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ORIGINAL RESEARCH PAPER

Generation of lycopene-overproducing strains of the fungus *Mucor circinelloides* reveals important aspects of lycopene formation and accumulation

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Abstract
Objectives To generate lycopene-overproducing strains of the fungus *Mucor circinelloides* with interest for industrial production and to gain insight into the catalytic mechanism of lycopene cyclase and regulatory process during lycopene overaccumulation.
Results Three lycopene-overproducing mutants were generated by classic mutagenesis techniques from a β-carotene-overproducing strain. They carried distinct mutations in the *carRP* gene encoding lycopene cyclase that produced loss of enzymatic activity to different extents. In one mutant (MU616), the lycopene cyclase was completely destroyed, and a 43.8% (1.1 mg/g dry mass) increase in lycopene production was observed in comparison to that by the previously existing lycopene overproducer. In addition, feedback regulation of the end product was suggested in lycopene-overproducing strains.
Conclusions A lycopene-overaccumulating strain of the fungus *M. circinelloides* was generated that could be an alternative for the industrial production of lycopene. Vital catalytic residues for lycopene cyclase activity and the potential mechanism of lycopene formation and accumulation were identified.

Keywords Car*RP* · Feedback regulation · Lycopene · Lycopene cyclas · *Mucor circinelloides*

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Introduction

Lycopene is the most potent antioxidant amongst all carotenoids that is widely used in health care products, food and cosmetics (Ciriminna et al. 2016). Recently, microbial lycopene has attracted much attention because microorganisms show rapid growth rate, and independence from geographic location and climate. *Mucor circinelloides,* which is a Mucoromycotina model, meets the accumulation carotenoids with the possibility to be genetically modified (Torres-Martinez et al. 2012). Carotenoid biosynthesis in *M. circinelloides* are controlled by three independent

regulatory mechanisms: light induction mediated by *mcwc-1c* gene (Silva et al. 2006), repression by *crgA* (Navarro et al. 2001), and repression by an unknown gene that is mutated in the MU218 strain (Zhang et al. 2016). Thus, deletion of the *crgA* gene in MU218 resulted in the generation of the strain MU606, which is a strong putative candidate for industrial production of β-carotene (Zhang et al. 2016) and also a starting strain to create overproducing lycopene strains if the *carRP* gene is disabled.

The *M. circinelloides carRP* gene codes for a protein with two distinct enzymatic activities, phytoene synthase and lycopene cyclase (Velayos et al. 2000). The P domain located in the *C*-terminal of the protein is responsible for phytoene synthase activity (Fig. 1), whilst the two R domains, located in the *N*-terminal, are in charge of the lycopene cyclase activity, which catalyses lycopene to β-carotene transformation (Fig. 1) (Velayos et al. 2000). Although the R domain of *M. circinelloides CarRP* is quite different to that of well-known enzymes from proteobacteria, cyanobacteria, algae and plants (Moise et al. 2013), little attention has been paid to it, resulting in a poor understanding of its structure and properties.

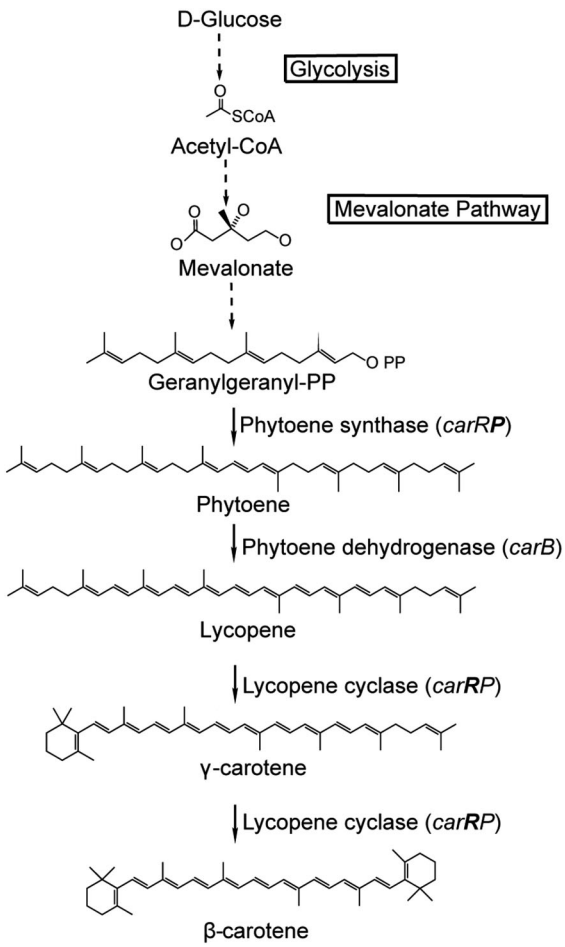

Fig. 1 Biosynthesis pathway of lycopene and β-carotene in *M. circinelloides*. The *letter in bold* of *carRP* gene indicates that the enzymes are encoded by either part of the gene

In this study, three red mutants derived from the β-carotene-overproducing MU606 strain were isolated that carried mutations in residues of the R domain of lycopene cyclase. The varied extent of lycopene cyclase activity loss in the mutants provides insight into the vital catalytic residues for the cyclisation activity and the potential mechanism of lycopene formation and accumulation. In addition, the mutant with the highest lycopene content could be of interest for biotechnological lycopene production.

Methods

Strains and media

The strains involved in this study are listed in Table 1. MU202 and MU224 are both lycopene-producing *M. circinelloides* strains obtained in previous studies. The MU202 *carRP* mutant was obtained after chemical mutagenesis of R7B, a leucine auxotrophic strain with wild-type carotenogenesis. MU224 was obtained by disrupting the *crgA* gene, which is the negative regulator of carotenogenesis in MU202. MU606 is a β-carotene-overproducing strain derived from MU218 after targeted disruption of the *crgA* gene. MU614, MU615 and MU616 are strains with mutations in the *carRP* gene that were obtained in this study by mutagenesis of MU606. *M. circinelloides* strains were grown on YPG or YNB medium. The pH of the media was adjusted to 3.2 or 4.5 for colony or mycelial growth, respectively.

DNA manipulations

Genomic DNA of *M. circinelloides* was prepared as described previously (Ruiz-Perez et al. 1995). The presence of mutations in the *carRP* gene was analysed by PCR amplification (Supplementary Table 1) and direct sequencing of PCR products, and comparison of the obtained sequence was made to the *M. circinelloides*

Table 1 Strains involved in this study

Strain	Phenotype	Genotype	Origin	Reference
R7B	Pale	*leuA*$^-$	CBS277.49	Roncero (1984)
MU202	Light red	*leuA*$^-$ *carRP*$^-$	R7B	Navarro et al. (1995)
MU224	Red	*carRP*$^-$ *crgA*$^-$	MU202	Nicolas-Molina et al. (2008)
MU218	Yellow	*leuA*$^-$	R7B	Navarro et al. (1995)
MU606	Bright yellow	*crgA*$^-$	MU218	Zhang et al. (2016)
MU614	Red	*crgA*$^-$ *carRP*$^-$	MU606	This study
MU615	Red	*crgA*$^-$ *carRP*$^-$	MU606	This study
MU616	Deep red	*crgA*$^-$ *carRP*$^-$	MU606	This study

CBS 277.49 reference genome (Corrochano et al. 2016). Total RNA was isolated using Trizol reagent (Invitrogen), and reverse transcription was performed with a PrimeScript RT reagent Kit with gDNA Eraser (Takara) following the manufacture's instructions. Real-Time PCR was carried out on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA) according to the supplier's instructions. Table S1 shows the primer sequences used in Real-Time PCR. The expressions of *carB* and *carRP* gene were calculated by $2^{-\Delta\Delta C_t}$ formula with *actin* (ID157034; scaffold_07: 2052804–2054242) as internal control gene.

Nitrosoguanidine mutagenesis and isolation of mutants

The mutagenesis process was carried out as described previously (Eslava et al. 1975). NTG-treated spores (survival rate below 10%) were spread on YNB plates and allowed to complete a full vegetative growth cycle to assure the expression of the recessive mutations and were harvested as independent recycled spore pools. To isolate *carRP* mutants, aliquots of spores from each independent spore pool were spread on YNB (pH 3.0) plates, and the red colonies were isolated.

Carotenoid analysis

Extraction of carotenoid was performed as described previously (Zhang et al. 2016). Collected carotenoid extractions were dried with Termovap Sample Concentrator and resolved in isopropanol and chloroform and for quantification with an HPLC system (UltiMate3000; Thermo Fisher Scientific, USA) equipped with a Hypersil GOLD C18 column (2.1 mm × 100 mm, 3 µm; Thermo Fisher Scientific) and a diode-array detector. The signal was detected at 450 and 470 nm. The mobile phase constituted acetonitrile/isopropanol (85:15, v/v) with a flow rate of 0.15 ml/min at 30 °C. Identification and quantification of the carotenes were performed with corresponding standards (Sigma, USA).

Results and discussion

Isolation and molecular characterisation of *M. circinelloides* strains that overaccumulate lycopene

The strategy followed to isolate mutants with high contents of lycopene was based on the chemical mutagenesis of a strain (MU606) that overaccumulates the final carotene (β-carotene; Fig. 1) due to the presence of regulatory mutations (Zhang et al. 2016). After 5×10^6 NTG mutagenised spores of MU606 were spread on YNB solid medium and incubated in the dark for 60 h, they were exposed to the light to stimulate carotenoid biosynthesis. Most colonies showed the bright yellow colour of the parental strain, but three colonies (MU614, MU615 and MU616) displayed a reddish colour (Fig. 2). The modification of the colour was similar to that in mutants (MU202, MS21 and MS35) that produce lycopene due to mutation in the R domain of CarRP protein (Navarro et al. 1995; Velayos et al. 1997).

To confirm that the red colour of the mutants was due to the presence of mutations in the *carRP* gene, the gene of the red mutants was PCR-amplified and sequenced directly without cloning to avoid the detection of mutations produced during the amplification. Analyses of the obtained sequences revealed that all three of the red mutants had missense

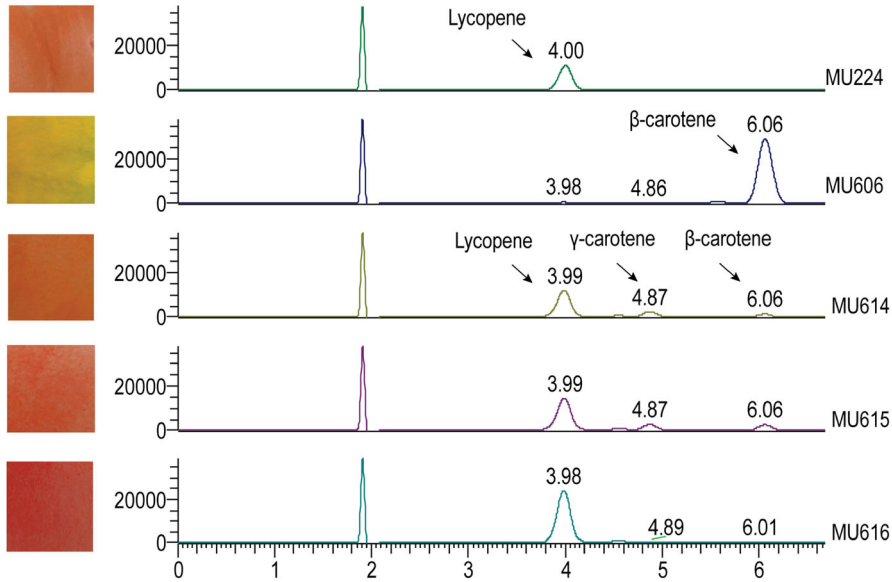

Fig. 2 Colours of reddish mutants and parental strain and corresponding HPLC analysis of carotenoids. Images correspond to a piece of mycelium grown in solid medium

mutations in the region of the *carRP* gene encoding the lycopene cyclase domain (R domain), particularly in residues conserved in most fungal lycopene cyclases (Fig. 3). Thus, MU614 has a valine instead of an alanine at position 43 of the protein (GCC→GTC), MU615 has a methionine instead of a valine at position 74 (GTG→ATG) and MU616 has a proline instead of a serine at position 61 (TCC→CCC). In a previous study, the activity of phytoene synthase was absent when the R domain was disrupted, while the lycopene cyclase remained active even though P domain was deleted, indicating that the protein encoded by *carRP* gene might not be cleaved into two products post-translationally as speculated (Velayos et al. 2000). As a consequence, the R domain were deduced to have the function of membrane anchoring of phytoene synthase besides the lycopene cyclase activity (Velayos et al. 2000). Residues at positions 43 and 74 both lay on the transmembrane domains, and, therefore, mutations in these residues (MU614 and MU615) might affect the anchoring point to the membrane, which was closely correlated to the activity of the protein. In particular, the residue at position 74 was located in "hPhEEhh-hhh" (h represents any hydrophobic amino acid residue) region, which is the distinctive motif in lycopene cyclase (Xie et al. 2015). The mutation at position 61 carried by MU616 caused a significant change in the properties of the corresponding amino acid because a hydrophobic amino acid (proline) was replaced by a polar amino acid (serine).

Other than the mutation sites observed in this study, the previously reported mutants MS21 and MS35 exhibited two different mutations at positions 78 and 86, respectively, and lycopene was accumulated as main carotenoid in both strains (Velayos et al. 1997). As shown in Fig. 3, the missense mutation at position 78 resulted in a noticeable amino acid change because a glutamic acid (negative charge) was replaced by a lysine (positive charge). More importantly, this change affects a residue that is absolutely conserved in the "hPhEEhhhhh" motif, and it thus almost leads to complete inactivation of the cyclisation function (Velayos et al. 1997). With respect to MS35, the replacement of threonine by isoleucine (T86I) resulted in a change of polarity in residue 86 that almost provoked a total loss of cyclase activity. In contrast to the mutants mentioned above that carried mutations in the first repeated R domain, the mutation (E219 K) of MU202 (the parental strain of MU224) occurred in the second repeated R domain. Actually, it was the same mutation present in MS35 but in the "hPhEEhhhhh" motif of the second R domain, demonstrating the great importance of this residue and the motif for the cyclase activity. As shown in Fig. 3, mutagenesis mainly took

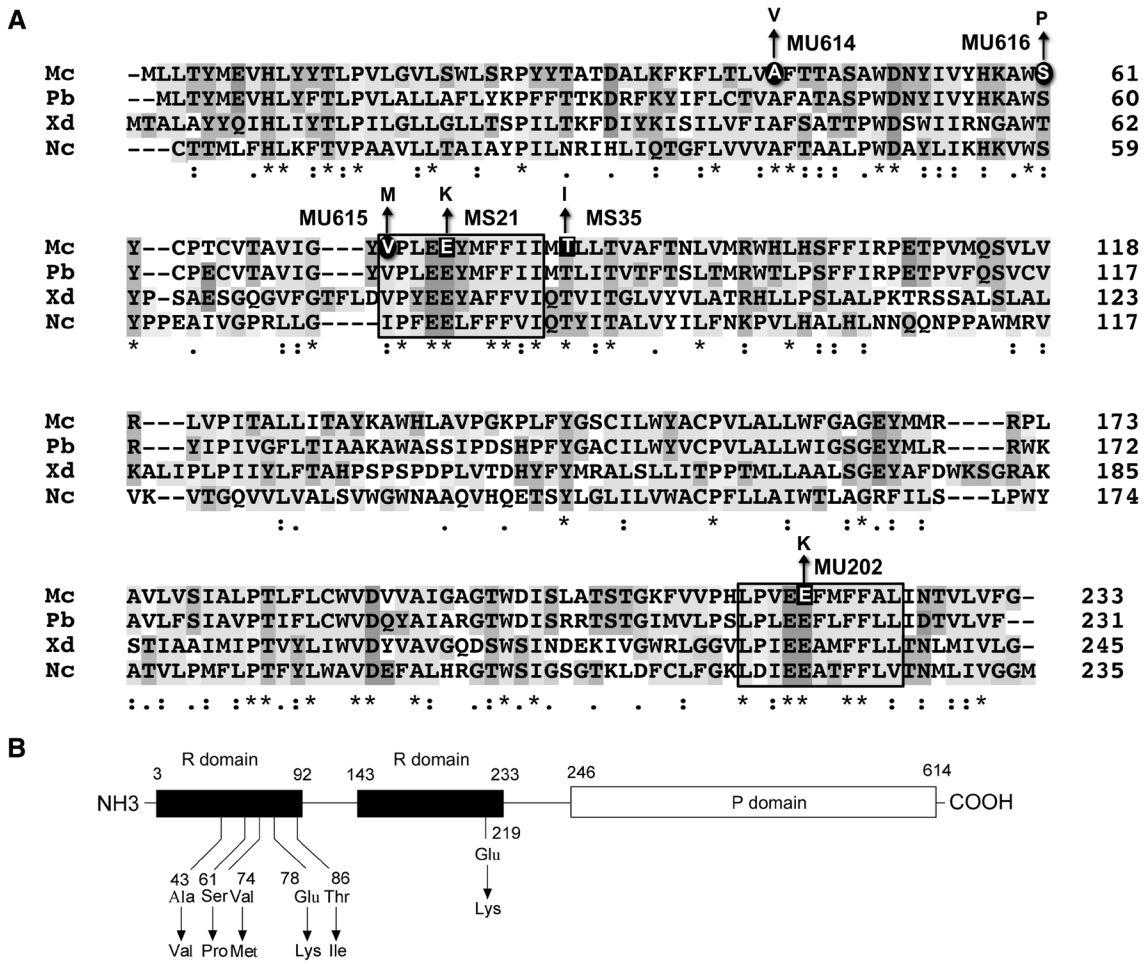

Fig. 3 Mutations in the *carRP* gene of reddish mutants. **a** Alignment of the amino acid sequences of the cyclase domain of proteins encoded by the *carRP* gene of *M. circinelloides*, the *carRA* gene of *P. blakesleeanus*, the *crtYB* gene of *X. dendrorhous* and the *al-2* gene of *N. crassa*. *White letters in black circles* indicate the missense mutations in different red mutants of *M. circinelloides* obtained in this study, *white letters in black squares* indicate the missense mutations in *carRP* mutants obtained in previous studies (Navarro et al. 1995; Velayos et al. 1997), *arrows* indicate the corresponding amino acid substitutions, and the *box* indicate the charactetistic motif of cyclase domain. **b** Scheme of the CarRP protein shows the positions of the R and P domains and identifies mutations that produced loss of cyclase function

place in the first R domain, which also occurred in *Saccharomyces cerevisiae* (Xie et al. 2015), suggesting the importance of this domain for cyclisation ability.

Carotene content and expression of carotenogenic genes in lycopene-overaccumulating strains

The carotene content in the *carRP* mutants isolated in this and previous studies was determined with an HPLC system (Fig. 2; Table 2). In accordance with the colour of the mycelia, all of the *carRP* mutants accumulated lycopene, although to different extents. In the case of MU614 and MU615, they produced 1.12 or 1.45 and 2.12 or 2.88 mg/g lycopene in the dark and light, respectively, amounts that are in a similar range as to that of MU224, which is a lycopene-overproducing strain that carries just the deletion of the *crgA* gene (Nicolas-Molina et al. 2008). However, apart from lycopene, these mutants accumulated 0.20 or 0.24 mg/g and 0.39 or 0.50 mg/g β-carotene in the dark and light, respectively. In addition, the intermediate between β-carotene and lycopene, γ-carotene, which is barely detectable in the parental strain

Table 2 Content and composition of carotenoids in reddish mutants

Strains	β-Carotene (mg/g dry mass)		γ-Carotene (mg/g dry mass)		Lycopene (mg/g dry mass)		Total carotenoids (mg/g dry mass)	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light
MU614	0.20 ± 0.02^{a}	0.39 ± 0.04^{b}	0.25 ± 0.03^{b}	0.43 ± 0.05^{b}	1.12 ± 0.1^{c}	2.12 ± 0.1^{c}	1.56 ± 0.2^{b}	2.94 ± 0.1^{b}
MU615	0.24 ± 0.07^{a}	0.50 ± 0.1^{a}	0.31 ± 0.04^{a}	0.53 ± 0.07^{a}	1.45 ± 0.1^{b}	2.88 ± 0.2^{b}	2.00 ± 0.1^{a}	3.92 ± 0.3^{a}
MU616	0.0073 ± 0.006^{b}	0.016 ± 0.006^{c}	0.015 ± 0.005^{c}	0.020 ± 0.003^{c}	1.90 ± 0.2^{a}	3.64 ± 0.3^{a}	1.92 ± 0.2^{a}	3.67 ± 0.2^{a}
MU224	nd	nd	nd	nd	1.14 ± 0.2^{c}	2.53 ± 0.2^{b}	1.14 ± 0.2^{c}	2.53 ± 0.2^{c}

[a–c] Values are mean ± standard deviation of at least three independent experiments. Values within a column with different superscript letters were significantly different ($p < 0.05$)

nd not detectable

MU606, was also found in MU614 and MU615 (Fig. 2). In summary, the proportion of lycopene in total carotenoids in both strains was around 70%, suggesting a partial loss of the cyclisation activity of lycopene cyclase and a similar relevancy of residues V74 and A43 in the cyclisation function. In contrast, the mutation S61P in MU616 almost completely destroyed the cyclisation function of lycopene cyclase, as only traces of β-carotene and γ-carotene were detected in it (Fig. 2; Table 2). This result indicates that the residue S61 together with residues E78, T86 and E219 are critical for cyclisation activity. In addition, the MU616 strain accumulates up to 1.90 and 3.64 mg/g of lycopene in the dark and light, respectively, corresponding to respective increases of 67 and 44% compared to MU224. And a similar increase was displayed in the total amount of carotenes in MU615 compared to MU224, while MU614 did not show the same increase which might be due to additional mutations in the genes involved in carotenogenesis or the declined activity of phytoene synthase caused by the change of conformation of R domain.

Interestingly, the HPLC peaks corresponding to β-carotene (retention time of 6.06 min) and γ-carotene (around 4.87 min) appeared in MU614, MU615 and even MU616, although they were very weak (Fig. 2), but were absent in MU224, which had the mutation located in the second R domain. The two cyclisations of both ends of lycopene to produce β-carotene are catalyzed independently by two R domains (Candau et al. 1991). Consequently, it is likely that the first R domain is responsible for the formation of the second β-ring (the step from γ-carotene to β-carotene) and that the second R domain controls the cyclisation of lycopene to γ-carotene. In addition, the trace, but not abundant, accumulation of γ-carotene in the mutants with a deficient first R domain revealed that the function of the second domain might depend on the first one and would be fully functional only when the first domain functions correctly.

High carotenoid production corresponds to high expression of carotenogenic genes (Navarro et al. 2001). To confirm that this also occurs in the isolated *carRP* mutants, the mRNA levels of the carotenogenetic structural genes *carB* and *carRP* (the primers were designed for the P domain) in the mycelia from mutants were investigated in the dark and in light and compared to the MU224 strain. MU614, MU615, MU616 and MU224 carried a deletion of *crgA* that increases the expression of carotenogenic genes, but in addition, MU614, MU615 and MU616 carried a second regulatory mutation that increases the expression of carotenogenic genes even further in strains that produce β-carotene (Zhang et al. 2016). Surprisingly, only strain MU616 and, to the least extent, MU615 showed higher *carB* and *carRP* mRNA levels than MU224. Even though MU614 presented lower *carB* and *carRP* mRNA levels than MU224 (Fig. 4), this suggested that mutant MU614 and probably MU615 carried additional mutations to those in the *carRP* gene as a consequence of random mutagenesis, which somehow repressed the accumulation of *carB* and *carRP* mRNAs. Despite this, the lycopene content and mRNA levels of carotenogenic genes exhibited a similar trend in the mutants (Table 2; Fig. 4). However, we should note that MU616 accumulated very high levels of *carRP* mRNA in light that did not

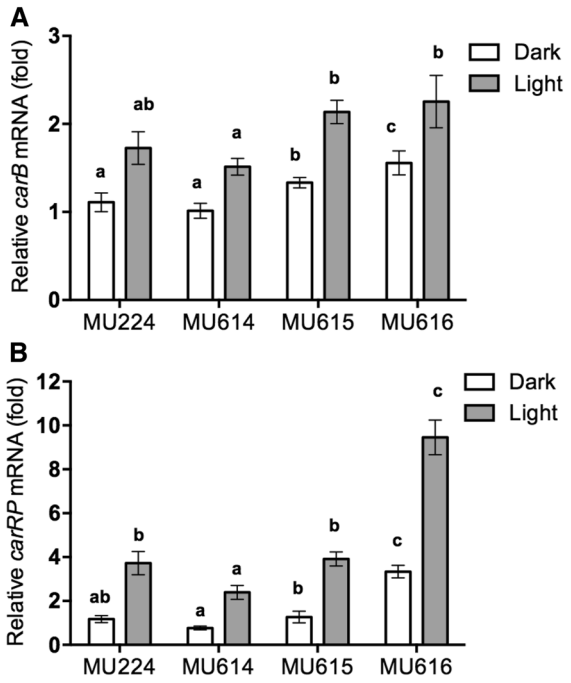

Fig. 4 Expression level of carotenogenic structural genes in *reddish* mutants. Different superscript letters within a group (*dark* or *light*) indicate a significant difference ($p < 0.05$)

correlate with the levels of lycopene, probably due to the *carB* mRNA level, which was similar to that of MU615. The great discrepancy between the mRNA levels of *carB* and *carRP* in MU616 in light may result in the deposition of phytoene, leading to a moderate metabolic process, and consequently, the lycopene was not synthesised to the expected level. Although alternative hypotheses cannot be discarded, we propose that high levels of lycopene could repress *carB* mRNA accumulation by end-product regulation, which is absent for β-carotene in *M. circinelloides* (Bejarano et al. 1988). Interestingly, an increase of *carB* expression either by blocking the proposed end-product regulation or by other means could result in the generation of strains with greater lycopene content that could be of interest for lycopene biotechnological production.

Conclusions

(1) This study generated a lycopene-overaccumulating strain of *M. circinelloides* as an alternative for the industrial production of lycopene. Mutation of the same site in the biofunctional gene *carRA* of commercial lycopene producer *Blakeslea trispora* may also create mutants deficient in lycopene cyclase, thus avoiding the addition of harmful chemical inhibitors of lycopene cyclase.

(2) In addition to the conserved "hPhEEhhhhh" motif, other conserved residues from this domain, such as alanine 43 and serine 61, are very important for the cyclisation function of lycopene cyclases and may represent new targets to be mutated to generate lycopene-overaccumulating strains.

(3) Feedback regulation might occur only when the lycopene concentration is high enough as in MU616.

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Supporting information Supplementary Table 1—Primer sequences.

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