Enhanced β-carotene production by promoting the multivesicular body (MVB) pathway in Yarrowia lipolytica

Fan Yang
Shaanxi Normal University
Liang Liu
Shaanxi Normal University
Shan Qiang
Xi'an Healthful Biotechnology Co
Ching Yuan Hu
Northwest Normal University; University of Hawaii at Manoa
Ying Li
China Agricultural University
Yong Hong Meng (mengyonghong@snnu.edu.cn)
Shaanxi Normal University

Research

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Abstract

Background: β-carotene is a precursor of vitamin A and has great commercial value as an additive in foods and feeds. Many pathways not directly related to the β-carotene synthesis affect β-carotene production since the interactions among metabolic fluxes of cells confer a complex regulatory network. Engineered *Y. lipolytica* strain has excellent potential for β-carotene production as oleaginous yeast. Optimizing indirectly metabolic pathways in *Y. lipolytica* may offer a new strategy for making the β-carotene production achieve a commercially viable yield.

Results: In this study, we found that the proper promotion of the multivesicular body (MVB) sorting pathway elevated the production of β-carotene by 1.58 fold when overexpressing one copy of the *Did2* gene in *Y. lipolytica*. Through the measurement of ATP, NADPH, the mRNA, and protein level of key genes in the β-carotene synthesis pathway, the reason for β-carotene elevated was deuced that the protein level of the key enzymes (tHMG and CarA) was increased. When overexpressing two copies of the *Did2* gene, the transcription level of the key genes was all elevated. However, the protein level of key enzymes in the β-carotene synthesis pathway was reduced compared with the overexpressing one copy of the *Did2* gene, which resulted in reduced β-carotene content.

Conclusion: This study suggests that the MVB sorting pathway is not responsible for sorting protein but has a crucial regulating effect on protein abundance in cells. Engineering the MVB sorting pathway could potentially increase the production of other high-value products. Moreover, manipulation of indirectly related metabolic pathways also is a critical strategy in synthetic biology research.

Background

β-carotene enhances the immune function of human, delays skin aging, and prevents cardiovascular diseases and cancers [1, 2]. β-carotene is widely used as a nutritional supplement and antioxidant in the food and pharmaceutical industries [3, 4]. The global market size of β-carotene was USD 466.7 million in 2017 [5]. The growth rate of β-carotene demand is 4.1%, which is estimated to continue over the forecast period. Since the consumers favor naturally derived β-carotene [6], using microbial fermentation to produce natural β-carotene has a promising future. Y.lipolytica is a generally-recognized-as-safe (GRAS) microorganism and has a lipid body, which is beneficial for the storage of β-carotene [7, 8]. For the production of β-carotene, the Y.lipolytica engineering strain is the right candidate.

Several strategies have been used to improve the production of β-carotene in the field of metabolic engineering. First, β-carotene production was improved by overexpressing key genes of the β-carotene synthesis pathway. For example, after integrating three copies of Thmg, two copies of Ggs1, five copies of CarRA, and one copy of CarB, the engineered Y. lipolytica strain produced 33 mg/g DCW β-carotene which is 100 fold of baseline construct [9]. Second, β-carotene production was improved by blocking the competitive pathway to prompt more precursors flow to β-carotene synthesis. Blocking the pentose phosphate pathway (PPP) has significantly increased the yield of β-carotene by 95% [10]. Third, β-

carotene production was improved by supplying more cofactors. For example, as the concentration of cofactors increased in the engineered tricarboxylic acid cycle (TCA), β-carotene production was improved by 39% [11]. All these strategies mainly focus on manipulating the pathway, which directly affects β-carotene synthesis. However, many pathways not directly related to the β-carotene synthesis have an important influence on its production.

The interactions among metabolic fluxes of cells confer a complex regulatory network. To further explore the potential of Y. lipolytica, it is critical to identify additional pathways that could increase β-carotene synthesis. Many genes from other pathways have significant and unexpected effects on β-carotene synthesis. The genes Cab1, Nsg1, Erg13, and Erg27 could promote β-carotene biosynthesis in S. cerevisiae as these genes involved in lipid biosynthesis [12]. The VOA1 improves the production of β-carotene in S. cerevisiae because VOA1 may result in a low pH of cell membranes [13]. Impressively, the gene Did2 related to protein metabolism increases β-carotene yield by 2.1 times in S. cerevisiae.

The amount of protein in cells is vital for the synthesis of β-carotene. The HMGR is a key enzyme of the β-carotene pathway. By substituting the entire sequence with a catalytic domain, the stability of protein HMGR was improved [14]. The production of β-carotene was significantly enhanced after overexpressing tHMG [15]. Overexpressing the Did2 gene has been shown to improve the production of β-carotene by promoting the transcription of β-carotene pathway genes (Hmg1, Erg12, Erg20, Erg8, Bts1, crtYB, and crtI). For example, the gene Did2, which is related to the protein trafficking, plays a role in the multivesicular body (MVB) sorting pathway. The MVB sorting pathway has two core components: ESCRTIII and Vps4. The Did2 recruits Vps4 to dissociate ESCRTIII from endosomes for the next cycle and increases the efficiency of the Vsp4 in the MVB sorting pathway [16]. However, the protein level in cells was not measured after overexpression of the Did2 gene, and the reason for the improved β-carotene synthesis by MVB sorting way was not given.

In the present study, we integrated both one copy and two copies of the Did2 gene into the Y. lipolytica chromosome to explore the effect of overexpressing the Did2 gene on the β-carotene synthesis and the mechanism of how the Did2 gene affects β-carotene synthesis. We discovered that overexpressing the Did2 gene prompted the MVB sorting pathway, subsequently improved the protein level of key enzymes in β-carotene synthesis, which led to the improvement of β-carotene production. We have provided evidence that Y. lipolytica cells coordinate transcriptional regulation and protein degradation to control protein abundance. Our study provides an excellent start to explore the potential of the yeast used for β-carotene synthesis by further manipulating other metabolic pathways in yeast cells. In addition, as the protein mediates most biological processes, our research also provides new knowledge for better utilization of the MVB sorting pathway to improve other high-valued biosynthetic products.

Methods

Strains and media

All strains used in this study are listed in Table S1. E. coli DH5α was used for routine cloning procedures, growing in Luria-Bertani (LB) medium at 37 °C, 220 rpm with 100 μg/mL of ampicillin when necessary. The Y. Lipolytica was cultivated in YPD medium or SD-Leu$^-$ medium. Y. Lipolytica was cultivated in YPD medium and incubated at 30 ℃, 150 rpm in 250 mL Erlenmeyer flasks containing 50% fermentation medium, and 2% inoculum. The Y. Lipolytica was precultured in test tubes containing 3 ml SD-Leu$^-$ medium at 30 °C, 200 rpm for 48 h, and then the cells were inoculated into a fresh medium with 2% inoculum.

Construction Of Recombinant Plasmids And Strains

All the plasmids and primers used in this study are listed in Table 1 and Table S1, respectively. For gene integration, the plasmid pJN44-Did2 was constructed that the gene Did2 (YALI0C10098g) was amplified with primers Did2-F/R from the Y.lipolytica genome and inserted at smaI/HindIII site of plasmid pJN44. Then the plasmid pURA-ΔGut2::Did2 and pURA-ΔGut2::2Did2 were formed that the expression cassette 'pTEF-Did2-CYC1t' was amplified with primers G-Did2-F/R from pJN44-Did2 and inserted into plasmid pURA-ΔGut2 at the site of speI. For western blot, a fusion StrepII tag is needed to add to the 3' end of tHMG, carRA, and carB gene. The tHMG, carRA, and carB gene were amplified with primers w-tHMG-tag-F/R, w-carB-tag-F/R, and w-carB-tag-F/R and inserted into plasmid pJN44 at the site of smaI to form plasmid pJN44-tHMG-StrepII, pJN44-carRA-StrepII, and pJN44-carB-StrepII.

Table 1
The content of β-carotene and the relative mRNA levels of the genes in the MVB sorting pathway (Did2, Vps4) and the β-carotene synthesis pathway (Thmg, Ggs1, CarRA, CarB) in engineered β-carotene strains YL-C31 and YL-C32.

Strains	mRNA level						β-carotene
	Did2	Vps4	Thmg	Ggs1	CarRA	CarB	
YL-C31	1.00	1.00	1.00	1.00	1.00	1.00	15.63 mg/g DCW
YL-C32	1.5	1.33	1.63	1.57	1.54	1.85	11.81 mg/g DCW

Lox/Cre-mediated genomic manipulate procedure was performed [8] as described using pURA for introducing gene constructs into Y. Lipolytica. We employed pURA-ΔGut2 for the gene integration at the target of gut2. The plasmids pURA-ΔGut2::Did2 and pURA-ΔGut2::2Did2 were linearized and transferred into the Y. Lipolytica strains, as described by Gao et al. Genomic integrations were confirmed by diagnostic PCR and DNA sequencing. All primers used for identification of the positive transformants are listed in Table S2.

Quantitative Pcr (qpcr) Analysis Of The Related Genes

Transcriptional levels of the key genes in the β-carotene synthesis pathway and the MVB sorting pathway were determined by qPCR following the previously published method with minor modifications [29]. Total

RNA was isolated firstly. Then the RNA was reverse transcribed into cDNA using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen; Beijing, China). qPCR was performed using the SYBR tip green qPCR super mix kit (Transgen; Beijing, China). The actin gene was used as the internal reference to normalize the different samples. The primer of key genes, Thmg, Ggs1, CarRA, CarB of the β-carotene synthesis pathway, and Vps4, Did2 of the MVB sorting pathway were designed for qPCR, and all the primer were listed in table S2. The analysis of relative transcription levels of these genes was conducted according to the published method [10].

Western Blot Assay

The protein expression level of tHMG, carRA, and carB were detected by Western blot. The Western blot assay was performed according to the method of Matthaus et al. with some modifications [30]. Total protein was extracted using a yeast protein lysate mix kit (BBproExtra; Beijing, China). 80 ug of denatured protein was loaded for electrophoresis to separate different molecular proteins, following the protein was transferred to the PVDF membrane at a current of 300 mA, 90 min. The PVDF membrane was blocked with skim milk powder for 2 h and subsequently incubated at 4 ℃ for 12 h with rabbit anti-Strep-tag II antibody (1:1000 in primary antibody dilution buffer; Abcam; Cambridge, UK). Then a second incubation for 1 h with goat anti-rabbit (H + L) HRP (1:10000 in HRP-conjugated antibody dilution buffer; Abbkine; California, USA) was carried out. Last, immunoreactivity was determined with the ECL method.

Measurement Of Farnesyl Pyrophosphate (fpp), Lycopene And β-carotene

FPP, one precursor of the β-carotene synthesis pathway, was measured following a previously published method with some modifications [31]. Alkaline phosphatase and Pyrophosphatase cleave the phosphoric acid moieties of FPP, convert FPP to farnesol. Using GC-MS detected the content of farnesol to reflect the amount of FPP. The cell pellets of 100 ml medium were harvested and suspended in a 2 ml buffer (1 M diethanolamine, 0.5 mM MgCl2, pH 9.8). After lysing cells with sonication for 30 minutes, the sample was centrifuged at 12000*g for 10 min. The supernatant was added to Pyrophosphatase (10 U) at 25 ℃ for 1 h, followed by the addition of Alkaline phosphatase (10 U) at 30 ℃ for 1 h. Finally, the farnesol was extracted by N-hexane for GC-MS detection. The β-carotene and lycopene were determined using a previously published method [9].

Determination Of Coenzyme Factor (atp, Nadph)

The ATP was determined using the ATP Assay Kit (Beyotime; Shanghai, China) with some modifications. The process that firefly luciferase catalyzes the production of fluorescence needs the participation of ATP. Using the fluorescence intensity reflects the amount of ATP. The content of NADPH was detected using NADP+ /NADPH Assay Kit (Beyotime; Shanghai. China), based on WST-8 color reaction with some modifications.

Statistical analysis

All experiments were repeated three times. Data from each treatment are presented as means ± standard deviation. Statistical analyses were conducted using SPSS 18.0 (SPSS Inc; Chicago, IL, USA). Data in Figs. 2, 3, 4, 5, 6, and 7 were analyzed using one-way ANOVA, followed by Duncan's multiple range tests to determine the significant difference. $P < 0.05$ was considered statistically significant. Origin software 8.0 (Origin Lab; USA) was used for graphs construction.

Results

Overexpression of the Did2 gene promoted β-carotene synthesis

The optimization of metabolic pathways indirect related to β-carotene synthesis improves the production of β-carotene. Engineered Y. lipolytica strain is oleaginous yeast, has excellent potential for β-carotene production, and a high capacity to store β-carotene [17]. To further explore the potential of engineered Y. lipolytica strain for producing β-carotene, we studied the effects of the indirectly related metabolic pathways on the β-carotene synthesis pathway. Several genes outside the β-carotene synthesis pathway have been shown to affect the production of β-carotene in S. cerevisiae [12, 18]. For example, the incorporation of the Did2 gene, a member of the MVB sorting pathway, led to the most significant improvement of β-carotene yield by engineered S. cerevisiae [13]. The YL-C1 strain is an engineered Y. lipolytica with basal β-carotene producing capacity. We integrated the Did2 gene into YL-C1 at the Gut2 site, resulting in stain YL-C31 to explore the effect of the Did2 gene on β-carotene synthesis in the engineered Y. lipolytica strain. The strain YL-C2, which the Gut2 gene was knocked out, was used as second control since the integration of the Did2 gene interrupted the Gut2 gene. β-carotene content was analyzed using HPLC after 96 h of fermentation. The β-carotene content in YL-C1, YL-C2, and YL-C31 strains was 9.85, 8.87, and 15.63 mg/g DCW, respectively; and the β-carotene concentration was 49.86, 51.45, and 80.65 mg/L, respectively (Fig. 1a, 1b). Both the content and concentration of β-carotene in the YL-C31 strain are highest. These results indicate that overexpressing the Did2 gene increased the β-carotene production in the engineered Y. lipolytica strain.

ATP and NADPH decreased after the overexpression of the Did2 gene

Coenzyme factors are essential for β-carotene synthesis. Synthesis of one mole of β-carotene requires 8 moles of ATP, 8 moles of CTP, and 16 moles of NADPH [11]. The levels of ATP and NADPH, coenzyme factors during the logarithmic growth phase in the engineered Y. lipolytica strains were examined to explore the reason that the overexpression of the Did2 gene improved the β-carotene synthesis. For ATP, the YL-C1, YL-C2, and YL-C31 strains produced 19.25, 138.34, and 45.19 nmol/g protein of ATP, respectively (Fig. 2a). For NADPH, the YL-C1, YL-C2, and YL-C31 strains produced 207.65, 162.35, and 127.49 nmol/g protein of NADPH, respectively (Fig. 2b). The ATP concentration was elevated ($P < 0.05$) in the YL-C2 with the Gut2 gene knocked out. The improved ATP status indicates that the interruption of the Gut2 gene might have diverted more G3P to the glycolytic pathway, which produced more ATP. Compared to YL-C2, both ATP and NADPH concentrations were lower ($P < 0.05$) in the YL-C31. This result indicates

that the overexpression of the Did2 gene led to a decrease in ATP and NADPH. The lower concentration might be attributed to that the ATP and NADPH were consumed for the synthesis of β-carotene.

Overexpression of the Did2 gene improved the mRNA level of the genes in the β-carotene synthesis pathway

We measured the relative mRNA level of Thmg, Ggs1, CarRA, and CarB, key genes in the β-carotene synthesis pathway, to explore the reason that the overexpression of the Did2 gene improved the production of β-carotene in Y. lipolytica. The actin gene was used as an internal reference [10]. The mRNA of key genes in YL-C1 was set as 1. For Thmg, the mRNA in YL-C31 was increased ($P < 0.05$) by 17% compared to YL-C1, increased ($P < 0.05$) by 30% compared to YL-C2 (Fig. 3a). For Ggs1, the mRNA in YL-C31 was increased ($P < 0.05$) by 45% compared to YL-C1, increased ($P < 0.05$) by 35% compared to YL-C2 (Fig. 3b). For CarRA, the mRNA in YL-C31 was increased ($P < 0.05$) by 78% compared to YL-C1, increased ($P < 0.05$) by 97% compared to YL-C2 (Fig. 3c). For CarB, the mRNA in YL-C31 was increased ($P < 0.05$) by 55% compared to YL-C1, increased ($P < 0.05$) by 91% compared to YL-C2 (Fig. 3d). So, the mRNA of Thmg, Ggs1, CarRA, and CarB genes were all higher ($P < 0.05$) in the YL-C31 strain with the overexpressed Did2 gene.

Overexpression of the Did2 gene improved the utilization of precursors in the β-carotene synthesis pathway

The utilization of the precursors is directly linked to β-carotene synthesis. The sesquiterpenes, diterpenes, triterpenes, and tetraterpenes all compete with β-carotene for the precursor FPP [19]. The lycopene is the direct precursor of β-carotene. To ascertain the effect of overexpression of the Did2 gene on the utilization of precursors, we measured the concentration of FPP and lycopene. The FPP and lycopene levels in YL-C1 were considered as 1. For FPP, the utilization of FPP in YL-C31 was increased ($P < 0.05$) by 34% compared to YL-C1, increased ($P < 0.05$) by 37% compared to YL-C2 (Fig. 4a). For lycopene, the utilization in YL-C31 was increased ($P < 0.05$) by 8.1% compared to YL-C1, increased ($P < 0.05$) by 7.9% compared to YL-C2 (Fig. 4b). The utilization of both FPP and lycopene in YL-C31 was highest ($P < 0.05$) among strains YL-C1, YL-C2, and YL-C31.

Overexpression of the Did2 gene increased the protein level of the key enzymes in the β-carotene synthesis pathway

We performed the Western blot assay to measure whether the amount of the key enzymes in the β-carotene synthesis pathway was affected by the overexpression of the Did2 gene. A fusion StrepII tag was chosen for co-expression of tHMG, carRA, and carB proteins. The fusion StrepII tag has been successfully used for analyzing the expression of carotenoid synthesis enzymes in Rb. Sphaeroides [10]. Plasmids pJN44-tHMG-s, pJN44-carRA-s, pJN44-carB-s were separately transformed into YL-C1, YL-C2, and YL-C31, respectively, resulted in YL-C1ts, YL-C2ts, YL-C31ts, YL-C1as, YL-C2as, YL-C31as, YL-C1bs, YL-C2bs, and YL-C31bs. tHMG-strepII (Fig. 5a) and carB-strepII (Fig. 5b) are identified by Western blotting. The protein bands were scanned. The expression amounts of the key enzymes in YL-C1ts, YL-C1bs were

regarded as 1. For tHMG-strepII, the protein level in YL-C31ts was increased ($P < 0.05$) by 37% compared to YL-C1ts, increased ($P < 0.05$) by 92% compared to YL-C2ts (Fig. 5c). For carB-strepII, the protein level in YL-C31bs was increased ($P < 0.05$) by 17% compared to YL-C1ts, increased ($P < 0.05$) by 25% compared to YL-C2ts (Fig. 5d). tHMG-strepII protein has the highest amount when the pJN44-tHMG-s was expressed in YL-C31. carB-strepII protein has the highest amount when the pJN44-carB-s was expressed in YL-C31. These results demonstrate that the overexpression of the Did2 gene increased ($P < 0.05$) the level of key enzymes (tHMG-strepII, carB-strepII). For carRA-strepII, the exact bands cannot be identified by Western blotting (As shown in Fig. S1). The reason may be that the stability of carRA-strepII is weak, and the protein was degraded during the extraction.

Overexpression of the Did2 gene increased the transcription level of the Vps4 gene in the MVB sorting pathway

The Did2 gene is a positive regulator of the MVB sorting pathway [20]. The Did2 protein, one subunit of the ESCRT protein complex, recruits Vps4 protein to bind ESCRT. Meanwhile, the Vps4 protein is a core factor of the MVB sorting pathway [21]. To explore the effect of the overexpression of the Did2 gene on the MVB sorting pathway, we measured the mRNA levels of the Vps4 gene and the Did2 gene. The mRNA level of the Did2 gene increased ($P < 0.05$) by 43% compared to YL-C1, and increased ($P < 0.05$) by 68% compared to YL-C2 (Fig. 6a). The mRNA level of the Vps4 gene increased ($P < 0.05$) by 28% compared to YL-C1, and increased ($P < 0.05$) by 47% compared to YL-C2 (Fig. 6b). These results demonstrate that the mRNA level of the Vps4 gene was increased ($P < 0.05$) by the overexpression of the Did2 gene.

Overexpression of two copies of the Did2 gene further stimulated the MVB sorting pathway but reduced the protein level of key enzymes in the β-carotene synthesis pathway

To further explore the effect of the MVB sorting pathway on the protein level of key enzymes in the β-carotene synthesis pathway, the MVB sorting pathway was promoted by overexpressing two copies of the Did2 gene. Two copies of the Did2 gene were integrated into the engineered Y. lipolytica strain (YL-C1) genome at the Gut2, resulted in YL-C32. The mRNA level of the Vps4 and Did2 genes in YL-C32 was further elevated ($P < 0.05$) by 50% and 33% compared to YL-C31 (Table 1), respectively. Meanwhile, compared to the overexpression of one copy of Did2 gene, the mRNA level of the Thmg, Ggs1, CarRA, and CarB, the key genes in β-carotene synthesis pathway, increased ($P < 0.05$) by 63%, 57%, 54%, and 85% (Table 1), respectively. However, the protein level of tHMG-strepII in YL-C32ts was reduced compared to YL-C31ts (Fig. 7). For carB-strepII, the protein level in YL-C32bs was also reduced compared to YL-C31bs (Fig. 7). These results indicate that the protein level of key enzymes (tHMG-strepII, carB-strepII) in the β-carotene synthesis pathway was lower in the YL-C32 strain with overexpressing two copies of the Did2 gene. Furthermore, the β-carotene content was reduced by 25% ($P < 0.05$) (Table 1).

Discussion

The optimization of the metabolic pathways indirect related to β-carotene synthesis makes the engineered Y. lipolytica strain beneficial for the expression β-carotene synthesis pathway. The engineered

Y. lipolytica strain is oleaginous yeast, has excellent potential for β-carotene production since a lot of acetyl-CoA and a lipid body used to store β-carotene [9]. Previous strategies for improving the production of β-carotene focused mainly on enhancing the isoprenoid flux toward carotenoid production [22]. Many genes indirectly related to carotenoid synthesis have been shown to improve the production of carotenoid. The knockout of the gdhA gene, the enzyme responsible for converting alpha-ketoglutarate to glutamic acid, led to the increase of lycopene in S. cerevisiae [23, 24]. Deletion of the genes Ser33 (related to amino acid synthesis), Prb1 (related to vacuolar protein degradation), and Rox1 (a transcription repress factor) improved the production of carotenoid [18]. Overexpression of the genes Tif 5 (a translation initiation factor), Voa1 (vacuolar H(C)-ATPase subunit 1), and Did2 (a subunit in the MVB sorting pathway) genes enhanced the production of β-carotene in S. cerevisiae [13]. Overexpression of the Did2 gene led to a 2.1-fold improvement of β-carotene production in S. cerevisiae [13]. In this study, we integrated the Did2 gene into the engineered Y. lipolytica strain and the production of β-carotene improved 1.58-fold. The reason for promotion could be deduced that the protein level of the key enzymes in the β-carotene synthesis pathway was increased by overexpression of Did2.

Proper promotion of the multivesicular body sorting pathway improves the protein level of key enzymes in β-carotene synthesis pathway. The cells control protein abundance by coordination of protein synthesis and degradation. When protein degradation was elevated, the cells regulate protein level by enhancing the mRNA expression to compensate for the effect of protein degradation [25]. The MVB sorting pathway plays a key role in protein degradation [26–28]. ESCRT, the key unit in the MVB sorting pathway, binds to endosomes to sort proteins for protein degradation or to transport to other organelles. The Did2 protein, one subunit of ESCRT protein complex, activates and recruits Vps4 to dissociate ESCRT from the endosome for the next cycle after the ESCRT accomplished sorting. In this study, the overexpression of the Did2 gene enhanced the mRNA level of the Did2 gene and the Vps4 gene. This result implies that the overexpression of the Did2 gene promoted the MVB sorting pathway. Both mRNA and protein of key enzymes (tHMG-strepII, carB-strepII) increased ($P < 0.05$), and the β-carotene content increased ($P < 0.05$) by 58% after overexpressing one copy of the Did2 gene. When overexpression of two copies of the Did2 gene, the transcription level of the key genes of the MVB sorting pathway (Did2 and Vps4) and β-carotene synthesis pathway (Thmg, Ggs1, CarRA, and CarB) were all elevated. However, the protein of key enzymes (tHMG-strepII and carB-strepII) was reduced. Analyzed the causes, we deduced that the transcription level of gene was not enough to compensate for the degradation of the corresponding protein though its mRNA level was elevated. Furthermore, the protein level of both tHMG-strepII and carB-strepII were all decreased, which resulted in the reduced the β-carotene content by 25%. Therefore, the overexpression of two copies Did2 gene was excessive for the promotion of the MVB sorting pathway. Furthermore, combined all results in this study, we can suggest the MVB sorting pathway is not responsible to protein degradation but has important regulating effects on protein abundance in cells.

Conclusion

In this study, we found the proper promotion of the MVB sorting pathway elevated the production of β-carotene in Y. lipolytica by overexpressing one copy of the Did2 gene. The reason for enhanced β-carotene production most likely is attributed to increased mRNA and protein levels of key genes, which resulted from the promotion of the MVB sorting pathway. These results suggest that engineering the MVB sorting pathway could potentially increase the production of other high-value products. Moreover, manipulating indirectly related metabolic pathways also is a critical strategy in metabolic engineering.

Abbreviations

MVB:Multivesicular Body; ESCRT:Endosomal sorting complex required for transport; FPP:Farnesyl pyrophosphate; PPP:Pentose phosphate pathway; TCA:Tricarboxylic acid cycle; tHMG:truncated hydroxymethylglutaryl-CoA reductase; HMGR:3-hydroxy-3-methylglutaryl-coenzyme-A reductase; carB:Phytoene synthase; carA:Bifunctional enzymes (lycopene cyclase, phytoene synthase); GGS1:Geranylgeranyl diphosphate synthase

Declarations

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Authors' contributions

YHM, YL, and FY designed the experiments. FY, LL and SQ performed metabolic engineering experiments and analyzed the data. FY, YHM, and QYH wrote the manuscript. All authors read and approved the final manuscript.

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Authors' information

a, Engineering Research Center of High Value Utilization of Western China Fruit Resources, Ministry of Education; National Research & Development Center of Apple Processing Technology; College of Food Engineering and Nutritional Science, Shaanxi Normal University, 620 West Chang’an Avenue, Changan, Xian 710119, P. R. China

b, Xi’an Healthful Biotechnology Co., Ltd. HangTuo Road, Changan, Xian 710100, P. R. China

Competing interests

Conflict of Interest SQ was employed by Xi'an Healthful Biotechnology Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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Supplementary Files Legend

Table S1. Strains and plasmids used in this study

Table S2. List of primers used in this study

Figure S1. The protein level of the key enzymes (carRA) in the β-carotene synthesis pathway after overexpression of the *Did2* gene. Western blot result of the carRA-strepII fusion protein in the strains YL-C1as, YL-C2as, and YL-C31as

Figures

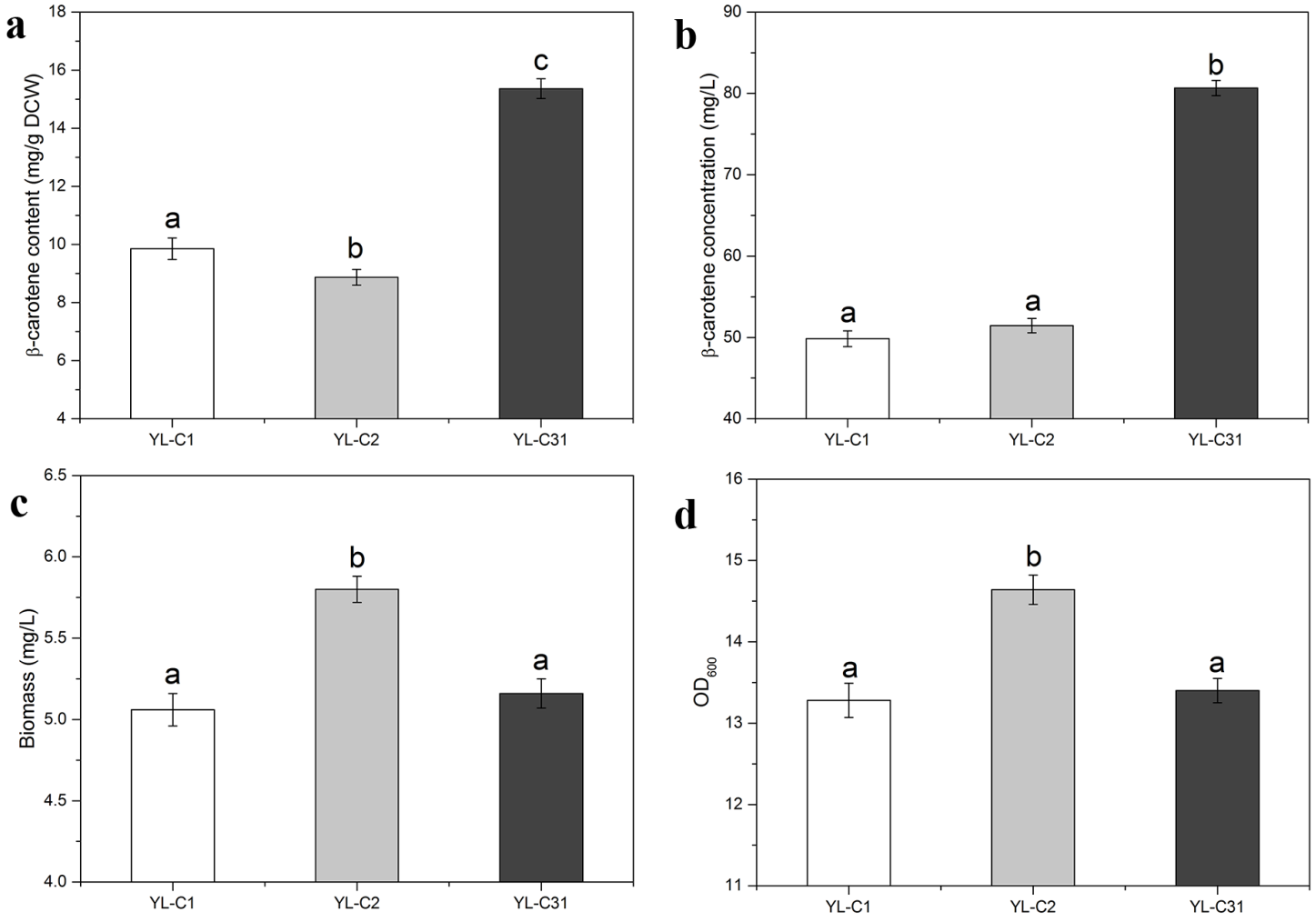

Figure 1

Overexpression of the Did2 gene promoted both the content and concentration of β-carotene (a, b) and decreased the biomass and OD600 (c, d). Error bars represent standard deviations (n = 3). Columns with different letters indicate a significant difference ($p<0.05$). YL-C31, the strain that integrated the Did2 gene at the Gut2 site based on the YL-C1; YL-C2, the strain that the Gut2 gene was knocked out.

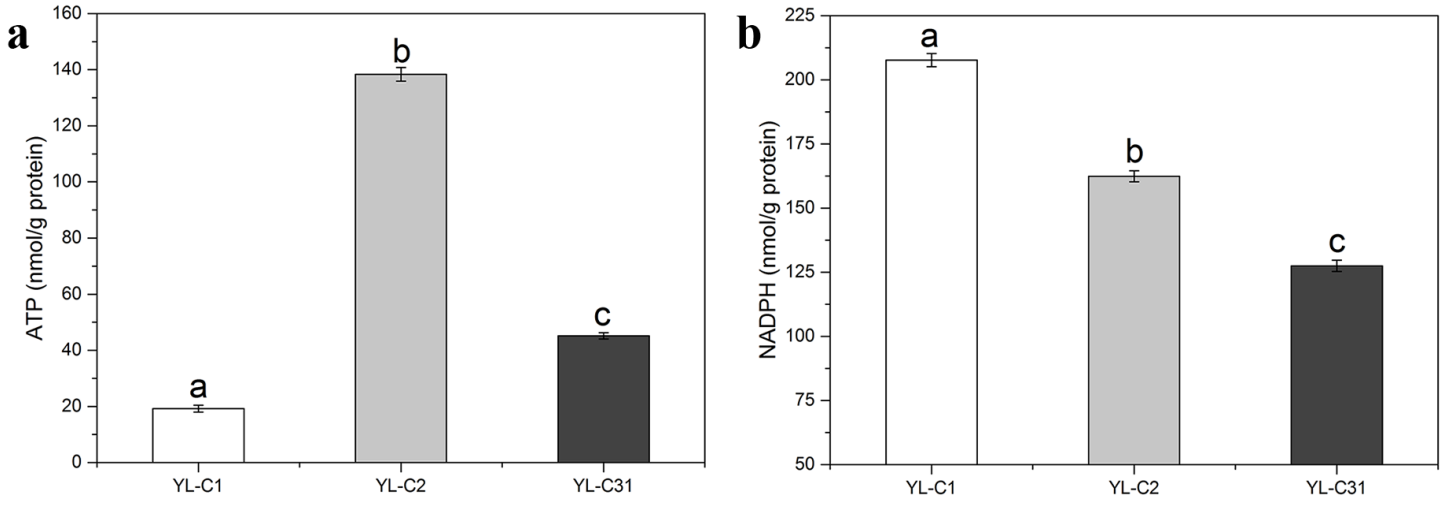

Figure 2

Overexpression of the Did2 gene decreased the content of the coenzymes factors. The content of ATP in engineered β-carotene strains YL-C1, YL-C2, and YL-C31 (a). The content of NADPH in engineered β-carotene strains YL-C1, YL-C2, and YL-C31 (b). Error bars represent standard deviations (n = 3). Columns with different letters indicate a significant difference ($p<0.05$).

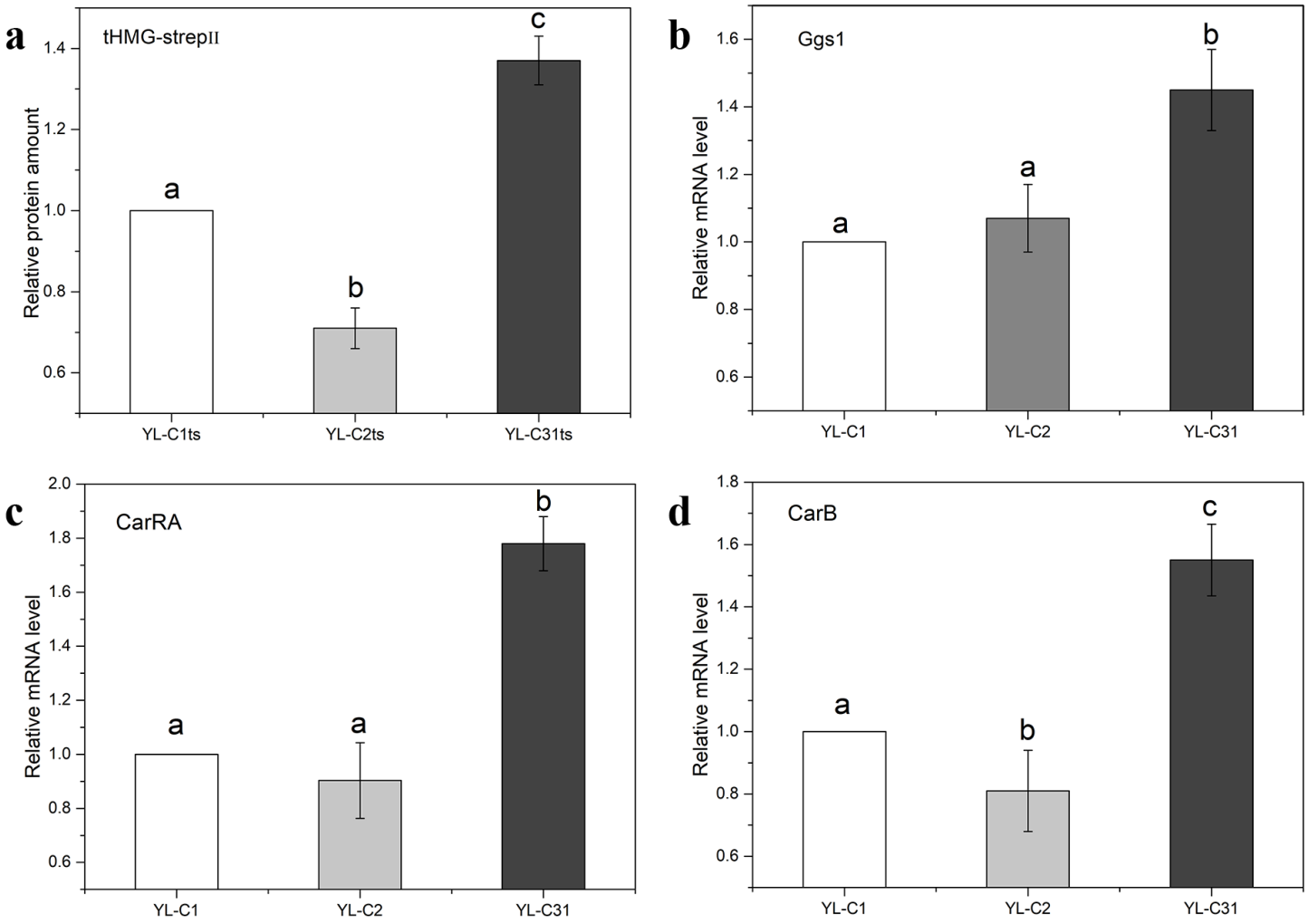

Figure 3

Overexpression of the Did2 gene improved the relative mRNA levels of Thmg (a), Ggs1 (b), CarRA (c), and CarB (d), key genes of the β-carotene synthesis pathway. Error bars represent standard deviations (n = 3). Columns with different letters indicate a significant difference ($p<0.05$).

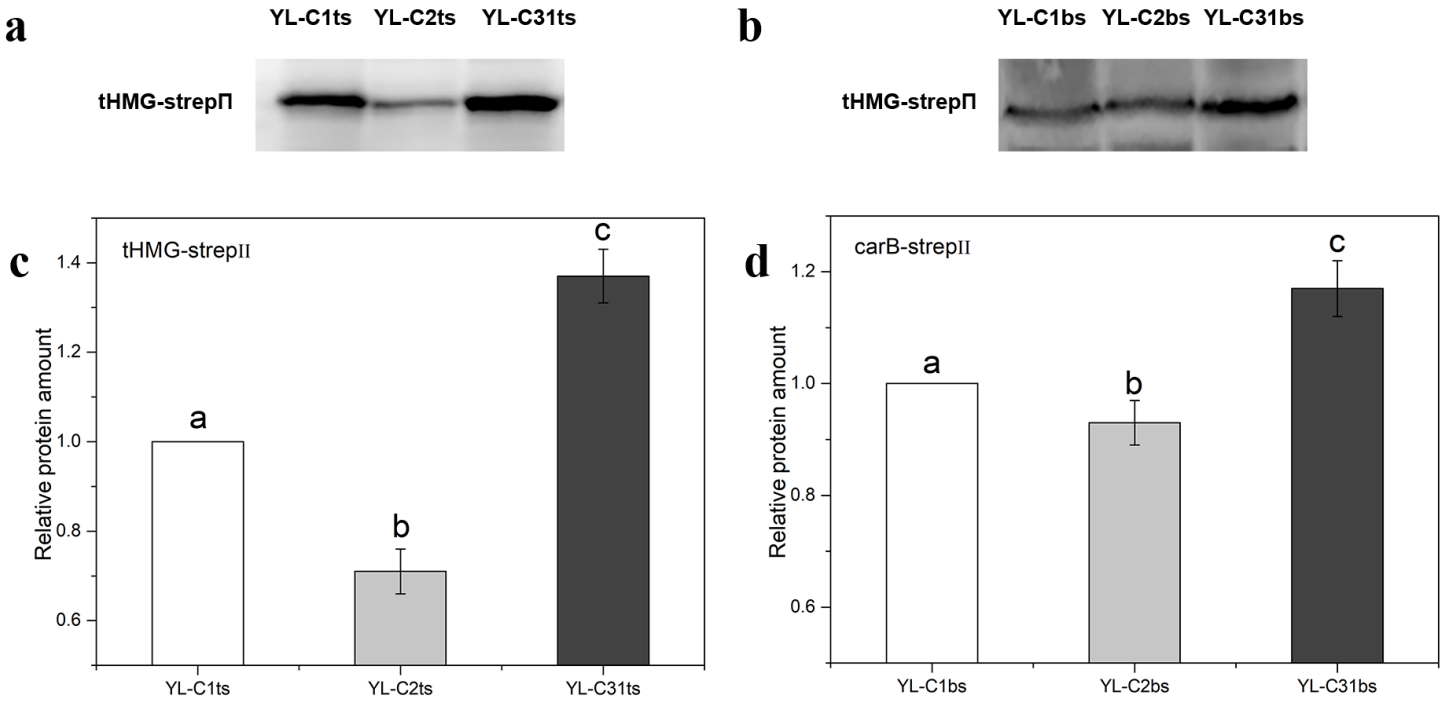

Figure 4

Overexpression of the Did2 gene improved the protein level of the key enzymes in the β-carotene synthesis pathway. Western blot result of the tHMG-strepII fusion protein in the strains YL-C1ts, YL-C2ts, and YL-C31ts (a), and the carB-strepII fusion protein in the strains YL-C1bs, YL-C2bs, and YL-C31bs (b). Scanned tHMG-strepII fusion protein in the strains YL-C1ts, YL-C2ts, and YL-C31ts (c), and the carB-strepII fusion protein in the strains YL-C1bs, YL-C2bs, and YL-C31bs (d). Error bars represent standard deviations (n = 3). Columns with different letters indicate a significant difference ($p<0.05$).

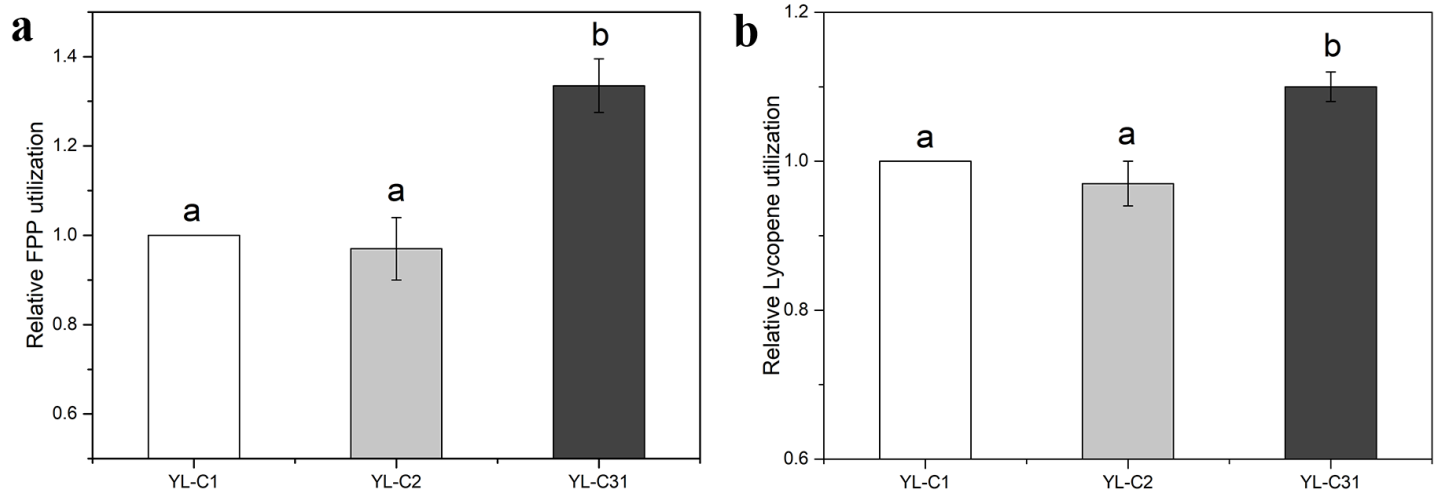

Figure 5

Overexpression of the Did2 gene improved the relative utilization rates of precursors of the β-carotene synthesis pathway. The utilization of FPP in engineered β-carotene strains YL-C1, YL-C2, and YL-C31 (a). The utilization of lycopene in engineered β-carotene strains YL-C1, YL-C2, and YL-C31 (b). Error bars represent standard deviations (n = 3). Columns with different letters indicate a significant difference ($p<0.05$).

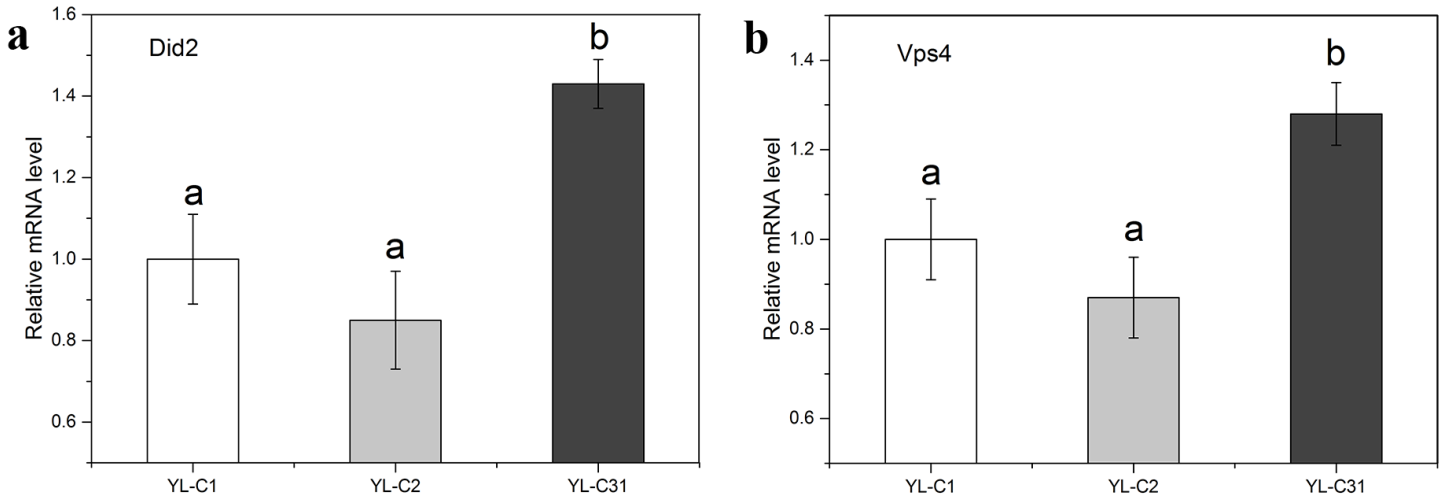

Figure 6

Overexpression of the Did2 gene elevated the relative mRNA levels of the Did2 and Vps4, genes of the MVB sorting pathway. The mRNA levels of the Did2 gene in engineered β-carotene strains YL-C1, YL-C2, and YL-C31 (a). The mRNA levels of the Vps4 gene in engineered β-carotene strains YL-C1, YL-C2, and YL-C31 (b). Error bars represent standard deviations (n = 3). Columns with different letters indicate a significant difference ($p<0.05$).

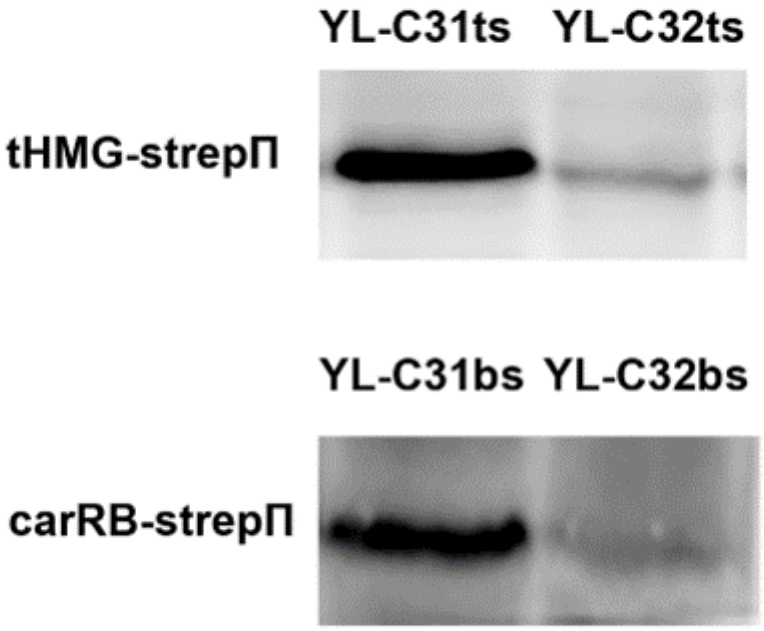

Figure 7

The protein level of the key enzymes reduced after overexpressing two copies of the Did2 gene compared with the overexpression of one copy of the Did2 gene. Western blot result of the tHMG-strepII fusion protein in the strains YL-C31ts and YL-C32ts and the carB-strepII fusion protein in the strains YL-C31bs and YL-C32bs.

Supplementary Files

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