# Characterization the carotenoid productions and profiles of three *Rhodosporidium toruloides* mutants from *Agrobacterium tumefaciens*-mediated transformation

**Running Head:** Carotenoid production and its profiles in three *R. toruloides* mutants

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**Abstract**:

The red yeast *Rhodosporidium toruloides* is a known lipid producer capable of accumulating large amounts of triacylglycerols and carotenoids. However, it remains challenging to study its carotenoid production profiles due to limited biochemical information and inefficient genetic tools. Here we used an *Agrobacterium tumefaciens*-mediated transformation (ATMT) to change its carotenoid production and profiles. We constructed *R. toruloides* NP11 mutant libraries with ATMT, selected 3 mutants with different colors, characterized their carotenoid products by high pressure liquid chromatography-mass spectrometry (HPLC-MS) analysis, and assured differences among those strains in terms of carotenoid production and its composition profiles. We then located T-DNA insertion sites by using the genome walking technology and provided discussions in terms of the new phenotypes. This is the first of its kind to change the carotenoid production profiles in *R. toruloides.*

**Key Words**: *Rhodosporidium toruloides*; Carotenoids; *Agrobacterium tumefaciens*-mediated transformation; T-DNA insertion mutants;

## Introduction

Carotenoids are attractive $C_{40}$ terpenoids with various physiological activities and functions. It is known that carotenoids can protect cells by quenching singlet oxygen, free radicals and reactive oxygen species. Therefore, they are widely used as nutritional supplements and food colorant (Ribeiro *et al.*, 2011). With increasing interests in carotenoid production, the industrial market is expected to expand to $1.4 billion until 2018 (Mannazzu *et al.*, 2015).

Carotenoids can be naturally synthesized by fruits, vegetables and microorganisms. Microbial production of carotenoids owns a series of advantages over other sources, such as no limitation by the seasonal and geographic variability, rapid growth ability and economic benefits of using low cost natural substrates as carbohydrates (Lin *et al.*, 2014). Nowadays, most commonly used microorganisms for producing carotenoids are *Blakeslea trispora* (Wang *et al.*, 2014), *Dunaliella salina* (Wichuk *et al.*, 2014) and red yeasts (Frengova *et al.*, 2009). The red yeasts belong to *Basidiomycota* phylum and have advantages of simple fermentation process and short growth cycle, due to their unicellular nature and high growth rate. Therefore, they are considered as potential candidates of carotenoid producers. However, the low carotenoid content per cell impedes their developments for industrial production.

*Rhodosporidium toruloides* belongs to red yeasts and is known for its ability to produce carotenoids. Over 90% of the carotenoids produced by *R. toruloides* were composed of torularhodin, torulene, γ-carotene and β-carotene (Buzzini *et al.*, 2007;Lee *et al.*, 2014). These carotenoids have important applications as food colorants and poultry feed (Buzzini *et al.*, 2001;Zoz *et al.*, 2015). In addition, *R. toruloides* not only has wide substrate utilization range (Lee *et al.*, 2014;Yang *et al.*, 2015), but also has good tolerance to inhibitors from lignocellulose biomass hydrolysis (Hu *et al.*, 2009). The whole genome of *R. toruloides* has been sequenced (Zhu *et al.*, 2012) and great progresses have been made in developments of genetic tools by *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Lin *et al.*, 2014;Liu *et al.*, 2013). These progresses facilitate using *R. toruloides* as a model organism to study the red yeasts' carotenoid producing pathway.

Mutagenesis is a common strategy to improve phenotypic profiles for high yields and different characteristics. Mutations can be generated by physical methods such as UV radiation or by chemical mutagens such as nitrosoguanidine (NTG) and ethyl methanesulfonate (EMS). An *Rhodotorula glutinis* mutant was isolated from samples treated with UV irradiation for increase in β–carotene production (Cutzu *et al.*, 2013) and mutants of *Xanthophyllomyces dendrorhous* were obtained with increases up to 100% in astaxanthin content (Stachowiak, 2013). Combined processes of mutagenesis have also been applied for *R. toruloides* to accumulated 0.23 g lipids/g DCW and 750 μg carotenoids/g CDW (Zhang *et al.*, 2016). ATMT is another mutagenesis method, and it takes advantages of other chemical or radiation mutagenesis, because the mutated genes are tagged by T-DNA, which can then be used to identify the disrupted genes or the flanking sequences. This "phenotype-to-genotype" strategy benefits in getting the expected mutants and explaining the reasons at the same time. ATMT has been widely used in yeast (McClelland *et al.*, 2005), fungi (Zhong *et al.*, 2007) and plants (Chao *et al.*, 2014). What's more, it supplies targets for further accurate engineering. However, there is rare study for carotenoid profile mutants of *R. toruloides* generated from ATMT.

In this study, we applied the ATMT to change the carotenoid production and profiles of *R. toruloides*. We constructed *R. toruloides* NP11 mutant library, selected mutants with different colors and characterized their carotenoids production and composition profiles. We then located the gene inserted positions by using the genome walking technology and discussed the reasons for the new phenotypes. To our knowledge, this is the first of its kind to change the carotenoid production profiles in *R. toruloides*.

## Materials and Methods

### Strains, culture conditions and reagents

Strains used in this study were listed in Table 1. *R. toruloides* NP11 GDMCC 2.224 was used as a host for ATMT. Yeast strains were grown at 28 °C in YEPD (20 g/L glucose, 10 g/L yeast extract and 10 g/L peptone, pH 6.0) with agitation of 200 rpm. *A. tumefaciens* AGL1-HYG1 and AGL1-HYG2 were cultivated in LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 ng/μL kanamycin, pH 7.0) at 28 °C with agitation of 200 rpm.

For carotenoid production, yeast strains were cultivated in culture containing 20 g/L glucose, 6.7 g/L yeast nitrogen base (without amino acids), pH 6.0. LA *Taq* DNA polymerase, *rTaq* DNA polymerase and pMD$^{TM}$ 18-T Vector Cloning Kit were supplied by TaKaRa (Dalian, China). Acetosyringone, hygromycin B and all primers used in this study (Table 2) were purchased from Dingguo Biotechnology (Beijing, China).

### Strains construction and verification

T-DNA inserted libraries were constructed by transformed *R. toruloides* NP11 with AGL1-HYG1 or AGL1-HYG2 according to a previous method (Lin *et al.*, 2014). A yellow transformant (*R. toruloides* NP11-4 GDMCC 2.226) was picked out from the PGK promoter's library. A white transformant (*R. toruloides* NP11-5 GDMCC2.227) and a red transformant (*R. toruloides* NP11-8 GDMCC 2.228) were picked out from the GPD promoter's library. These transformants underwent molecular analysis of T-DNA insertion by PCR. HYG protein expressions by western-blot and mitotic stability tests were done according to the former report (Lin *et al.*, 2014).

### Cultivations in shake-flasks

All the strains were maintained on YEPD agar plates in less than one week. Pre-cultures were made by inoculating strains into 50 mL SD liquid culture for 36 h at 28°C with 200 rpm agitation. Carotenoids fermentations were initiated upon 45 mL of SD culture mediums inoculated with 5 mL of pre-culture in 250 mL Erlenmeyer flask at an optical density ($OD_{600}$) of 0.496. All the experiments were done in triplicates and data were presented as mean value ± standard deviation.

**Analytical methods**

The residual glucose concentrations were determined using an SBA-40E glucose analyzer (Shandong Academy of Sciences, Jinan, China). Culture samples were harvested by centrifugation at 7800 *g* 8 min and washed twice with distilled water. The washed cells were dried at 105°C for 24 h to a constant dry cell weight (DCW) and the biomass was determined gravimetrically.

**Carotenoid extraction, characterization and quantitation**

Carotenoids were extracted according to a previous report (Xie *et al.*, 2014) with some modifications. Two milliliters of cell cultures were centrifuged for 8 min at 7800 *g* at 4°C, and then washed by water. Cell precipitates were collected and suspended in 1 mL of 3 M HCl, after 20 min, heated for 3 min in boiled water and then cooled for 3 min in ice water. The mixture was centrifuged at 7800 *g* for 10 min at 4°C. Then, 2 mL acetone was added to the precipitates and the mixture was vortexed followed by centrifugation at 7800 *g* for 10 min at 4°C. The supernatants were removed and the procedure was repeated until the supernatants became colorless. All the supernatant fractions were combined for carotenoid characterization and quantitation.

Characterization and quantitation of carotenoids were determined with photodiode array detection by HPLC-MS equipped with C18 column (5 μm, 150 mm × 4.6 mm). Samples were filtered through 0.45 μm microporous film before injections. Elution began with the mobile phase composed of 89% acetone for 7 min and changed to a gradient from 89% to 100% acetone for 6 min followed by staying isocratic for 2 min. The flow rate was 1 mL/min and the total elution time was 25 min. Standard solutions of β-carotene with concentrations between 0.05 and 0.1 μg/mL were used to obtain a calibration curve. The β-carotene's milli-absorbance units (mAU) obtained from the HPLC profile were quantified. The γ-carotene was identified on the basis of retention time in the previous study (Buzzini *et al.*, 2007) and further confirmed with the extract ion profile at 537 m/z which represent the 2 isobaric compounds of β-carotene and γ-carotene (Figure S3). Similarly, the presents of torularhodin and torulene were identified by HPLC retention time profiles and confirmed at the extract ion profile at 565 m/z and 535 m/z, respectively (Figure S3). Amounts of the four peaks were summed to provide the total carotenoid production and the content of carotenoids

was expressed as microgramme per gram of dry cell weight (μg/g DCW).

**Determine the copy number by real-time quantitative PCR analysis**

For the determination of the copy number of the inserted gene cassette, qPCR experiments were carried out by amplification of each promoter sequence in the gDNA sample with primers listed in Table 2. All experiments were done with two technical replicates. The PGK/GPD gene was a single-copy gene of *PZPK-pPGK/pGPD-HYG-Tnos* (containing one copy of T-DNA) and *R. toruloides* chromosomal DNA, and the FBA gene was a single-copy gene of *R. toruloides* chromosomal DNA. Consequently, quantification of sample with the PGK/GPD-set and FBA-set represents corresponding amount of T-DNA and *R. toruloides* chromosomal DNA. A 10-fold serial dilution series of each plasmid (see Table 1) containing the corresponding promoter sequences ranging from $2\times10^1$ to $2\times10^6$ copies was used to construct the standard curve. The concentration of the plasmid was measured using a fluorometer on Thermal Cycler Dice™ Real Time System II (TaKaRa). The gDNA of the wild-type *R. toruloides* and the mutants were used as templates, and all the gDNA concentration were adjusted to a final concentration of 30-40 ng/μL with nuclease-free water. PCR reaction mixture contained 12.5 μL SYBR Premix Ex TaqII (Ti RNaseH Plus) (TaKaRa), 1 μL each forward and reverse primer (10 μM), 1 μL gDNA template, and nuclease-free water to a final volume of 25 μL. PCR conditions were as follows: 95°C for 30 s, (95°C for 5 s, 60°C for 30 s) for 40 cycles, 95°C for 15 s, 55°C for 15 s, 95°C for 15 s. The copy number of the pPGK or pGPD cassettes (represented as T-DNA insertion number) were calculated based on qPCR data according to a known method (Lee *et al.*, 2006).

**T-DNA location identified by genome walking**

Total genomic DNA was extracted according to a former study (Lin *et al.*, 2014). The thermal asymmetrical interlaced-polymerase chain reaction (TAIL-PCR) was used to identify flanking sequence of T-DNA as described by Liu (Liu *et al.*, 2007). Four arbitrary degenerated primers (LAD-1, LAD-2, LAD-3, LAD-4) and three specific right border (RB) primers (RB-0-a, RB-1-a, RB-2-a) were used. The four arbitrary degenerate primers and the RB-0-a were used in pre-amplification. The AC1 primer which shared a common sequence with LAD primers was used in the primary TAIL-PCR and the secondary TAIL-PCR cooperated with RB-1-a and RB-2-a, respectively. The reaction conditions and thermal cycling settings were the same

as the previous report (Liu *et al.*, 2007). PCR products were analyzed on 1.0% (w/v) agarose gels. By comparing the three PCR results, the highest intensity and the most specific product was purified with SanPrep Column DNA Gel Extraction Kit of Sangon Biotech (Shanghai, China). The purified DNA fragment was ligated into pMD$^{TM}$ 18-T Vector and transformed into *Escherichia coli* DH10B. Transformants were picked out by colony PCR and the sequences between the T-cloning sites were determined by Sangon Biotech. T-DNA location was identified by comparing the sequences with published *R. toruloides* genome (Zhu *et al.*, 2012) by using the BLAST programs.

**Results and discussion**

**Selection of transformants with different colors from ATMT mutant library**

*Agrobacterium tumefaciens*-mediated transformation is an efficient way to insert exogenous genes into a host's genome (Lin *et al.*, 2014;McClelland *et al.*, 2005;Wang *et al.*, 2016;Zhong *et al.*, 2007). Two mutant libraries were constructed by transforming *R. toruloides* NP11 with AGL1-HYG1 or AGL1-HYG2. *R. toruloides* is able to accumulate carotenoids and shows pink or orange clones on the plates. Therefore, mutants with different colors can be easily observed. Transformants were obtained on plates with 50 ng/μL hygromycin B. Three mutants were picked out, with strains NP11-4 showing light orange color from the PGK promoter's library, NP11-5 showing white color from the GPD promoter's library and NP11-8 showing dark pink color from the GPD promoter's library (Figure S1). After three times' purifications, all these strains exhibited stable phenotypes. These mutants were subjected to genotype verification by PCR. The results showed that all the transformants contained T-DNA fragment. qPCR results showed that there were 2.31 copies of the promoter sequences of pPGK for NP11-4, 2.14 and 1.91 copies of the promoter sequences of pGPD for NP11-5 and NP11-8, respectively, indicating that each mutant had only one copy of the T-DNA cassette in the genome. It was consisted with other reports (Michielse *et al.*, 2005;Wang *et al.*, 2016) demonstrated that T-DNA integration mainly resulted in single-copy in yeasts and fungi. Furthermore, HYG protein expressions were also confirmed the successful insertions of exogenous genes (data not shown). These mutants maintained their hygromycin resistance after being sub-cultured for five generations on YEPD which showed mitotic stability.

**Growth and carotenoid production by *R. toruloides* strains**

The growths of the mutants and carotenoid productions were investigated in the SD culture medium. Previous experiments and other studies (Lee *et al.*, 2014) showed that the carotenoids accumulation in *R. toruloides* were stable until 8-9 days, therefore, all the fermentations were stopped at 216 h and the results were shown in Figure 1. The wild strain *R. toruloides* NP11 accumulated 940.1 μg/g DCW carotenoids at the end of the fermentation. In Buzzini's study (Buzzini *et al.*, 2007), five-day-old cells of *R. toruloides* produced 122.6 μg/g DCW carotenoids. In Zhang's report (Zhang *et al.*, 2016), *R. toruloides* NP11 produced 350 μg/g DCW carotenoids for 144 h. Compared with these studies, it was suggested that the production of carotenoids may increase with time. At the end of 216 h fermentation, the mutants' colors (Figure S2 a b) were consistent with their phenotypes on the plates (Figure S1). The biomass of NP11-4 and NP11-5 were similar to the wild strain NP11, which were 2.2 g/L, 2.3 g/L and 2.5 g/L respectively. However, the NP11-8 showed a slightly lower biomass of 1.9 g/L. The mutants' carotenoid contents were consistent with their colors. The white NP11-5 didn't produce any carotenoids. NP11-8, which was the reddest one, had the highest carotenoid content of 1136.7 μg/g DCW and was 1.2 times of that in wild strain NP11. The NP11-4, which appeared light orange, only produced carotenoids equivalent to 64% of that in NP11.

**Characterization and Quantitation of Carotenoids profiles by HPLC-MS**

To further characterize the carotenoids in these *R. toruloides* strains, the carotenoid compositions were identified and quantitated by HPLC-MS (Table 3). Our analysis showed that strain NP11-5, the white color strain, had none of the four pigments, which matched its colorless phenotype. In the wild strain NP11, torularhodin was the major pigment which was 518.3 μg/g DCW, followed by 361.6 μg/g DCW β-carotene, 43.5 μg/g DCW γ-carotene and 16.8 μg/g DCW torulene. For strain NP11-4 showing a light orange to yellow color, the amounts of torularhodin and β-carotene were about 42% and 80% of those in the wild strain. However, the γ-carotene was 1.6 times as that in strain NP11 and consisted 11% of the total carotenoids, which was 2.4 times more than the wild strain. The γ-carotene is a yellow pigment, therefore, its accumulation gave the strain NP11-4 a light orange to yellow color as shown in Figure S1 and Figure S2 a b. For strain NP11-8, the torularhodin produced was 64%

of that in the wild type, while the torulene and β-carotene produced were 9 times and 1.7 times, respectively, of those produced by the wild type. The higher amounts of torulene, β-carotene and total carotenoids produced by strain NP11-8 gave it a darker red color.

**Identification the T-DNA insertion sites in *R. toruloides* mutants by genome walking**

To find out the reasons for the changes in carotenoid profiles in strains, locations of the T-DNA fragment in the mutants were determined by genome walking. Further confirmations by PCR suggested the validity of the genome walking results (data not shown). Results revealed that none of the insertion events happened at identical chromosomal sites (Table 4). The strain NP11-4's insertion was in an intron of a hypothetical protein (RTHO_00032) which consisted of 414 amino acids. T-DNA insertion in strain NP11-5 was located at the exon of basic leucine zipper (bZIP) transcription factor of 601 amino acids. For strain NP11-8, the insertion site was in the promoter of DUF1479 domain protein (RTHO_07650) of 503 amino acids. These inserted T-DNA flanking sequences were compared with each other and no homology was found among them. This suggested that T-DNA integrations occurred at random position in *R. toruloides* genome by a process of non-homologous recombination, which has been reported previously (Lin *et al.*, 2014;McClelland *et al.*, 2005;Wang *et al.*, 2016;Zhong *et al.*, 2007). Thus, ATMT could be used as a potential method for generating traceable *R. toruloides* mutants.

Insertion in the exon of bZIP transcription factor (RTHO_07952) in the NP11-5 strain caused the inactivity of this protein. At the same time, the carotenoid pathway was blocked and no carotenoids were detected. From the carotenoid pathway, we could hypothesize that the RTHO_07952 gene might influence the synthesis from the colorless phytoene to the red neurosporene (Figure 2) or blocked the pathways upstream, such as GGPP to phytoene step. This phenomenon was similar to the bZIP induction by ethylene regulatory control in tomato fruit. In the process of using ethylene for the control of tomato ripening, Alba observed that the transcription factor bZIP1 was induced, while the tomato turned red and the carotenoids content in fruit was increased (Alba *et al.*, 2005). The bZIP transcription factor plays critical roles in the organismal responded to the environment and is associated with resistance to reactive oxygen species (ROS), hyperosmotic stress or antifungals (Yin *et al.*, 2013). The bZIP transcription factor may response to ROS and then induced the carotenoid synthetic

pathway. When the bZIP transcription factor were damaged by T-DNA insertion, as in strain NP11-5, the mutant failed to respond to ROS and biosynthesis of carotenoids was decreased.

In strain NP11-4, the γ-carotene increased significantly and the amounts of following pathway metabolites, such as β-carotene, torularhodin and torulene, were greatly decreased. Therefore, T-DNA insertion site of RTHO_00032 may involve in the synthesis or regulation γ-carotene's downstream pathway (Figure 2) which may be reduced and result in accumulation of γ-carotene.

In the strain NP11-8, the insertion in the promoter of DUF1479 domain protein caused the accumulations of torulene and β-carotene, while the amount of torularhodin was decreased. It suggested that RTHO_07650 (Figure 2) might down-regulate the expression of genes responsible for the oxidation of torulene to torularhodin. This insertion might also have effects on increasing the metabolic flux for carotenoid synthesis.

In this study, carotenoid pathway was firstly changed in *R. toruloides* by ATMT to obtain three strains with different color from the wild strain. Total carotenoids and carotenoid profiles were detected to be changed in these mutants. The strain NP11-4' γ-carotene showed a 2.4 times increase compared with the wild strain, while the strain NP11-8 had a 9 times of the torulene and 1.7 times of β-carotene. The mutants' T-DNA insertion sites were identified. These findings increase our knowledge of carotenoid synthetic pathway and the insertion sites may be used as further engineering targets in the future.

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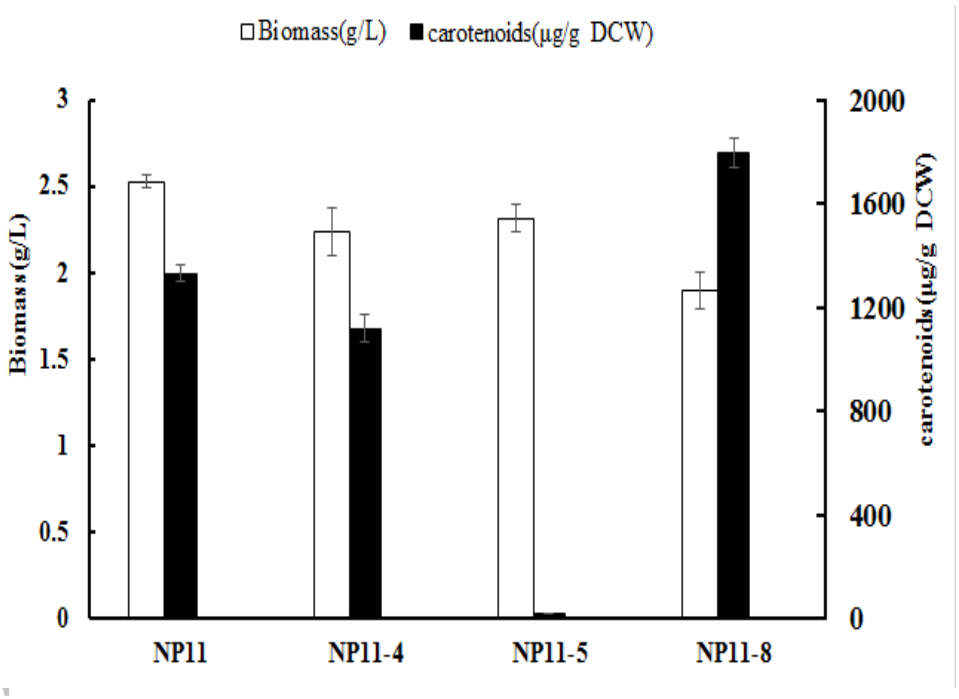


**Figure 1.** Biomass of *R. toruloides* strains after growth in 250 mL shake flasks containing 50 mL medium and the carotenoid production by these strains for 216 h. The error bars represent standard deviation calculated from triplicate experiments. Strain NP11 is the wild type while strains NP11-4, NP11-5 and NP11-8 were mutants.

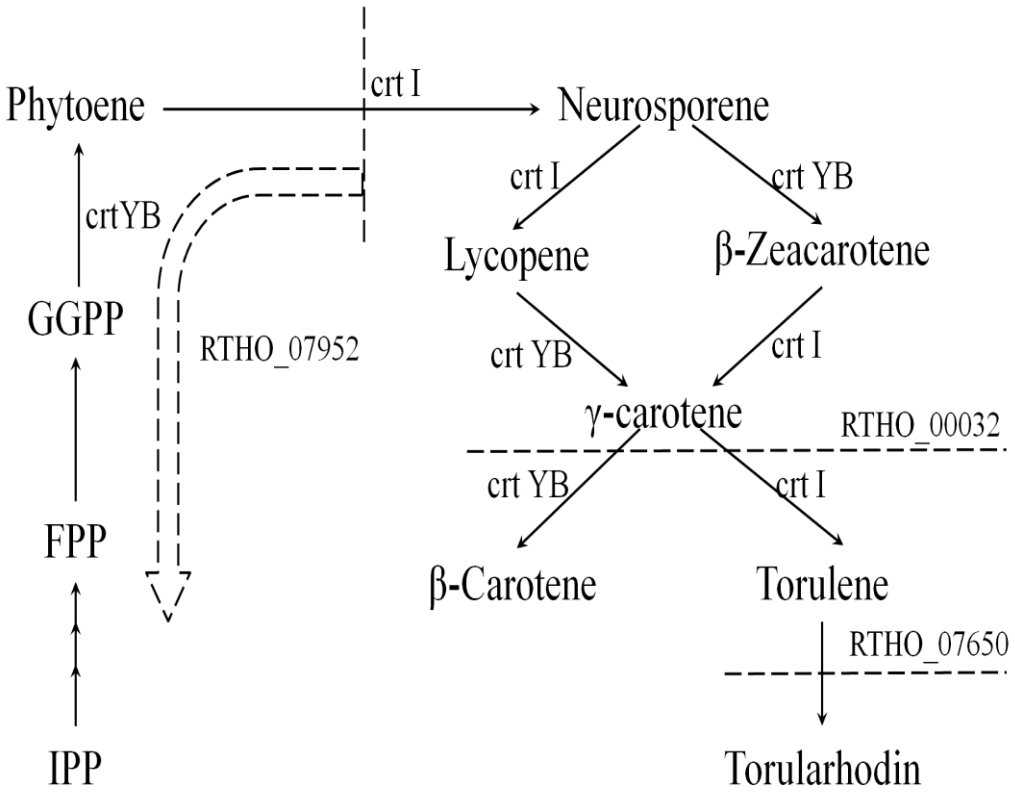


**Figure 2.** Model for T-DNA insertion sites in regulation of carotenoid pathway (Kot *et al.*, 2016) in *R. toruloides*.

**Table 1. Strains used in this study**

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *Strains* | | |
| *R. toruloides* NP11 GDMCC[a] 2.224 | *MAT A* | (Zhu *et al.*, 2012) |
| *R. toruloides* NP11-4 GDMCC 2.226 | p*PGK-HYG*-Tnos, $HYG^R$ | This study |
| *R. toruloides* NP11-5 GDMCC 2.227 | p*GPD-HYG*-Tnos, $HYG^R$ | This study |
| *R. toruloides* NP11-8 GDMCC 2.228 | p*GPD-HYG*-Tnos, $HYG^R$ | This study |
| *A. tumefaciens* AGL1-HYG1 | *PZPK-pPGK-HYG-Tnos* | (Lin *et al.*, 2014) |
| *A. tumefaciens* AGL1-HYG2 | *PZPK-pGPD-HYG-Tnos* | (Lin *et al.*, 2014) |
| *Plasmids* | | |
| *PZPK-pPGK-HYG-Tnos* | *pGPD–HYG–Tnos in PZPK* | (Lin *et al.*, 2014) |
| *PZPK-pGPD-HYG-Tnos* | *pGPD–HYG–Tnos in PZPK* | (Lin *et al.*, 2014) |
| *PZPK–pFBA–HYG–Tnos* | *pFBA–HYG–Tnos in PZPK* | (Wang *et al.*, 2016) |

[a]: Guangdong Microbiology Culture Center

**Table 2. Primers used in this study**

| Primer | Sequence(5'–3') | Function |
|---|---|---|
| LAD-1 | ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA | Primer used for Pre-amplification of TAIL-PCR |
| LAD-2 | ACGATGGACTCCAGAGCGGCCGCBNBNNNGGTT | |
| LAD-3 | ACGATGGACTCCAGAGCGGCCGCVVNVNNNCCAA | |
| LAD-4 | ACGATGGACTCCAGAGCGGCCGCBDNBNNNCGGT | |
| RB-0-a | GGCAATAAAGTTTCTTAAGATTGAATCCTGT | |
| AC1 | ACGATGGACTCCAGAG | Primer used for primary and secondary TAIL-PCR |
| RB-1-a | ACGATGGACTCCAGTCCGGCCTGTTGCCGGTCTTGC GATGATTATCA | Primer used for primary TAIL-PCR |
| RB-2-a | GTAATGCATGACGTTATTTATGAGATGGGTT | Primer used for secondary TAIL-PCR |
| PMD-p1 | GGCTTTACACTTTATGCT | Primer used for identification the successful ligation of T-vector |
| PMD-p2 | ACAGATGCGTAAGGAGA | |
| PGK-F | CTGTCTAGCCTGTCAGCGAATG | Amplification of PGK gene by qRT–PCR |
| PGK-R | GCGAGTGCGAGTGAGAAGTG | |
| GPD-F | CGCAGGAAGCCGTTACAAG | Amplification of GPD gene by qRT–PCR |
| GPD-R | TGATGGGAAAGAGGGCAAA | |
| FBA-F | CACACTCTCGCTTCTCCTGCT | Amplification of FBA gene by qRT–PCR |
| FBA-R | TGACATCCCAGTCGTCCTTG | |

**Table 3. Production of total and individual carotenoids by strains in the present study**

| Strains | carotenoids composition(μg/g DCW) | | | |
|---|---|---|---|---|
| | Td | Tn | γ-Car | β-Car |
| NP11 | 518.3[a] | 16.8[b] | 43.5[b] | 361.6[b] |
| NP11-4 | 220.0[b] | 26.5[b] | 68.2[a] | 287.5[c] |
| NP11-8 | 334.0[b] | 148.9[a] | 47.3[b] | 606.5[a] |

Note: Td, torularhodin; Tn, torulene; γ-Car, γ-carotene; β-Car, β-carotene. Values within a column with different letters are significantly different ($P<0.05$) among average values.

**Table 4. T-DNA insertion informations of the mutants in this study**

| Strain | T-DNA inserted position | Functional annotation | Amino acid sequence length | Inserted region |
|---|---|---|---|---|
| NP11-4 | RTHO_00032 | hypothetical protein | 414 | Intron |
| NP11-5 | RTHO_07952, | bZIP transcription factor | 601 | Exon |
| NP11-8 | RTHO_07650 | DUF1479 domain protein | 503 | Promoter |