972 and JQ342973 GenBank sequences (Fig. 3 and S1). The amino acid substitutions in the investigated sequences did not affected the oxygen-binding site (^{339}AGYETS^{344}) and heme-binding (^{488}FISGPRACFG^{497}) domains, nor the domain involved in the maintenance of the three dimensional structure (^{394}ESLR^{397}), which determined Álvarez et al. (2006). At the same time, one of the four potential *N*-glycosylation motifs [NX(T/S)] (Álvarez et al. 2006) was concerned by amino acid substitutions at position 42 of the JQ342971, JQ342972 and JQ342973 sequences (Fig. 3).

The *crtR* of CBS 6938 gene and the deduced amino acid sequence differed in six nucleotides and two amino acids, respectively, from those the corresponding sequences of ATCC 24229 and SZMC 9073 and those determined by Alcaíno et al. (2008) (Figs. 4, S2). The amino acid substitutions in CBS 6938 did not affect the FMN, FAD and NAD(P)H binding domains (the P450 binding regions are located in the FMN binding domain) or the amino terminal transmembrane region, described by Alcaíno et al. (2008).

Previously, Palágyi et al. (2006) determined the carotenoid content the *X. dendrorhous* strains used in this study, where CBS 6938, ATCC 24229 and SZMC 9073 (named ATCC 24229/S119 in that study) had 260, 177 and 756 μg g^{-1} (dry weight) total carotenoid and 98, 41 and 274 μg g^{-1} (dry weight) astaxanthin content, respectively. The astaxanthin/total carotenoid quotient (A/TC) value of CBS 6938 is 0.377, which is significantly higher, than that of ATCC 24229 (A/TC = 0.232) supposing an active CrtR in that strain. Non-functional protein must be lead to β-carotene accumulating strain: mutations in the *X. dendrorhous* CBS 6938 *crtR* gene lead to astaxanthin non-

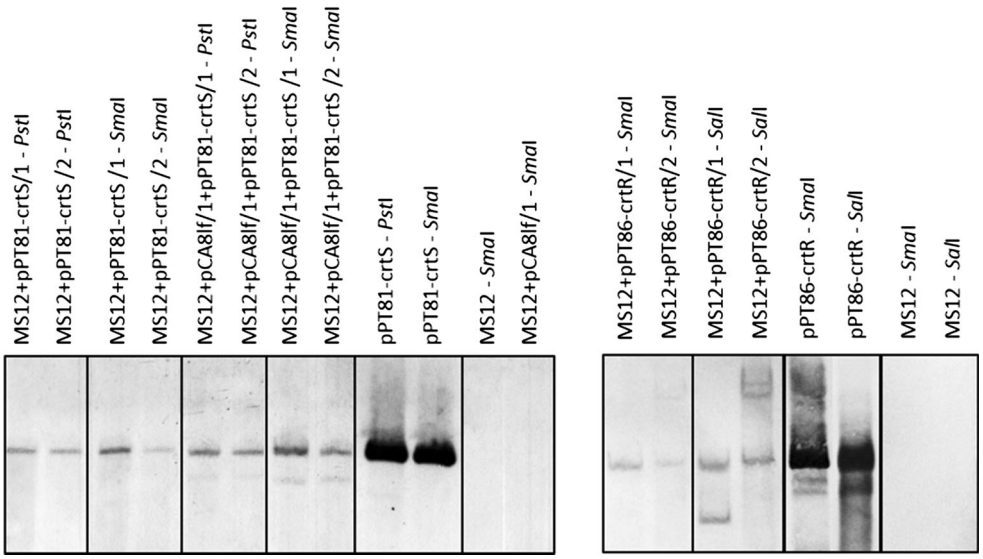

Fig. 5 Southern hybridization patterns. The total DNA of transformants MS12 + pPT81-crtS, MS12 + pPT86-crtR, MS12 + pPT81-crtS + pPT86-crtR and MS12 + pCA8lf/1 + pPT81-crtS was digested with *Sma*I, *Pst*I, or *Sal*I. XantcrtS1–XantcrtS2 and XantcrtR1–XantcrtR2 primer pairs were used for the synthesis of gene probes. Digested plasmids were used as positive control, while the MS12 and MS12 + pCA8lf/1 as negative controls. Bands with same size indicate that the transformants maintain the introduced DNA in circular plasmids, but plasmid rearranges can be also presumed

producing and β-carotene accumulating mutant strain (Alcaíno et al. 2008). It was also concluded by Alcaíno et al. (2008) that the *X. dendrorhous* CBS 6938 strain could be haploid and our results strongly suggest also, that the genome of this strain harbours only one *crtR* gene copy.

The astaxanthin production ability of SZMC 9073 (A/TC = 0.362), which is a derivative of the wild-type ATCC 24229 made by γ-irradiation followed by UV irradiation, is significantly higher, than that of the wild-type strain (A/TC = 0.232; Palágyi et al. 2006). Amino acid sequences of the putative CrtS and CrtR proteins of these two strains did not differ, indicating that mutations in other genes may be in the background of the different astaxanthin producing abilities (Figs. 3, 4).

Frequency of transformation with pPT81-crtS and/or pPT86-crtR plasmids was found to be similar to those detected in our previous works where autonomously replicating vectors were used to transform *M. circinelloides* (Csernetics et al. 2011). The transformants proved to be mitotically stable after ten consecutive cultivation cycles on minimal (YNB) and complete (MEA) media. The relative plasmid copy number varied between 1 and 10 copy/genome and fluctuations in the copy number was observed after the consecutive cultivation cycles (Table 4). Previously, it was shown that transformants of *M. circinelloides* and closely related species (i.e. *Rhizopus oryzae*) carrying autonomously replicating vectors are often unstable and the copy number of the circular plasmid remains low (Anaya and Roncero 1991; Ibrahim and Skory 2006; Mertens et al. 2006). In contrast with these results, we found that *M. circinelloides* transformants are mitotically stable and cultivation on complete medium did not result in a decrease in the plasmid copy number, however, fluctuations were observed generally, irrespectively of that the transferred gene was homologous or heterologous (Csernetics et al. 2011). Transformation of the MS12 with autonomously replicating plasmids containing the *Paracoccus* sp. N81106 *crtW* gene and endogenous isoprene biosynthesis gene resulted in 0.07–1 and 1–7 plasmid copy per host genome, respectively (Csernetics et al. 2011). It seems that copy number of the plasmid, which harbours heterologous fungal gene are similar to those in *Mucor*, which carries endogenous gene. The relative transcript level of the *crtS* and *crtR* genes (varied between 0.002 and 1.32) was low in comparison with the *Mucor actA* gene. Interestingly, when the *crtS* and *crtR* or the *crtS* and *crtW* genes were co-expressed (MS12 + pPT81-crtS + pPT86-crtR/1–10 and MS12 + pCA8lf/1 + pPT 81-crtS/1–10 transformants), the relative transcript level of the *crtS* gene increased 3–50 fold (relative transcript level was 0.1–3, referred to *actA*) in comparison with expression of the single *crtS*. However, overexpression of endogenous genes or the expression the *crtW* led to significantly higher relative transcript levels (Csernetics et al. 2011). In spite of the relatively high copy number detected by qPCR, rearranges may prevent the transcription in a major part of the plasmids. Actually, the Southern hybridization patterns verified the presence of rearranged plasmids (Fig. 5). Indeed, rearrangements of the introduced DNA were often verified in *Mucor* transformants (Monfort et al. 2003; Csernetics et al. 2011).

Two genes are responsible for the conversion of β-carotene to astaxanthin in *X. dendrorhous*: the *crtS* encoding a cytochrome-P450 hydroxylase (astaxanthin synthase) is responsible for the addition of hydroxyl and keto groups to the β-ionone rings of β-carotene and the *crtR* encoding a cytochrome-P450 reductase, as electron donor, is required to the CrtS for addition of functional groups to the substrate (Álvarez et al. 2006; Ojima et al. 2006; Alcaíno et al. 2008). Overexpression of the *crtS* gene in *X. dendrorhous* resulted in higher level of astaxanthin production (Contreras et al. 2013), while point mutations in the gene led to astaxanthin non-producing and β-carotene accumulating yellow mutants (Álvarez et al. 2006; Ojima et al. 2006; Barbachano-Torres et al. 2014). Deletion of the *crtR* gene was not lethal, suggesting an alternative electron donor in *X. dendrorhous*, but the transformants were not able to accumulate astaxanthin, indicating that the *crtS* and *crtR* genes are also necessary for the conversion of β-carotene to astaxanthin (Alcaíno et al. 2008). In contrast with previous findings, where only the β-carotene hydroxylase activity of CrtS was demonstrated by expression of the coding gene in *M. circinelloides* (Álvarez et al. 2006), in our study, the β-carotene ketolase activity was also detected, although the ketolase activity of the enzyme was very weak. The carotenoid composition of the transformants MS12 + pPT81-crtS/1–10 were nearly equal; minor differences may occur in consequence of the fluctuation of the plasmid copy number. The average accumulated β-cryptoxanthin [43 μg g^{-1} (dry mass)] and zeaxanthin [18 μg g^{-1} (dry mass)] was about 1.8–2.2 times higher in these isolates in comparison with the untransformed MS12 [24 μg g^{-1} (dry mass) and 8 μg g^{-1} (dry mass), respectively] (Table 4). Expression of the *crtS* of *X. dendrorhous* VKPM Y2410 in *M. circinelloides* led to increased β-cryptoxanthin (140–160 %) and zeaxanthin (190–330 %) content (Álvarez et al. 2006). Rise in zeaxanthin accumulation was similar, but in β-cryptoxanthin was slightly higher (130–220 %) in MS12 + pPT81-crtS/1–10 than in those transformants. In contrast with the findings of Álvarez et al. (2006), low amounts of canthaxanthin, echinenone (β,β-carotene-4-on) and astaxanthin were also detected in these transformants (Table 4); the amount of ketolated β-carotene derivatives was near equal in all transformants. In our previous study, expression of the *Paracoccus* sp. N81106 *crtZ* gene (encoding a β-carotene hydroxylase) under the regulation of *gpd1* promoter and terminator regions on autonomously replicating plasmid in *M. circinelloides* resulted in 41 μg g^{-1} (dry mass) β-cryptoxanthin and 10 μg g^{-1} (dry mass) zeaxanthin content (Papp et al. 2006), while expression of the bacterial *crtW* (encoding a β-carotene ketolase) gene under the same conditions resulted in 6–10 μg g^{-1} (dry mass) canthaxanthin, 11–17 μg g^{-1} (dry mass) echinenone and 3 μg g^{-1} (dry mass) astaxanthin (Papp et al. 2006; Csernetics et al. 2011). In comparison with these results, similar or higher zeaxanthin and β-cryptoxanthin, but significantly lower canthaxanthin, echinenone and astaxanthin accumulation could be achieved by expression the *crtS* in MS12. Besides the increased total carotenoid and hereby xanthophyll content, co-expression of *crtS* and *crtR* in *M. circinelloides* lead to a slight increase in the proportion of xanthophylls referred to total carotenoid in comparison with transformants harbouring the single pPT81-crtS plasmid (Table 4). Low amounts of canthaxanthin, echinenone and astaxanthin could be also detected in MS12 + pPT81-crtS + pPT86-crtR/1–10 transformants (Table 4).

β-Carotene accumulation was achieved by heterologous expression of the *X. dendrorhous crtI* (encoding a phytoene desaturase) and *crtYB* (encoding a phytoene synthase/lycopene cyclase) genes in the carotenoid non-producing *Saccharomyces cerevisiae* and was enhanced by overexpression of the *S. cerevisiae BTS1* gene (encoding the geranylgeranyl pyrophosphate synthase). Co-expression of the *crtS* and *crtR* genes in that β-carotene overproducing mutant led to low amount of astaxanthin accumulation [3 μg g^{-1} (dry weight), which is significantly lower, than expression of the *crtZ* and *crtW* genes in the same mutant], however expression only the *crtS* gene, did not resulted conversion of β-carotene to astaxanthin (Ukibe et al. 2009). Ukibe et al. (2009) presumed that CrtS has high specificity for its own reductase: the endogenous *S. cerevisiae* cytochrome-P450 reductase was not effective for functional expression of *X. dendrorhous* CrtS. However, activity of *crtS* in *M. circinelloides* did not required the presence of the *crtR* gene and co-expression of the *crtS* and *crtR* genes only slightly boosted the xanthophyll accumulation suggesting an active cytochrome-P450 reductase capable to provide the CrtS with electrons. Although, expression of the *crtS* and *crtR* genes in *S. cerevisiae* and *M. circinelloides* led to significantly less effective conversion of β-carotene to astaxanthin as in *X. dendrorhous*.

By co-expressing *crtS* and *crtW* in the MS12 + pCA8lf/1 + pPT81-crtS/1–10 transformants, significantly increased canthaxanthin and echinenone content could be achieved [170 and 132 μg g^{-1} (dry mass), respectively, the amount of canthaxanthin reached 190 μg g^{-1} (dry mass)] in comparison with MS12 + pCA8lf/1 (Table 4).

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