Appl Microbiol Biotechnol (2006) 69: 526–531
DOI 10.1007/s00253-005-0026-6

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Heterologous expression of astaxanthin biosynthesis genes in *Mucor circinelloides*

Received: 4 February 2005 / Revised: 5 May 2005 / Accepted: 19 May 2005 / Published online: 21 July 2005

Abstract Most *Mucor* species accumulate β-carotene as the main carotenoid. The *crtW* and *crtZ* astaxanthin biosynthesis genes from *Agrobacterium aurantiacum* were placed under the control of *Mucor circinelloides* expression signals. Expression vectors containing the bacterial genes were constructed, and PEG-mediated transformations were performed on a selected *M. circinelloides* strain. Transformants that exhibited altered carotene production were isolated and analyzed. Southern analysis showed that all plasmids behave as autoreplicative elements. Northern analysis detected the actual heterologous transcription products, whereas thin layer chromatography and high-performance liquid chromatography studies revealed the presence of new carotenoid compounds and intermediates among the transformants.

Introduction

Carotenoids are one of the most widely distributed groups of natural pigments. They are used in the food, pharmaceutical, and cosmetic industries and also as color additives in feedstuffs. Their effective antioxidant property (Naguib 2000; Paiva and Russel 1999), linked to the potential preventive action against various types of cancer (Nishino et al. 2002; Chew et al. 1999) and an enhancement of the immune response (Hughes 1999), makes them important in the human diet.

Most of the current industrial carotenoid production is performed by chemical methods, and only a few natural carotenoid compounds are derived from cheap plant sources (Bhosale 2004). Mixtures of natural carotenoids seem to be more advantageous to suppress carcinogenesis than single carotenoids, for example, β-carotene (Paiva and Russel 1999). All these factors contributed to the renewed and increasing interest in new sources of carotenoids of microbial origin and especially the valuable orange-red ketocarotenoids (such as astaxanthin and canthaxanthin).

In zygomycetes fungi (e.g., in *Blakeslea trispora* and *Phycomyces blakesleeanus*) β-carotene is the predominant carotenoid, whereas astaxanthin is accumulated in the basidiomycetous yeast *Xanthophyllomyces dendrorhous*. Besides the several advantages, the usage of these fungi for industrial carotenoid production has some drawbacks. For example, there is a decrease in carotene accumulation of *P. blakesleeanus* in shaken culture, therefore, surface culture method must be elaborated; the growth temperature for *B. trispora* and *P. blakesleeanus* must be below 25°C. Low temperature is also an important factor for *X. dendrorhous*; moreover, this latter fungus has thick cell wall, so it must be partially lysed before using as animal feed (Iturriaga et al. 2005).

In the recent years, great progress has been made to clarify the carotenoid biosynthesis and it's regulation in the β-carotene producer zygomycete *Mucor circinelloides*. Several reasons make *M. circinelloides* a good candidate for carotene production studies: (1) Species of the genus *Mucor* are presently applied for industrial production of various enzymes, such as proteases, lipases, or rennin. (2) Structural genes involved in the carotenogenic pathway have been cloned and characterized (Velayos et al. 2000a,b, 2003), a regulatory gene also has been isolated and ana-

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lyzed (Navarro et al. 2000). (3) Morphological dimorphism of *M. circinelloides* is a great advantage in relation to potential biotechnological applications. Yeast morphology is preferred by the fermentation industry because it allows the growth in submerged condition; the biomass production is higher, and the cells are more easily separated from the media. Moreover, yeast cells grow well at about 30°C (Iturriaga et al. 2001). (4) Classical genetic techniques are well established in *B. trispora* and *P. blakesleeanus*. However, it is quite difficult to apply the molecular techniques to these fungi, because of the lack of an efficient transformation system. The existence of well-developed transformation systems (Appel et al. 2004; Wolff and Arnau 2002; Arnau and Strøman 1993; van Heeswijck and Roncero 1984) and the ability to express exogenous genes (Benito et al. 1992; Iturriaga et al. 1992) make *M. circinelloides* more amenable to molecular techniques than the other zygomycetes traditionally used in carotenogenic studies (Iturriaga et al. 2000).

At present, more than 150 structural carotenogenic genes have been isolated from bacteria, algae, plants, and fungi (Schmidt-Dannert et al. 2000). This progress in the molecular biology of carotenoid biosynthesis has opened up the possibility of modifying and engineering the carotenoid pathway in microorganisms (Sandmann 2001). Metabolic engineering of the astaxanthin biosynthetic pathway of *X. dendrorhous* was described recently by Verdoes et al. (2003). Carotenoid biosynthesis was successfully performed in otherwise colorless organisms, such as *Escherichia coli* (Steiger and Sandmann 2004; Schmidt-Dannert et al. 2000; Lee et al. 2003; Wang et al. 1999; Takaichi et al. 1996; Misawa et al. 1995), *Saccharomyces cerevisiae*, or *Candida utilis* (Misawa and Shimada 1998), by introduction and modification of heterologous carotenogenic genes. The objective of the present work was to modify the carotenoid production of *M. circinelloides* by expressing heterologous genes for the production of oxygenated β-carotene derivatives.

Agrobacterium aurantiacum is a marine, astaxanthin-producing bacterium. Misawa et al. (1995) isolated the carotenoid biosynthesis gene cluster of this organism. The products of two of the genes in this cluster (*crtZ*, encoding β-carotene hydroxylase, and *crtW*, encoding β-carotene ketolase) mediate the oxygenation reactions converting β-carotene to astaxanthin (Fig. 1). These two bacterial genes were placed under the control of the regulatory regions of the *M. circinelloides* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd1*; Wolff and Arnau 2002) and used in our study to transform *M. circinelloides*.

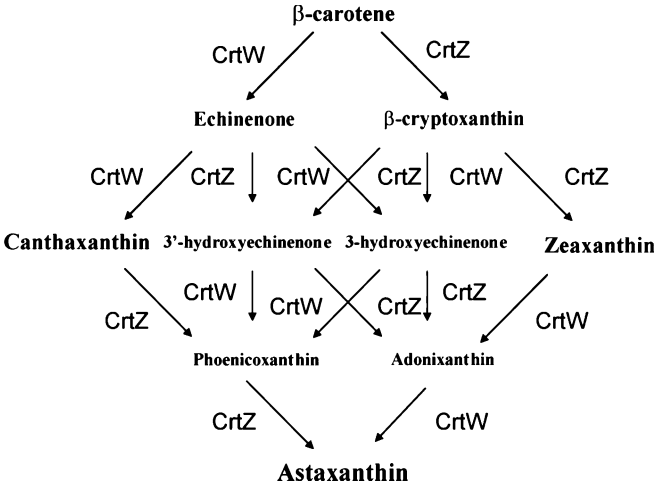

Fig. 1 Astaxanthin biosynthetic pathways and various possible intermediates in *A. aurantiacum*, on the basis of Misawa et al. (1995)

Materials and methods

Strains and growth conditions

MS12, a *leuA*$^{-}$*pyrG*$^{-}$ mutant (Benito et al. 1992) derived from the wild-type *M. circinelloides* strain CBS277.49, was used in the transformation experiments. For both nucleic acid and carotenoid extraction, *Mucor* strains were cultured in minimal medium (YNB: 5% glucose, 0.05% yeast nitrogen base without amino acids, 0.15% $(NH_4)_2SO_4$, 0.15% glutamic acid), supplemented with leucine or uracil (200 or 400 μg ml^{-1}, respectively) as required, and grown for 4 days under continuous light.

E. coli DH5α was used for all cloning experiments and plasmid amplifications.

Molecular techniques

The genomic DNA of *M. circinelloides* was prepared from mycelia disrupted with pestle and mortar in liquid nitrogen and purified in a 0.5 mg ml^{-1} bis-benzimide–CsCl density gradient (Iturriaga et al. 1992). Total RNA was extracted by the guanidine isothiocyanate method (Choi et al. 1988). General procedures for plasmid DNA preparation, cloning and transformation of *E. coli*, and Southern and Northern blotting were performed following standard procedures (Sambrook et al. 1989). The nucleic acids for hybridization were labeled with the digoxigenin-based "PCR DIG Probe Synthesis Kit" (Roche), following the instructions of the manufacturer. "CDP-Star" chemiluminescent alkaline phosphatase substrate (Roche) was used for the immunological detection of nucleic acid blots, according to the conditions recommended by the supplier.

Construction of expression vectors

Expression vectors were constructed by inserting *crtW* and *crtZ* genes of *A. aurantiacum* (Misawa et al. 1995) into the *E. coli* vector pBluescript II, respectively. To assure the strong expression in *Mucor*, promoter and terminator regions of the *M. circinelloides* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd1*; Wolff and Arnau 2002) were placed upstream and downstream of the bacterial genes (e.g., *crtW* and *crtZ*), respectively. For the construction of pPT43, the *gpd1* promoter and terminator regions (EMBL

Accession No. AJ293012, nucleotides 13 to 799 and 1,831 to 2,470, respectively) were amplified by PCR from *M. circinelloides* chromosomal DNA using *Not*I- and *Xho*I-modified specific primers and then cloned into pBluescript II SK+ (Stratagene). The genes of *A. aurantiacum* (*crt* gene cluster, EMBL Accession No. D58420) were amplified from the plasmid pAK96K (Misawa et al. 1995; kindly provided by N. Misawa), by PCR with similarly modified primers, and then cloned into the *Not*I-*Xho*I site of pPT43, giving rise to pPT43-crtW and pPT43-crtZ, respectively. The *M. circinelloides pyrG* gene encoding orotidine-5′-monophosphate decarboxylase (Benito et al. 1992; EMBL Accession No. M69112) was cloned into pPT43-crtZ as selection marker, while at the same time, the *Mucor leuA* gene encoding α-isopropylmalate isomerase (Roncero et al. 1989) was introduced into pPT43-crtW. These steps resulted in the transforming vectors pPT50 and pPT51, respectively.

Transformation

Protoplasts of *M. circinelloides* were prepared as described earlier (Iturriaga et al. 1992). Washed germlings were treated with 1.5 mg ml^{-1} $Novozyme_{234}$ (Novo Industries) and 4–12 U ml^{-1} Streptozyme in the presence of 0.5 M sorbitol and 10 mM sodium phosphate buffer, pH 6.5. Polyethylene glycol mediated transformations of protoplasts were performed according to Van Heeswijck and Roncero (1984). Transformants were selected on the basis of auxotrophy complementation and color change.

Carotenoid extraction and analysis

Carotenoids were extracted from 500 mg mycelial powder with 500 μl acetone and vortexing. This extraction step was repeated until the pellet was found to be devoid of pigments. Extracts were combined and partitioned with an equal volume of 10% diethyl ether in petroleum ether. To facilitate the separation and to remove dissolved acetone, 1 ml distilled water was added. The petroleum ether fractions were combined and dried under nitrogen. The pigments obtained were subjected to thin layer chromatography (TLC) on silica gel ($60F_{254}$, Merck), which was developed with acetone/petroleum ether (20:80). For high-performance liquid chromatography (HPLC), samples and standards were analyzed by using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with an autoinjector, a DR5 solvent-delivery system, a column thermostat, and a diode-array UV detector. The dried samples were redissolved in 1 ml tetrahydrofuran directly before the analysis, and 5 μl was subjected to HPLC analysis on a Zorbax SB C_8 5 μm column (5×250 mm). Isocratic separation was performed with acetonitrile/acetone (9:1, v/v) as mobile phase, at a flow rate of 0.6 ml min^{-1}. The detection wavelength was 480 nm. To identify the purified carotenoids, the following standards were used: synthetic astaxanthin and β-carotene (Sigma), echinenone, β-cryptoxanthin, zeaxanthin (DHI Water and Environment), and canthaxanthin (Roche).

Results

Transformation

The main data and the results of the transformation experiments are presented in Table 1. The yellow color of the fungus changed to orange in transformants harboring pPT51 or both plasmids, but not in those with pPT50 alone. The fact that maintenance of the selective conditions was necessary for the transformants to retain the phenotypes, as well as Southern blot analysis, suggests that plasmids in all of the transformants tested were autoreplicative (data not shown). Cotransformant strains maintained both plasmids also under selective conditions. To our knowledge, this is the first report on the efficient cotransformation of a *Mucor* strain with the use of more than one plasmid.

Analysis of carotenoid production in the transformants

Transcription of the genes introduced in the transformants was demonstrated through Northern analysis (Fig. 2). TLC revealed the existence of new derivatives of β-carotene in the transformant strains, but all the transformants still produced β-carotene as the main carotenoid. Table 2 lists the average carotenoid contents of the different types of transformants, measured by HPLC. The transformants denoted MS12-Z (harboring pPT50) produced zeaxanthin (3,3′-dihydroxy-β,β-carotene) and β-cryptoxanthin (3-hydroxy-β,β-carotene) in considerable amounts. Interestingly, the same carotene pattern could also be observed in the case of the wild-type strain, MS12. This result is in agreement with an earlier observation (A. Velayos, unpublished data), where these derivatives were detected in *M. circinelloides* by HPLC analysis. The fact that wild-type *M. circinelloides* produces the hydroxylated derivatives of β-carotene suggests the existence of β-carotene hydroxylase activity in *M. circinelloides*. As the previous studies on the

Table 1 Data on the transformation experiments

Expression vector	Introduced gene	Phenotype of transformants	Number of transformants per 10^5 protoplasts
pPT50	*crtZ*	Leu^-, yellow	51
pPT51	*crtW*	Ura^-, orange	25
pPT50, pPT51[a]	*crtW*, *crtZ*	Prototrophic, orange	93

[a]Cotransformation

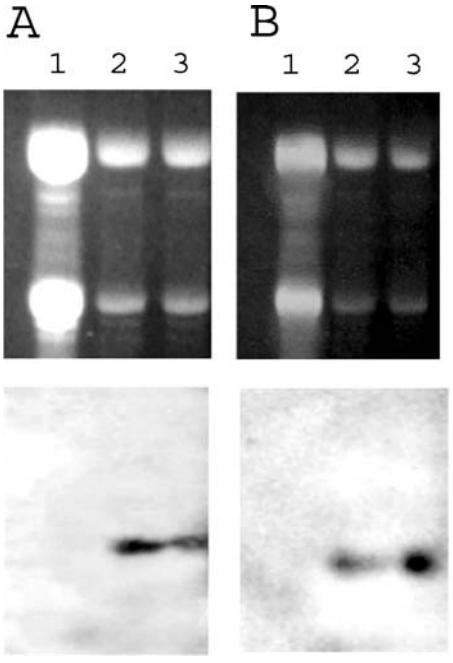

Fig. 2 Northern hybridization with the digoxigenin-labeled *crtW* (**a**) and *crtZ* (**b**) gene probes. **a** Lanes MS12, MS12-W, MS12-ZW. **b** Lanes MS12, MS12-Z, MS12-ZW

carotenoid pathway in *Mucor* concentrated to the formation of β-carotene, the carotenoid detection methods have been optimized according to this goal, and further conversion processes involving β-carotene have not yet been detected. The presumable β-carotene hydroxylase of *M. circinelloides* also remains to be characterized. The MS12-Z transformants produced zeaxanthin and β-cryptoxanthin in significantly higher quantity than did MS12, suggesting the expression of the bacterial gene.

Both the transformants containing pPT51 (MS12-W) and the cotransformants harboring pPT50 and pPT51 (MS12-ZW) produced β-carotene, β-cryptoxanthin, echinenone (β,β-carotene-4-one), canthaxanthin (β,β-carotene-4,4′-dione), and a small amount of astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione). MS12-W produced astaxanthin and β-cryptoxanthin, which also requires the existence of natural *M. circinelloides* hydroxylase activity.

Discussion

Introduction of only a β-carotene ketolase gene seems then to be enough to force astaxanthin production in *M. circinelloides*, because β-carotene and its hydroxylated derivatives are already present in *M. circinelloides* and could serve as substrates for this conversion. Table 2 reveals that MS12-W contained more astaxanthin and less canthaxanthin and β-cryptoxanthin than did MS12-ZW. No zeaxanthin was detected in either MS12-W or MS12-ZW. Fraser et al. (1997) characterized the activity and substrate specificity of astaxanthin biosynthetic enzymes from *A. aurantiacum* and *Alcaligenes* sp. strain PC-1: the product of gene *bkt* (encoding β-carotene oxygenase) of *Haematococcus pluvialis* and the product of *crtZ* of *Erwinia uredovora*, in vitro. They demonstrated by means of specific enzyme assays that all these enzymes are bifunctional. This means that the CrtZ enzymes can form zeaxanthin from β-carotene via β-cryptoxanthin and also astaxanthin from canthaxanthin via phoenicoxanthin (3-hydroxy-β,β-carotene-4,4′-dione), and CrtW/BKT enzymes can form canthaxanthin from β-carotene via echinenone as well as adonixanthin (3,3′-dihydroxy-β,β-carotene-4-one) from zeaxanthin. However, one or other substrate may be preferred. In previous experiments, only small increments in the canthaxanthin level led to the elimination of zeaxanthin formation by the CrtZ enzyme of the marine bacterium (Fraser et al. 1997). Assumption of a similar situation could explain why zeaxanthin was not detected in the *crtW*-harboring *M. circinelloides* transformants. Interestingly, the β-cryptoxanthin level was comparable with that of MS12.

Proper expression of the bacterial enzymes (e.g., *crtZ* and *crtW*) requires that the genes encoding them be modified to resemble *Mucor* genes (proper promoters, polyadenylation signals, etc.). The genes were placed under the control of the regulator sequences of the highly expressing *M. circinelloides gpd1*. Promoter of this gene has reported previously as able to drive the expression of the bacterial *Tn5*-derived kanamycin resistance gene in *Mucor* (Appel et al. 2004). To force a high-level expression of the introduced genes, 5% glucose was used in the media, because the expression of *gpd1* is regulated by the carbon source (Wolff and Arnau 2002). However, the ability of the transformants to synthesize new derivatives was limited as compared with their β-carotene production. The effective synthesis possibly requires coordination in the expression among the heterologous and the host genes of the pathway. In *M. circinelloides*, the expressions of the specific genes of β-carotene are coordinated and induced by blue light (Velayos et al. 2000a). A recent model of the β-carotene synthesis in *M. circinelloides* proposes an aggregate of carotenogenic enzymes constituted by two molecules of phytoene synthase/lycopene cyclase and four phytoene

Table 2 Average carotene production of transformants (μg g^{-1} dry weight)

Strain	Total carotenoids	β-Carotene	Astaxanthin	Cantaxanthin	Zeaxanthin	β-Cryptoxanthin	Echinenone
MS12	223	128	–	–	4	26	–
MS12-Z	249	146	–	–	10	41	–
MS12-W	231	155	3	6	–	20	17
MS12-ZW	210	114	2	13	–	31	17

dehydrogenase units. This aggregate would be anchored in the membrane by the N-terminus of the carRP enzyme (Iturriaga et al. 2001). If this hypothesis is true, maybe the physical separation of the introduced enzymes and the β-carotene solubilized in the lipid bilayer of the membrane may also hinder the conversion of β-carotene to astaxanthin. In some cases, the different codon usage or G+C content could also decrease the expression level of a gene from a widely divergent origin; however, availability of such data concerning *M. circinelloides* is very limited.

In this study, autoreplicative expression vectors containing bacterial astaxanthin biosynthesis genes were constructed, and a β-carotene-producing *M. circinelloides* strain was transformed with them. Analysis of the carotene production in the transformants revealed the existence of new oxygenated derivatives of β-carotene, demonstrating that the *crtZ* and *crtW* genes of *A. aurantiacum* might well be suitable for the production of astaxanthin in *Mucor*, but further investigations are required to optimize their expression in the new host. We are currently developing an expression system where astaxanthin biosynthesis genes will be forced to integrate into the host genome and/or fused to the phytoene synthase/lycopene cyclase encoding genes.

Acknowledgements This research was supported by grants BIO2001-2040 (Spanish Ministerio de Ciencia y Tecnología); SA067/01 (Junta de Castilla y León, Spain); and the Hungarian Scientific Research Fund (OTKA) grants T037471, F046658, and D048537. Dr. N. Misawa is acknowledged for the plasmid pAK96K.

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