Among the different species of the genus, *Rhodotorula* transformation [11], the production of biosurfactants [12] has been recently explored for copper bio-

…counteract fungal pathogens in postharvest [6], and the production of enzymes, lipids, biodiesel, fragrances and car-

The *Pucciniomycotina* represents a subphylum within the basidiomycetes that recently underwent revision based on combined molecular and phylogenetic approaches [1]. Yeasts belonging to the genus *Rhodotorula*, which now also includes most of the species that were known as *Rhodospori-

INTRODUCTION

The *Pucciniomycotina* represents a subphylum within the basidiomycetes that recently underwent revision based on combined molecular and phylogenetic approaches [1]. Yeasts belonging to the genus *Rhodotorula*, which now also includes most of the species that were known as *Rhodosporidi-

Among the different species of the genus, *Rhodotorula mucilaginosa* has been recently explored for copper bio-

and unsaturated fatty acids [13]. Moreover, *R. mucilaginosa* is a good candidate for the production of carotenoids, mainly β-carotene, torulene and torularhodin, also from low-cost carbon sources [14–16]. The carotenoid biosynthetic pathway has been extensively investigated in many biological systems and has been described in red yeasts [17]. In this regard, genes coding for key enzymes in the carotenogenic pathway have been isolated in *Xanthophyllomyces dendrorhous* [18–21]. Although great advances have been achieved in the study of the regulation of carotenogenic genes in *X. dendrorhous* at a transcriptomic level [22, 23], nothing is known about the tight and complex regulation of the carotenogenic pathway in *Rhodotorula* yeasts. This is due, at least in part, to the limited amount of available genomic data and functional annotation. Indeed, the release of the draft genome sequence of *R. mucilaginosa* [24] and other related species [25], the
development of tools for the genetic manipulation of red yeasts [26–29] and for their proteomic analysis [30] will contribute to filling this gap of knowledge and to gathering information on the molecular mechanisms that underlie features of interest in these yeasts.

Here, with the aim of shedding light on the molecular basis of carotenoid biosynthesis in *R. mucilaginosa*, the structure of the CAR gene cluster and the transcript level of carotenogenic genes were analysed for the first time.

**METHODS**

**Microbial strain**

*R. mucilaginosa* C2.5t1, kindly provided by Professor M. Ciani (Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy) was isolated by cacao seeds (*Theobroma cacao* L.) sampled in Cameroon, and is deposited at the DBVPG Industrial Yeast Collection (University of Perugia, Italy) with accession number DBVPG 10619. This strain, previously assigned as *Rhodotorula glutinis*, was characterized for its capability to produce biomass and carotenoids when grown in glycerol-containing media [31, 32] and further characterized as reported in [24] and [30].

**Bioinformatics analysis**

*R. mucilaginosa* C2.5t1 sequencing was performed as previously reported [24]. Raw reads were subjected to quality filter analysis using the NGS QC toolkit [33], and a de novo assembly was performed using the software Abyss 2.0 [34] with k-mer size 37 and with at least three paired reads confirming the produced scaffolds. Scaffolds shorter than 500 bp were removed from the final dataset. Gene prediction was initially performed with Augustus using a *U. maydis* training set [35] and Genemark-ES using the built-in fungus specific algorithm [36]. The identified transcripts were used as evidence of gene annotation to perform the Maker gene prediction pipeline (v.2.31, [37]) together with the training set [35] and Genemark-ES using the built-in prediction was initially performed with Augustus using a *U. maydis* training set [35] and Genemark-ES using the built-in fungus specific algorithm [36]. The identified transcripts were used as evidence of gene annotation to perform the Maker gene prediction pipeline (v.2.31, [37]) together with the training set [35] and Genemark-ES using the built-in fungus specific algorithm [36]. Similar, also the models in fungi and to the closely related *Rhodotorula* yeast species (R. toruloides NP11 [42] and ATCC204091 [43], R. graminis WP1 [44], Rhodotorula sp. GJ-1b [45]).

For the identification of the transcripts whose protein product is known to be involved in the last steps of the carotenoid biosynthetic pathway, the *R. graminis* WP1 and *R. toruloides* NP11 proteins Car0 (carotenoid dioxygenase), Car1 (phytoene dehydrogenase) and Car2 (phytoene synthase/lycopene cyclase) were retrieved from GenBank and the corresponding transcripts were subjected to tBLASTn search against the *R. mucilaginosa* CDS predictions, and (ii) BLASTx analysis also in *Rhodotorula* sp. JG-1b. Since it is known that these genes are organized in cluster (A. Idnurm, personal communication), the regions identified in *R. graminis* WP1, *R. mucilaginosa* C2.5t1, Rhodotorula sp. JG-1b and *R. toruloides* NP11 were retrieved and subjected to synteny analysis using the ACT Artemis software [46].

**R. mucilaginosa growth conditions**

Culture media were: YEPGLY (2 % glycerol, 2 % peptone, 1 % yeast extract); YEPD (2 % glucose, 2 % peptone, 1 % yeast extract, 2 % agar, when required) and YEPGLY 40 % (as YEPGLY with 40 % glycerol). Yeast cells were precultured in 20 ml of YEPGLY and incubated at 30 °C under shaking conditions (180 r.p.m.). After 12 h, 10^6 cells ml^-1 were inoculated in 250 ml baffled flasks containing 50 ml of YEPGLY and cultured under shaking conditions (180 r.p.m.) at 30 °C. Cell growth was monitored by evaluating total cell count, dry weight of biomass, or viable cell count. Yeasts were maintained at 4 °C on YEPD for short-term storage and in YEPGLY 40 % at −80 °C for long-term storage.

**Carotenoid extraction and quantification**

Carotenoid extraction was carried out according to [47], and modified as indicated by Cutzu and colleagues [32]. Briefly, cells contained in 1–3 ml aliquots of yeast cultures were harvested, washed twice in sterile distilled water, re-suspended in 1 ml acetone, centrifuged for 3 min at 3500 g and let dry briefly. The cell pellet was re-suspended in 2 ml DMSO pre-heated at 40 °C, added with 0.5 g acid washed glass beads (ø 212–300 µm), shaken for 5 min and incubated at 40 °C for 10 min. 2 ml acetone, 2 ml petroleum ether (added with hydroxytoluene butylate, 0.25 %) and 2 ml tBLASTn analysis in the *R. mucilaginosa* coding DNA sequence (CDS) prediction. Similarly, also the *R. mucilaginosa* genes coding for mitochondrial manganese superoxide dismutase, catalase A, actin and tubulin, (SOD2, CTA1, ACT1 and TUB2) were identified through tBLASTn analysis using as queries the respective *S. cerevisiae* encoded proteins. A second gene coding for a catalase-like protein, from now on indicated *CalP*, was retrieved. Collinearity studies with NP11 did not provide additional evidences for the identification of this protein. To assess the accuracy of the automated gene prediction, retrieved DNA sequences were subjected to BLASTx analysis on the non-redundant protein sequences available in GenBank and, where necessary, manual corrections were made according to protein models in fungi and to the closely related *Rhodotorula* yeast species (R. toruloides NP11 [42] and ATCC204091 [43], R. graminis WP1 [44], Rhodotorula sp. GJ-1b [45]).
NaCl 20% were added, the mixture was vigorously vortexed for 5 min and centrifuged at 3500 g for 5 min. The organic phase containing carotenoids was transferred to a fresh tube. When required, 2 ml DMSO were added to the cell pellet and all the steps described above were repeated to obtain a second aliquot of petroleum ether containing carotenoids that was added to the first one. All the stages following the addition of DMSO were carried out in the dark under red light. Total carotenoid concentration was evaluated using a SmartSpecTM plus spectrophotometer (Bio-Rad, Hercules, CA, USA) according to [48] and expressed as β-carotene-equivalents in respect to a calibration curve obtained by utilizing pure β-carotene (Sigma Adrich). Data were obtained in triplicate from at least three biological replicates. Statistical analyses of the data were performed using ANOVA followed by Tukey–Kramer honest significant difference test (all pair comparison) using the JMP version 3.1.5 software (SAS Institute).

RNA extraction, reverse transcript and quantitative PCR (qPCR)

Total RNA extraction was carried out by using PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer instructions. RNA was treated with DNase I, Amplification Grade, Thermo Fisher Scientific), visualized on 1% agarose gel and subject to PCR amplification using primers specific for the TUB2 gene to assess the presence of residual genomic DNA. RNA was quantified using a SmartSpecTM plus spectrophotometer (Bio-Rad, Hercules, CA, USA) according to [48] and expressed as β-carotene-equivalents in respect to a calibration curve obtained by utilizing pure β-carotene (Sigma Adrich). Data were obtained in triplicate from at least three biological replicates. Statistical analyses of the data were performed using ANOVA followed by Tukey–Kramer honest significant difference test (all pair comparison) using the JMP version 3.1.5 software (SAS Institute).

Table 1. PCR primers utilized for q-RT PCR

<table>
<thead>
<tr>
<th>Molecular target</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
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<tr>
<td>HMG1</td>
<td>HMG1F</td>
<td>5'-TCACGCTCCACTCGCTCAAC-3'</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>HMG1R</td>
<td>5'-CGAGGACAAGATGGGGTTGG-3'</td>
<td></td>
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<tr>
<td>ERG12</td>
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<td>5'-CAGTGGGCGGAGGCTTCTT-3'</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>ERG12R</td>
<td>5'-GGACGCGTGCGAGTAGAGC-3'</td>
<td></td>
</tr>
<tr>
<td>CAR2</td>
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<tr>
<td></td>
<td>CAR2R</td>
<td>5'-CGTTGTTGCGCTACAGGAGG-3'</td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>CAR1R</td>
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</tr>
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<td>5'-GGCCGTTAAGCGTGATG-3'</td>
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<td>ACT1AR</td>
<td>5'-CGGCAATGAAACCCCTCT-3'</td>
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<td>TUB2</td>
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<tr>
<td></td>
<td>TUB2R</td>
<td>5'-CAGACGCCGGAACAG-3'</td>
<td></td>
</tr>
</tbody>
</table>

For accurate quantification of the qPCR products, at least three technical replicates of five biological replicates were carried out.

Primers specific for qPCR were designed on the revised sequences of the R. mucilaginosa genes HMG1, ERG12, CAR0, CAR1, CAR2, SOD2, CTA1 and CaLP. To this aim, the Primer3 software was utilized and primer pairs with an alignment temperature of 60°C were selected. ACT1 and TUB2 genes (coding for actin and β-tubulin, respectively) were considered as possible housekeeping genes for R. mucilaginosa. ACT1 gene expression proved stable at all the sampling times analysed with a 2% variation in the threshold cycle variation. Thus, the relative abundance of the original template for the examined genes between samples was calculated compared to that of ACT1 by considering the percentile amplification efficiency (PAE) of each gene [49]. Considering the small sample sizes, PAE-adjusted ANOVA for repeated measures was chosen for statistical analysis of the data (P<0.05) [49]. Assumption of sphericity was assessed with Mauchly’s test of sphericity. For a given gene, Fisher’s least significant
Several assembly algorithms were tested with the aim to determine which means are significantly different from which others at the 95.0 % confidence level.

RESULTS

Genome annotation

Several assembly algorithms were tested with the high-quality R. mucilaginosa assembly previously reported [24]. In this regard, the software Abyss produced as few as 707 scaffolds featuring an N50 of 63,566 bp and a total of 19,976,055 bp in the final assembly (earlier assembly was composed of 1034 scaffolds featuring N50=45,031) (Table 2). Gene prediction revealed a dataset of 5715 protein-coding genes of which 4845 passed our quality filter (e.g. transcript sequence starting with ATG, ending with a canonical stop codon and featuring an integer number of nucleotide triplets). Functional annotation carried out with the bioinformatics software Blast2GO assigned a GO term to 4033 of the predicted proteins (~70 % of the total).

Implementation of molecular tools for the study of the carotenogenic pathway

An initial set of R. mucilaginosa genes predicted to be involved in different steps of the mevalonate biosynthetic pathway was retrieved through BLAST searches using S. cerevisiae orthologues (Fig. 1). The predicted function of the identified R. mucilaginosa genes was confirmed through reciprocal best blast hit approach on GenBank and Blast2GO annotation, and based on alignments to orthologous genes of closely related species, the original gene model was manually improved. Among the genes identified, HMG1, ERG12, CAR2, CAR1 and CAR0 were selected for further analyses due to their key role in the carotenoid biosynthetic pathway (Fig. 1). Sequences of these genes were used to design specific primers for qPCR analyses during carotenogenesis.

Genes CAR1, CAR2 and CAR0 coding for phytoene desaturase, phytoene synthase/lycopene cyclase and carotenoid dioxygenase, respectively, proved to be clustered within a region of ~10 kb. We integrated the information available on such a cluster in red yeast species Sporobolomyces sp. IAM 13481 and R. graminis WP1 (A. Idnurm, personal communication) by searching the homologous regions in R. toruloides NP11 and Rhodotorula sp. GJ-1b and used them for comparative analysis with that of R. mucilaginosa C2.5t1. As shown in Fig. 2, while the genes CAR2 and CAR0 have the same orientation in the four examined clusters, the CAR1 gene has opposite orientation in R. mucilaginosa and Rhodotorula sp. GJ-1b compared to R. graminis and R. toruloides. Notably, in the R. mucilaginosa C2.5t1 and Rhodotorula sp. GJ-1b CAR cluster, another gene (OPT1) is also present (Fig. 2). The OPT1 gene, which codes for an oligopeptide transporter, does not seem to have any obvious connection with the biosynthesis of carotenoids. The accurateness of the genome assembly performed was also confirmed by PCR analysis and sequencing (data not shown), indicating that the presence of the OPT1 gene is not an artifact.

Growth kinetics and carotenoid production on a glycerol-containing medium

In order to establish the time points at which to evaluate the level of transcription of the genes of interest, the kinetics of growth and of carotenoid production were analysed in R. mucilaginosa C2.5t1 during batch cultivation on YEPGLY that proved to be a well-suited substrate for carotenoid production [32]. On this medium, C2.5t1 reached the stationary phase at 40 h growth. As already observed for X. dendrorhous during cultivation on non-fermentable carbon sources [50], carotenoid production showed no lag phase but it accompanied cell growth, progressively increasing during exponential phase (Fig. 3a). Further increases in carotenoid content were observed at early stationary phase (48 h) with minor variations afterwards. Indeed, the carotenoid production rate peaked at 48 h, when cells were in stationary phase (Fig. 3b). Based on these results we decided to evaluate the level of transcription of the genes involved in carotenoid biosynthesis and in the antioxidant response at different sampling times during exponential phase (16 and 24 h) at the entry in stationary phase (40 h) and at early and late stationary phase (48 and 72 h).

Transcript levels of HMG1, ERG12, CAR2, CAR1 and CAR0

To gather information on the expression kinetics of the selected carotenogenic genes during carotenoid accumulation, qPCR was performed. The transcript levels of HMG1 and ERG12 (coding for 3-hydroxy-3-methylglutharyl-CoA reductase and mevalonate kinase, respectively) showed a comparable trend with significant increases at 24 h and a peak at the end of exponential growth phase (40 h) (Fig. 4). At 48 h there was a significant decrease of the transcript levels of both genes with this trend being maintained during stationary phase (Fig. 4).

Contrary to that observed for HMG1 and ERG12, CAR2 and CAR1 genes (coding for late enzymes of the carotenogenic pathway) did not show a clear trend of induction. CAR2 maintained transcript levels that ranged between 0.8- and 1.5-fold of the transcript level of the housekeeping gene. CAR1 showed higher expression levels in respect to CAR2.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison of original (JWTJ01.1) and herein presented (new draft) assemblies of the R. mucilaginosa C2.5t1 genome</th>
</tr>
</thead>
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<tr>
<td>Parameter</td>
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<td>#Predicted transcripts</td>
<td>6412</td>
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<tr>
<td>#Anomalous transcripts</td>
<td>883</td>
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</table>
Expression of the CAR0 gene (coding for carotenoid dioxygenase) was low at exponential phase, but it significantly increased at 40 and 48 h while decreasing at 72 h (Fig. 5).

Transcript levels of SOD2, CTA1 and CaLP genes

The transcript level of the SOD2 gene, coding for mitochondrial manganese superoxide dismutase, significantly increased during growth with a peak at 40 h after which it dramatically decreased although maintaining high expression levels (Fig. 6). Among the catalase coding genes, CTA1, coding for catalase A, showed a peak of expression at 48 h. The CaLP gene, coding for a catalase-like protein that shows 46 and 49 % identities with Ctt1 and Cta1 of S. cerevisiae, respectively, significantly decreased its transcript level in the first 48 h until reaching a plateau at stationary phase (Fig. 6). SOD2 and CTA1 genes were highly expressed at all sampling times, while the CaLP gene, although varying significantly during growth, ranged between 1.4- and 3.8-fold of the transcript level of the housekeeping gene (Fig. 6).

DISCUSSION

Here, an improved de novo assembly of the genome of R. mucilaginosa C2.5t1 and its complete functional annotation are presented. Moreover, the identification of the genes involved in the biosynthesis of carotenoids and their transcript accumulation during carotenogenesis are reported.

Several assembly algorithms were tested to improve the final draft of the C2.5t1 genome which resulted in a 32 % reduction in the number of scaffolds and a 1.4-fold increase of N50, while assembling approximately the same number of bases. Genome annotation, previously based on the prediction generated by the software Augustus [24], was considerably improved here by analysing the final assembly with the combined approach described in Methods. In fact, the number of assembled scaffolds was reduced from 1034 to 707 whereas N50 was increased from 45 031 to 62 566. This resulted in an increase of both the average and the median scaffold length (Table 2).

Accordingly, the search for the genes involved in carotenoid biosynthesis on the newly annotated genome led to the identification of most of the genes of interest, and those having a pivotal role in this biosynthetic pathway were selected for further analyses. Among these, HMG1 codes for 3-hydroxy-3-methylglutharyl-CoA reductase; ERG12 codes for mevalonate kinase; CAR2: phytoene synthase/lycopene cyclase; CAR1: phytoene dehydrogenase; CAR0: carotenoid dioxygenase.

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Fig. 1. Diagram of the carotenoid biosynthetic pathway. HMG-CoA: 3-hydroxy-3-methylglutharyl-CoA; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPP: geranyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate. HMG1: 3-hydroxy-3-methylglutharyl-CoA (HMG-CoA) reductase; ERG12: mevalonate kinase; CAR2: phytoene synthase/lycopene cyclase; CAR1: phytoene dehydrogenase; CAR0: carotenoid dioxygenase.

(three to fivefold of the housekeeping gene). However, also in this case no marked variations were observed during growth (Fig. 5). Thus, under the culture conditions tested in R. mucilaginosa there is no correlation between the transcript level of genes coding for late carotenogenic enzymes and the production of carotenoids.
Carotene. CAR0 codes for a carotenoid dioxygenase very likely implicated in the turnover of carotenoids and in the maintenance of carotenoid homeostasis. In addition, SOD2, CTA1 and CaLP genes were also selected. They encode the mitochondrial superoxide dismutase, catalase A and a catalase-like protein, respectively, and are likely involved in the antioxidant response of yeasts that, according to Martinez-Moya and colleagues [23], accompanies carotenoid biosynthesis.

Analyses of the transcript levels of carotenogenic genes revealed that in R. mucilaginosa carotenoid biosynthesis is accompanied by a transient induction of HMG1 and ERG12. These genes are not exclusively involved in this biosynthetic pathway. However, their role in the production of carotenoids has already been discussed in other species. In particular, it was reported that HMG1 transcription is induced during carotenoid production [53, 54] and that the overexpression of ERG12 determines an increase of heterologous carotenoid production in recombinant S. cerevisiae [54]. Thus, the rise in the mRNA levels of these two genes in exponential phase could be functional to the production of precursors in the mevalonate pathway. The drop at the following sampling times, in concomitance with a marked increase in the intracellular carotenoid content, is compatible with a high stability of the corresponding enzymes at stationary phase.

The low level of transcription of CAR2 and CAR1 genes during carotenoid accumulation is in accordance with the low expression of carotenogenic proteins [30, 55] and suggests that, similar to what was observed in X. dendrorhous, the amount of carotenoids produced by R. mucilaginosa may depend more on the regulation of enzyme activity than on the transcript levels of carotenogenic genes [50]. Carotenoid production was also accompanied by a significant increase in CAR0 expression. Carotenoid dioxygenase catalyses the selective oxidative cleavage of carotenoids to produce apocarotenoids [56]. In mammals, this enzyme is involved in carotenoid degradation to protect mitochondrial membranes from the pro-oxidant effect of carotenoids [57]. In fact, β-carotene may work as a pro-oxidant in the
presence of high oxygen concentrations [58] and its intracellular accumulation determines an increase in the sensitivity to reactive oxygen species (ROS) [59]. This is in accordance with the low expression of CAR0 shown by R. mucilaginosa at early exponential phase, when the intracellular carotenoid content is still low, its increase at 40 and 48 h, when carotenoid content and production rate significantly increase, and its further decline at 72 h when carotenoid biosynthesis slows down. Similarly, transcript levels of SOD2 and CTA1 genes also rose during carotenoid accumulation and decreased in concomitance with the reduction of carotenoid production rate. These results agree with the role of antioxidant genes in the protection of mitochondria from the oxidative damage caused by respiration and are in accordance with the upregulation of enzymes involved in the stress response during the carotenoid production observed by other authors [23, 60]. The abundance of enzymes involved in the response to stress is related to the induction of carotenogenesis [23]. Indeed, β-carotene and other carotenoids induce ROS production, depolarize the mitochondrial membrane in a dose-dependent manner [57] and cause membrane stress [61] thus resulting in a marked increase in mitochondrial superoxide dismutase in carotenoid oxygenase deficient mutants [62]. Hence, the induction of SOD2 and CTA1 genes, together with that of the CAR0
Fig. 5. Relative expression of $\text{CAR2}$, $\text{CAR1}$ and $\text{CAR0}$ genes during growth on YEPGLY. Relative expression data are the mean±SD of at least three technical replicates of five independent experiments. Where not seen, bars lie within the symbols. Same letters indicate not significant differences among means as determined by one-way repeated ANOVA measures followed by Fisher’s LSD procedure ($P<0.05$).

Fig. 6. Relative expression of $\text{SOD2}$, $\text{CTA1}$ and $\text{CaLP}$ genes during growth on YEPGLY. Relative expression data are the mean±SD of at least three technical replicates of five independent experiments. Where not seen, bars lie within the symbols. Same letters indicate not significant differences among means as determined by one-way repeated ANOVA measures followed by Fisher’s LSD procedure ($P<0.05$).
gene, leads to the involvement of these genes being hypothesized in the protection of cell structures from oxidative stress during carotenoid biosynthesis.

Since other authors have already described a cluster of CAR genes in red yeast species (A. Idnurm, personal communication) the discovery of the same cluster in R. mucilaginosa was not unexpected. However, we observed here that this CAR cluster and that of Rhodotorula sp. GJ-1b differ from those of R. graminis WP1 and R. toruloides NP11 for the different orientation of the CAR1 gene and the presence of an OPT1 orthologue flanking the CAR1 gene. Indeed, in R. toruloides NP11 also, a gene apparently not related to carotenogenesis (NA) is present between CAR0 and CAR1. However, this is not an orthologue of OPT1 and it has an unknown function. Moreover, we found the OPT1 gene in different genomic locations in phylogenetically related red yeast species (data not shown). Based on these evidences it may be hypothesized that a gene rearrangement event, that led to the translocation of the OPT1 gene in the CAR cluster and to the inversion of CAR1, had occurred in an ancestral species of R. mucilaginosa and Rhodotorula sp. GJ-1b.

In conclusion, here we obtained new data suitable for the study of the carotenoid biosynthetic pathway, one of the most relevant in R. mucilaginosa. Moreover, the de novo assembly and annotation of the genome sequence of C2.5t1 will contribute to boosting the biotechnological potential of R. mucilaginosa by improving the outcome of further research efforts aimed at also exploring other features of interest in this yeast.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This article does not contain any studies with human participants or animals performed by any of the authors.

References


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