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Lycopene over-accumulation by disruption of the negative regulator gene *crgA* in *Mucor circinelloides*

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Abstract Lycopene has become one of the most interesting antioxidant compounds, especially in relation to human health. This work describes a genetic strategy to modify the carotenoid biosynthesis pathway to develop a lycopene-overproducing strain. The *crgA* gene, a negative regulator of carotenogenesis, was disrupted in the *Mucor circinelloides* strain MU202, which lacks the lycopene cyclase activity and accumulates lycopene instead of β-carotene. The resultant strain, MU224, demonstrated increased transcriptional levels of the carotenogenesis structural genes *carRP* and *carB* compared to the parental strain MU202. As a consequence, strain MU224 accumulated 5 mg/g of dry weight of cells in liquid cultures, a sevenfold increase with respect to the parental strain. Moreover, when lycopene production was examined in a complex enriched medium, biomass increased tenfold compared to that obtained in synthetic minimal medium. In this complex medium, the production rate of lycopene by strain MU224 reached 54 g/l. These results illustrate how a combination of genetic manipulation and optimized culture conditions can be utilized to enhance the production of commercially desirable compounds such as lycopene.

Keywords Lycopene · *crgA* · *Mucor circinelloides* · Gene regulation

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Introduction

Lycopene is an open-chain hydrocarbon containing 11 conjugated and 2 non-conjugated double bonds arranged in a linear array. Lycopene and other carotenoids have been widely used in industry as nutrient supplements, food colourants and in animal feeds. As well as its dietary role, lycopene has attracted much attention because of its antioxidant and antiproliferative properties, which have been demonstrated in animal and in vitro studies; although similar activity in humans remains controversial (reviewed in Rao and Rao 2007).

Initially, industrial carotenoid production was based on natural sources of pigments, such as the production of β-carotene from an over-accumulating *Blakeslea trispora* strain (Ciegler et al. 1963). However, natural sources were later rejected due to their inability to compete with chemically synthesized carotenoids. Nowadays, biological production is attracting new attention because of public opinion regarding synthetic food additives and also as a result of improvements in carotenoid production by fungi, algae and bacteria. Carotenoid biosynthesis in these organisms is regulated by the level and activity of carotenoid biosynthetic enzymes and the total carbon flux. Thus, over-accumulation can be achieved by modifying both the level and activity of these enzymes using molecular approaches (Schmidt-Dannert 2000; Sandmann 2001).

Carotenoid biosynthesis has been widely studied in filamentous fungi such as the ascomycete *Neurospora crassa* and the zygomycetes *Phycomyces blakesleeanus* and *Mucor circinelloides* (Ruiz-Vázquez and Torres-Martínez 2003). The carotenoid biosynthetic pathway begins with the condensation of two molecules of geranylgeranyl pyrophosphate by the phytoene synthase enzyme to generate a molecule of phytoene. The next step is carried out by the

phytoene dehydrogenase, which introduces four dehydrogenations into phytoene to produce lycopene. The final product, β-carotene, is obtained through two cyclizations of lycopene by the lycopene cyclase enzyme. In *M. circinelloides*, the *carB* gene encodes the phytoene dehydrogenase enzyme (Velayos et al. 2000a), whereas the *carRP* gene codes for a protein with two different enzymatic activities located in two different domains: the R domain, at the N terminus of the protein, where the lycopene cyclase activity is located; and the P domain at the C terminus, which contains the phytoene synthase activity (Velayos et al. 2000b). Blue light regulates the carotenoid biosynthetic pathway in these three fungi. The carotenoid content remains at a basal level under dark conditions, and after illumination, a significant increase in the expression of the carotenogenic structural genes results in carotenoid accumulation. Only a few regulatory genes implicated in this light response have been identified and characterized. In *N. crassa*, the white-collar proteins, WC-1 and WC-2, have been identified as transcriptional activators required for carotenogenesis and most other blue-light-regulated responses in this organism (Ballario et al. 1996; Linden and Macino 1997). In *M. circinelloides*, a negative carotenogenesis regulatory gene, *crgA*, has also been cloned and characterized (Navarro et al. 2000; Navarro et al. 2001). Recent functional studies have revealed that the CrgA protein contains two ring-finger domains and a polyglutamine-rich region that are essential for accurate light regulation of carotenogenesis, indicating a role for the ubiquitin–proteosome pathway in the light regulation of carotenogenesis in this fungus (Lorca-Pascual et al. 2004; Murcia-Flores et al. 2007).

Filamentous fungi are widely used in industry for the production of primary and secondary metabolites (Peberdy 1994). The advantages of using filamentous fungi instead of yeast or bacteria are the high levels of metabolite produced as well as the final biomass. Among zygomycetes, *M. circinelloides* is one of the few that is amenable to molecular techniques. The availability of a carotenogenesis mutant collection, together with an efficient genetic transformation system (Van Hesswijck and Roncero 1984; Navarro et al. 1995) and the recently described gene silencing mechanism triggered by self-replicative transgenes (Nicolas et al. 2003), make this fungus a suitable organism for the genetic manipulation of carotenoid biosynthesis. In this paper, we present a strategy to obtain lycopene-superproducing strains by combining a mutation in the lycopene cyclase gene with a disruption in the negative regulatory gene *crgA*. Through this approach, a new lycopene-superproducing strain of *M. circinelloides* has been developed that accumulates a substantial amount of lycopene in a complex liquid medium, illustrating how this research could lead to the optimization of lycopene production in already improved industrial strains.

Materials and methods

Strains and transformation conditions

The leucine auxotroph R7B (Roncero 1984), derived from *M. circinelloides f. lusitanicus* CBS 277.49 (syn. *Mucor racemosus* ATCC 1216b; Schipper 1976), was used as the wild-type strain. The MU202 strain, a carotene-production mutant affected in the lycopene cyclase gene, was obtained by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis of the R7B strain (Navarro et al. 1995). Strain MU221 is a *crgA* null mutant derived from R7B (Navarro et al. 2001). The MU224 strain used in this work was obtained by disruption of the *crgA* gene in the MU202 genetic background. Both the R7B and MU202 strains were transformed with the pLEU4 plasmid (Roncero et al. 1989) to obtain an identical $LeuA^+$ phenotype for comparative studies with the MU224 strain. Transformation was carried out as described previously (Van Heeswijck and Roncero 1984) except for the protoplast formation step, which was performed by incubating 2.5×10^8 germinated spores with 5 mg/ml chitosanase RD (US Biologicals) and 0.5 mg/ml Lysing Enzymes (L-1412; Sigma) during 90 min at 30°C.

Growth and lycopene measurements

Cultures were grown in yeast nitrogen base (YNB) minimal medium (Lasker and Borgia 1980), with or without L-leucine (20 mg/ml), or in Mucor Milk Medium (MMM; 40 g/l dehydrated milk, 15 g/l seeds oil, 15 g/l saccharose). The pH of the YNB medium was adjusted to 4.5 and 3.2 for mycelial and colonial growth, respectively. Submerged cultures were carried out by inoculating 2.5×10^6 spores into 50 ml of liquid medium and were shaken at 300 rpm. Unless specified, mycelia were grown under illumination conditions described previously (Quiles-Rosillo et al. 2003). For lycopene measurements, mycelia were washed with distilled water, dried between paper towels, frozen on liquid nitrogen, ground with a pestle and mortar to a fine powder and lyophilized. Approximately 5 mg of this powder was suspended in 1 ml of methanol and extracted four times with 1 ml of petroleum ether. The resultant fractions were mixed, and carotenes were quantified using the absorption coefficients given by Davies (1976).

Plasmids

Plasmid pVEN172 (Navarro et al. 2001) harbours a 4.4-kb *Pst*I fragment of pLEU4 (Roncero et al. 1989) flanked by DNA regions neighbouring the *crgA* gene (Fig. 1). This *Pst*I fragment contains the wild-type *leuA* allele, which complements the *leuA* mutation present in MU202 and the wild-type strains used in this work.

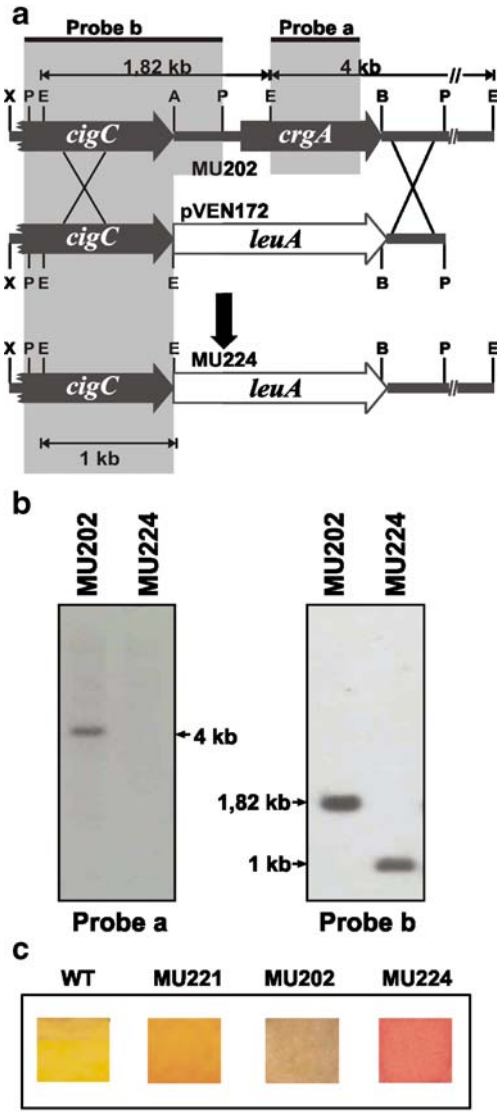

Fig. 1 Disruption of the *crgA* gene. **a** Schematic representation of the disruption of the *crgA* gene by integration of the 6.5-kb *Pst*I fragment of plasmid pVEN172. *Probes a* and *b* were used for Southern analysis. *Probe a* corresponds to the internal 0.8-kb *EcoR*I-*Sac*I fragment of the *crgA* gene, and *probe b* corresponds to the 1.6-kb *Pst*I fragment of the *crgA* upstream region (*crgA* accession number AJ250998). Only relevant restriction sites are shown: *X*, *Xho*I; *P*, *Pst*I; *E*, *EcoR*I; *A*, *Apa*I; *B*, *BamH*I. **b** Southern hybridization analysis demonstrating the replacement of the *crgA* gene by homologous recombination. MU224 was obtained after transformation of MU202 with the 6.5-kb *Pst*I fragment of pVEN172. Genomic DNA (1 μg) was digested with *EcoR*I and hybridized with *probes a* (*left*) and *b* (*right*). The expected sizes of the *EcoR*I fragments detected by the probes are indicated by *arrows*. **c** Colour phenotype of the carotenogenesis wild-type strain R7B (WT), the *crgA* mutant MU221, the *carR* mutant MU202 used as parental strain, and the double *carRcrgA* mutant MU224. Approximately 1 cm^2 of mycelia grown in solid YNB medium for 3 days under illumination conditions was excised from the media and dried between paper towels before being photographed

Plasmid pMAT1256 contains a 2.7-kb DNA fragment of the carRP genomic sequence (accession number AJ250827). This fragment was amplified from genomic DNA by polymerase chain reaction (PCR) using the oligonucleotide pairs CarRPB (5′-TTGGGATGTCTGCTGCTCC-3′) and pcarBApa4 (5′-GCGAGAGTTGGAAAAGAGAAAGA GATAGGG-3′). The amplified fragment was cloned into pGEM-T (Promega) to generate plasmid pMAT1256. The *Escherichia coli* strain DH5α (Hanahan 1983) was used for all cloning experiments.

Nucleic acid isolation and analysis

Genomic DNA from *M. circinelloides* was prepared as described previously (Ruiz-Perez et al. 1995). Total RNA was isolated using Trizol reagent following the instructions of the supplier (Gibco-BRL). Standard recombinant DNA manipulations were performed (Ausubel et al. 1989, Sambrook and Russell 2001). Southern and Northern blots were hybridized to radioactively labelled probes at 65°C in 0.9 M NaCl, 1% sodium dodecyl sulfate (SDS) and 0.1 g/ml dextran sulfate. Filters (Hybond N+, Amersham Biosciences) were washed in 2× standard saline citrate (SSC), 0.1% SDS at room temperature for 5 min, twice in 2× SSC, 0.1% SDS at 65°C for 20 min, and once in 0.1× SSC, 0.1% SDS at 65°C for 20 min. Probes were labelled with [α-32P]dCTP using Ready-to-Go DNA labelling beads (Amersham Biosciences), following the instructions of the supplier. The *carB* probe corresponds to the 1.8-kb *EcoR*I-*Xho*I fragment of the *carB* cDNA (accession number AJ238028) and was isolated from plasmid pGCMM20 (kindly provided by Prof. A. P. Eslava, University of Salamanca, Spain). The *carRP* probe corresponds to the 2.7-kb CarRPB-pcarBApaI fragment of the *carRP* gene and was amplified from plasmid pMAT1256 by PCR. The 25S rRNA probe corresponds to a 450 bp fragment containing the 3′ region of the 25S rRNA gene of *M. circinelloides*. The filter was exposed to Kodak X-OMAT MS film with an intensifying screen at −70°C. After exposure, the film was scanned using an Image Scanner (Amersham Biosciences), and the images were analyzed with Image Quant TL software (Amersham Biosciences).

Results

Disruption of the *crgA* gene

Mutants affected in the *crgA* gene, a negative regulator of carotenogenesis, accumulate large amounts of β-carotene under both dark and light conditions (Navarro et al. 2001). The *M. circinelloides* strain MU202 lacks lycopene cyclase activity, accumulating lycopene instead of β-carotene

(Navarro et al. 1995). To increase lycopene biosynthesis in the strain MU202, disruption of the *crgA* gene was conducted. The plasmid pVEN172, a knockout vector designed to disrupt the *crgA* gene, contains the *leuA* gene of *M. circinelloides* flanked by sequences adjacent to the *crgA* gene (Fig. 1a). This plasmid was digested with the enzyme *Pst*I, and the resulting 6.5-kb fragment was used to complement the leucine auxotrophy of the MU202 strain. Integration of this fragment by homologous recombination was expected to result in the replacement of the endogenous *crgA* gene by the *leuA* gene. A total of 23 transformants were obtained, which showed a phenotype for lycopene production similar to the parental strain MU202. After several cycles of vegetative growth under selective pressure, ten transformants were analysed for a stable Leu^+ phenotype, as an indicator of exogenous DNA integration. One homokaryotic transformant, demonstrating a 100% stable Leu^+ phenotype, was finally selected for Southern analysis. This homokaryotic transformant, named MU224, possessed a lycopene-superproducing phenotype (Fig. 1c), and results confirmed that the *crgA* gene was disrupted in this transformant. DNA from the parental strain MU202 and the lycopene-over-accumulating strain MU224 was digested with *Eco*RI and probed with the central region of the *crgA* gene (Fig. 1b, probe a). The absence of the expected 4-kb endogenous band in the transformant MU224 indicated that the integration event had occurred at the *crgA* locus. To confirm this result, the same digested DNA was hybridized with a probe localised to a region upstream of *crgA* (Fig. 1b, probe b). The expected endogenous 1.8-kb band was present in the parental strain MU202 but not in the lycopene-superproducing strain MU224, where a 1-kb band was observed, thus confirming the successful disruption of the *crgA* gene.

Transcription levels of the carotenogenic genes *carB* and *caRP*

Light-inducible carotenogenesis in *M. circinelloides* is a consequence of light-regulated transcription of the carotenogenic structural genes, as has been shown for the *carB* and *carRP* genes (Velayos et al. 2000a, b). To assess the effect of the *crgA* disruption on the expression of the carotenogenic genes, transcription of genes *carB* and *carRP* was analysed in the *crgA* null strain MU224. Total RNA from the null *crgA* mutant MU224, the parental strain MU202 and the wild-type strain R7B, isolated either from dark-grown mycelium or mycelium exposed to a light pulse, was hybridized to probes corresponding to the *M. circinelloides* genes *carB* and *carRP*. Unlike the wild-type strain, the null *crgA* mutant MU224 accumulated a significant amount of *carB* mRNA in the dark, showing an eightfold increase with respect to the wild-type R7B under the same conditions (Fig. 2a). Light further stimulated expression of *carB* in the $crgA^-$ genetic background, with levels of *carB* mRNA showing an additional increase with respect to the wild-type and parental strains (Fig. 2a). Similar results were observed in the expression analysis of the *carRP* gene, which increased its mRNA levels both in the dark (50-fold) and the light (twofold) in the $crgA^-$ genetic background with respect to the wild-type and parental strains (Fig. 2b).

Effect of the *crgA* disruption on lycopene content

The new strain MU224, which contains a disrupted *crgA* gene, presented a lycopene-superproducing phenotype,

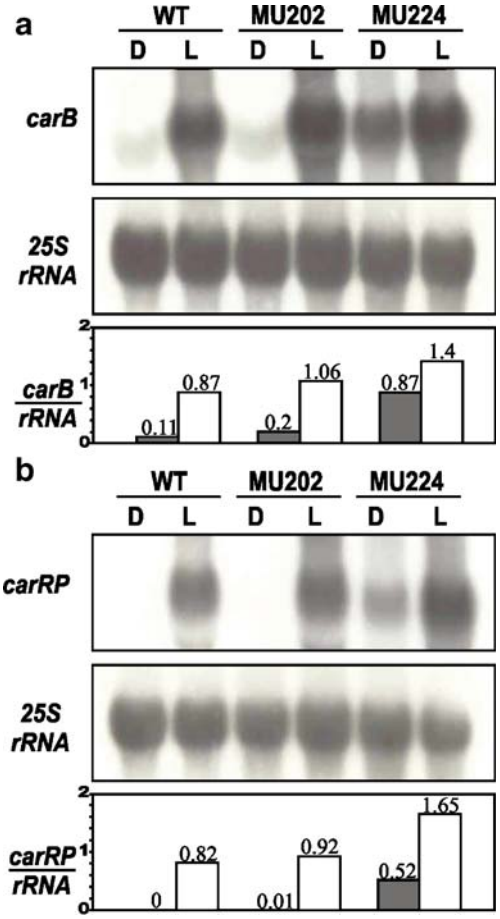

Fig. 2 Regulation of *carB* and *carRP* expression by *crgA*. **a** Levels of *carB* RNA in dark-grown (*D*) and light-pulsed (*L*) mycelia of the *crgA* mutant MU224, the wild-type strain R7B (WT) and the parental strain MU202. An aliquot (5 μg) of total RNA was loaded in each lane and hybridized with the *carB* probe. Equal loading was verified by re-probing the membrane with the gene for 25S rRNA. **b** Levels of *carRP* RNA were detected as in **a**, but the membrane was hybridized with the *carRP* probe and re-probed with the gene for 25S rRNA to confirm equal loading. Illuminated mycelia (*L*) were exposed to blue light for 4 min (at 4 W/m^2) and incubated in the dark for 20 min before RNA isolation. Densitometric analysis was used to estimate the mRNA levels. *Numbers* indicate absolute values of the ratio between *carB* mRNA and rRNA

showing an intense red pigmentation (Fig. 1c). Quantitative analysis of the lycopene content of the *crgA* mutant MU224 and its parental strain MU202 was carried out in solid and liquid YNB minimal medium (Fig. 3). After 3 days of growth in solid YNB medium, the lycopene content of the null *crgA* mutant MU224 was fivefold higher than that of the parental strain MU202. This increase was even greater when cells were grown in liquid culture. Different culture conditions were tested, and the highest rate of lycopene production was obtained when 5×10^4 spores/ml were inoculated into 500 ml flasks containing 50 ml of YNB medium, shaken at 300 rpm (data not shown). Under these conditions, the parental strain MU202 did not show a significant increase in lycopene production compared to levels observed in solid medium. However, the lycopene content of the MU224 strain increased significantly when grown in liquid medium, reaching a value of 5 mg/g of dry weight of cells (DWC), representing a sevenfold increase in lycopene synthesis with respect to the MU202 parental strain (Fig. 3).

Lycopene production on a complex medium

Minimal medium, which supports growth of the fungus at a constant specific growth rate, is required for metabolic and genetic investigations. However, minimal media are associated with the low production of metabolic compounds as a consequence of poor final biomass yields. To test the ability of the *M. circinelloides crgA* strain to improve biomass yield and in consequence, the total amount of lycopene produced, a complex medium was designed (MMM, "Materials and methods"), and a comparative study of the biomass and lycopene production in minimal and complex medium was undertaken (Fig. 4). The biomass production in MMM was approximately tenfold higher than in minimal medium after 5 days of growth, reaching 100.9 g/l (Fig. 4a). Thus, even with the reduction of

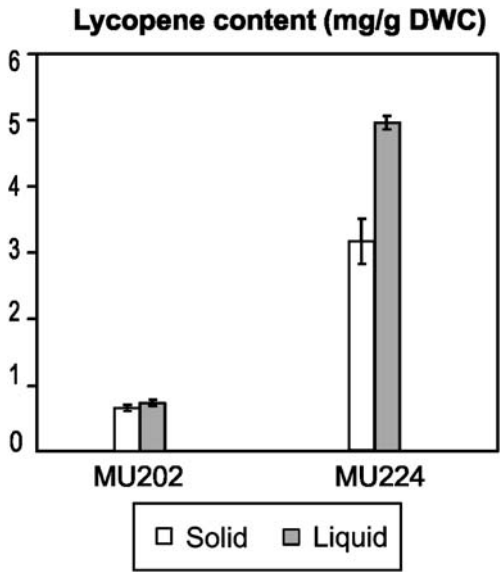

Fig. 3 Effects of the *crgA* disruption on lycopene accumulation. The parental strain MU202 and the *crgA* mutant MU224 were inoculated in solid (10^4 spores per millilitre) or liquid (5×10^4 spores per millilitre) YNB medium and grown for 72 h. The values are mean± standard errors of three independent experiments. *DWC* Dry weight of cells

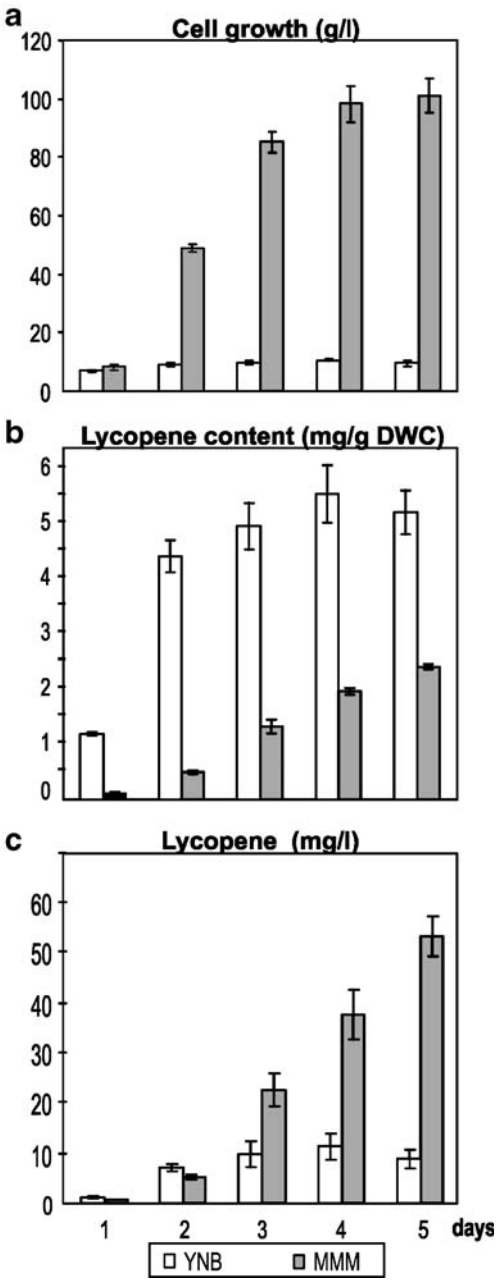

Fig. 4 Time course of the lycopene content and cell growth of the *crgA* mutant MU224. Approximately 5×10^4 spores per millilitre were inoculated into 50 ml of minimal YNB medium or complex MMM medium. Cultures were incubated at 28°C for 1 to 5 days, and at different times, mycelia were harvested, weighted for cell growth measurements (**a**) and lyophilised for lycopene measurements (**b**). **c** Total lycopene production (milligrams per litre of medium) was calculated from data presented in **a** and **b**

lycopene content shown by mycelia grown in complex medium when compared with that grown in minimal medium (Fig. 4b), the global lycopene production (*w*/*v*) was fivefold higher in cultures grown in MMM medium than in those grown in minimal medium, with a maximum of 54 g/l after 5 days of growth (Fig. 4c). These results indicate that *M. circinelloides* can be grown in high-biomass-producing media to significantly increase the amount of lycopene produced.

Discussion

Among filamentous fungi, some species of the order Mucorales such as *B. trispora*, *P. blakesleeanus* and *M. circinelloides* possess a carotenogenesis pathway and accumulate β-carotene as the final product. In all these species, lycopene-accumulating strains affected in the lycopene cyclase enzyme have been obtained by classic mutagenesis (Murillo et al. 1978; Navarro et al. 1995; Mehta et al. 2003). Lycopene cyclase mutants in the otherwise wild-type genetic background of *P. blakesleeanus* produce 2.47 mg of lycopene per gram DWC (Murillo et al. 1978). Similar strains have been obtained in *M. circinelloides* and *B. trispora*, which accumulate 0.25 mg/g DWC (Navarro et al. 1995; Mehta et al. 2003). Although *P. blakesleeanus* has proved to be a good model organism for studying the genetics of carotenogenesis (Cerda-Olmedo 2001) and *B. trispora* is the only Mucoral that is used in the industrial-scale production of β-carotene (Ruiz-Vázquez and Torres-Martínez 2003), only *M. circinelloides* is amenable to genetic transformation and gene replacement (Van Heeswijck and Roncero 1984; Navarro et al. 2001). These genetic techniques have enabled the development of the novel strain MU224, that accumulates 5 mg of lycopene per gram DWC; a 20-fold increase with respect to the original strain MU202.

M. circinelloides mutants affected in the R domain of the *carRP* gene, which determines lycopene cyclase activity, are red and accumulate the intermediate product lycopene instead of β-carotene (Velayos et al. 1997, 2000a). Mutants affected in the *crgA* gene accumulate high amounts of β-carotene both in dark and light, indicating that *crgA* acts as a negative regulator of carotenogenesis (Navarro et al. 2001). We combined these two mutations to generate a lycopene-over-accumulating strain.

Disruption of the *crgA* gene was carried out by homologous recombination using a DNA fragment containing the *leuA* marker, flanked by sequences that corresponded to the upstream and downstream regions of the *crgA* gene. The *crgAcarR* strain obtained by gene replacement, produced high amount of lycopene and demonstrated increased mRNA levels of the two carotenogenic structural genes *carB* and *carRP*. This increase in mRNA levels probably results in high enzymatic activities of the proteins encoded by these two genes, explaining the lycopene-superproducing phenotype. In a similar way, the over-expression of the key *HMG* gene, involved in the isoprenoid pathway of *Candida utilis*, increased the lycopene content up to fourfold compared to the control strain (Shimada et al. 1998). The disruption of the *crgA* gene in the wild-type strain increases the *carB* mRNA level resulting in a constitutive phenotype, with accumulation of high amounts of β-carotene both in dark and light conditions (Navarro et al. 2001). This work demonstrates that the negative regulatory role of *crgA* also affects mRNA levels of *carRP* gene, illustrating the involvement of *crgA* gene throughout all the steps of the carotene biosynthesis pathway.

Aside from the transcriptional regulation of carotenoid biosynthesis genes, final carotenoid content is governed by culture conditions (Bhosale 2004). Distinct culture conditions result in different morphological and physiochemical characteristics of fungal hyphal elements and thereby their capability to produce and accumulate metabolic compounds. Of particular relevance is the amount of biomass produced in submerged cultures, one of the key bioprocess parameters that determines the final amount of metabolic compound produced. Lycopene production of the MU224 strain was examined under several culture conditions. A final lycopene production rate of 54 mg/l was obtained from the submerged culture of MU224 grown in complex MMM medium. To our knowledge, this is the highest amount of lycopene produced by a filamentous fungus. Although only certain species of *Mucor* are used nowadays in industrial processes, the results described in this paper demonstrate that the potential exists to further optimize lycopene production in already improved industrial strains. Furthermore, the existence of an ortholog of the *M. circinelloides crgA* gene in the zygomicete *B. trispora* (Quiles-Rosillo et al. 2005), which is used for the industrial production of β-carotene, suggests that the genetic approach described here could be applied to this fungus to develop a lycopene-superproducing strain of *B. trispora*.

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