

Transcriptome sequencing and global analysis of blue light-responsive genes provide clues for high carotenoid yields in *Blakeslea trispora*

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Abstract

Blakeslea trispora has great potential uses in industrial production because of the excellent capability of producing a large quantity of carotenoids. However, the mechanism of light induced carotenoid biosynthesis even the structural and regulatory genes in pathways remain unclear. In this paper, we reported the first transcriptome study in *B. trispora* in which we have carried out global survey of expression changes of genes participated in blue light response. We verified that the yield of β -carotene reaching to 3-fold when transferred from darkness to blue light for 24 h and the enhancement of transcription levels of *carRA* and *carB* presented a positive correlation with the increase in carotenoid production. RNA-seq analysis revealed that 1124 genes were upregulated and 740 genes were downregulated respectively after blue light exposure. Annotation through GO, KEGG, Swissprot and COG databases showed 11119 unigenes compared well with known gene sequences, 5514 unigenes were classified into Gene Ontology, and 4675 unigenes were involved in distinct pathways. Among the blue light responsive genes, 4 genes (*carG1*, *carG3*, *carRA* and *carB*) identified to function in carotenoid metabolic pathways were dominantly upregulated. We also discovered that 142 TF genes belonging to 45 different superfamilies showed significant differential expression ($p \leq 0.05$), 62 of which were obviously repressed by blue light. The detailed profile of transcription data will not only allow us to conduct further functional genomics study in *B. trispora*, but also enhance our understanding of potential metabolic pathway and regulatory network involved in light regulated carotenoid synthesis.

Introduction

Carotenoids, widely known as lycopene, astaxanthin and other terpenoid compounds. represent a group of naturally occurring pigments with red, orange or yellow color. They have a broad market demand and are used extensively as natural colorants in food for years. But more importantly, they have strong antioxidant effect and are beneficial to human health. Using microorganisms to produce carotenoids is an environmentally friendly strategy compared to traditional methods of chemical synthesis and direct extraction from plants (Foong et al. 2021; Johnson and Schroeder 1996; Mussagy et al. 2021). *Blakeslea trispora*, a member of Mucorales species, is featured by the sexual phase between the plus and minus mating partners among heterothallic species. It produces zygospores during the sexual development and accumulates carotenoids within the hypha through the whole growth cycle (Lee and Idnurm 2017). The strain has great potential uses in industrial production because of the excellent capability of producing a large quantity of lycopene during sexual reproduction (Lopez-Nieto et al. 2004). It has been confirmed in *B. trispora* that lycopene cyclase that mediates lycopene cyclization and lycopene dehydrogenase that converts phytoene to lycopene encoded respectively by *carRA* and *carB* are the crucial enzymes for carotenoid biosynthesis (Breitenbach et al. 2012; Rodriguez-Saiz et al. 2004; Schmidt et al. 2005). In the past few years, researchers have identified several genes involved in mating by cDNA-AFLP, but there have little reports on the regulation mechanism of carotenoid production (Kuzina et al. 2008). In addition to sexual reproduction, light also affected carotenoids synthesis with a decrease in carotenoid production under continuous light exposure and an increase in production after dark cultured and then under light (Quiles-Rosillo et al. 2005). Conversely, the carotenoid synthesis was significant improved by continuous light exposure in *Mucor circinelloides* and *Phycomyces blakesleeanus* (Almeida and Cerda-Olmedo 2008; Quiles-Rosillo et al. 2005), indicating that there existed obviously difference in light signal reception and transduction .

Blue light is an important environmental signal for various organisms affecting their developmental and physiologic processes mediated by photoreceptors (Corrochano 2019; Fuller et al. 2015). Our knowledge about

photoreceptors involved in these responses has been derived from different fungal species, including *Neurospora crassa*, *Aspergillus nidulans*, *Fusarium fujikuroi*, *P. blakesleeanus*, etc (Corrochano and Garre 2010; Kim et al. 2014; Purschwitz et al. 2008; Ruiz-Roldan et al. 2008). The photoreceptors identified include the White Collar proteins and cryptochromes for blue light, opsins for green light, and phytochromes for red light (He and Liu 2005; Wang et al. 2018). The best-described blue light photoreceptors in fungi are WC-1 and WC-2 which serves as the central components in the blue-light signal transduction pathway and have been reported in ascomycetes, basidiomycetes and zygomycetes (Corrochano and Garre 2010; Liu et al. 2003; Yang et al. 2016). After receiving the light signals, white collar complex (WCC) formed by WC-1 and WC-2 interaction *in vivo* subsequently binds to the promoters of light-regulated genes in order to rapidly activate transcription in response (Smith et al. 2010; Wang et al. 2015). The typical structure of WC proteins was slightly different. In addition to a GATA zinc-finger DNA-binding motif, WC-1 possesses three PAS domains while WC-2 usually exhibits only one PAS which is involved in the protein-protein interaction (Cheng et al. 2002). Multiple copies of WC are widespread in *Mucorales* species. Since *P.blakesleeanus* is quite sensitive to light stimulation, it has aroused great interests of researchers and was used as a model species for researching blue light signal transduction since 1969 (Bergman et al. 1973; Polaino et al. 2017). After three *wc-1* and four *wc-2* genes have been identified in *P.blakesleeanus*, Using light-responsive blind mutants, MadA and MadB were also shown to form a WCC-like complex that plays a dominant role in all known light responses, including phototropism of the sporangiophores, activation of carotenoid synthesis, and inhibition of sexual reproduction (Sanz et al. 2009; Shakya and Idnurm 2017). However, since efficient genetic manipulation including gene knockout or overexpression towards *P.blakesleeanus* was not available, functional studies on WC proteins have been hampered for a long time other than MadA and MadB (Polaino et al. 2017; Tagua et al. 2020). Another blue light receptor, CryA, belonging to the cryptochrome-DASH family, has been well investigated in *P. blakesleeanus*, which has complete photocleavage synthase activity and can repair CPD damage on single- and double-stranded DNA (Tagua et al. 2015). Functional studies on the multi-copy WC proteins were mainly carried out in *M. circinelloides*, that is another representative strain of zygomycetes, just because of the development of molecular tools to genetically manipulate this strain (Nagy et al. 2017; Navarro et al. 2013). With the help of gene knockout, the light response of the three WC-1 proteins were emerged: McWC-1a and McWC-1c were respectively involved in mediating phototropism and light-driven carotenoid synthesis, while McWC-1b regulated carotenoid production independent to light signal (Navarro et al. 2013; Silva et al. 2006). CrgA, another transcription factor regulating carotenoids synthesis, was initially discovered as a negative regulator. It was subsequently shown that carotenoids accumulated in a *crgA*-deletion strain both under light and dark conditions (Lorca-Pascual et al. 2004; Silva et al. 2008). Furthermore, light enhanced the transcription level of carotenoid synthesis genes and consequently improved the carotenoids content. Homologous protein of CrgA in *B. trispora* was also functionally clarified through gene complement in *M. circinelloides* Δ *crgA* strain as well as gene knockout in *B. trispora* (Luo et al. 2020; Quiles-Rosillo et al. 2005). The phenotype analysis combining the double deletion mutants of genes *Mcwc-1* and *crgA* showed that CrgA negatively regulate carotenoid synthesis in a way independent of McWC-1c. Further studies showed that, as an E3 ubiquitin ligase, CrgA inhibits the transcriptional activation of carotenogenic genes by the ubiquitination modification of McWC-1b (Silva et al. 2008). Recent studies have shown that *crgA* deficiency alters intracellular metabolic flow, and the increase in carotenoid production is only part of its effect on intracellular metabolism (Luo et al. 2020).

As compared to many loss-of-function mutants obtained by physical and chemical mutagenesis methods in *P. blakesleeanus*, very few mutants of *B. trispora* have been reported so far (Bergman et al. 1973; Tagua et al.

2020). More important, genetic manipulation for this multinucleate fungus is extremely difficult to achieve due to the heterokaryons would appear after the exogenous genes were transferred into any mycelia. Consequently, the study on either the mechanism of carotenogenesis in this strain or effort for genetic improvement is seriously hindered for years. It is widely accepted that RNA-seq technology appears to be the most powerful tool for transcriptome analysis and has great potential to investigate the genes involving in alteration of specific factors. The aim of the study was to decipher the transcriptome changes that take place in light response and the regulatory mechanisms of candidate genes involved in light-induced carotenoid biosynthesis. Our work for the first time provided gene expression differences between blue light and dark conditions at a whole genome level. Because the lack of stable and efficient genetic manipulation system as well as very high rates of heterokaryon formation for *B. trispora*, the results offer significant information and new research targets for investigators to further clarify gene functions *in vitro*.

Materials And Methods

Strains, medium and growth conditions

In this study *B. trispora* NRRL2896(-), used as the wild type strain, was cultured in PDA medium at 28°C for 2-4 days to achieve mycelial growth. Sporangiospore were harvested from mycelia grown at 5th day grown on YpSs agar medium and kept in glycerol (40% w/v in water) at -80 °C. For blue light illumination, plates inoculated with 10^4 spores were previously incubated in the dark for 60 h and then exposed to continuous blue light at 1.34 W/m² for different times intervals. The mycelia were collected in darkness after irradiation and then frozen immediately in liquid nitrogen at the selected time points. Mycelia without blue-light illumination were also obtained in the same procedure and used for controls.

Carotenes Extraction and Chemical Analyses

Carotenes were extracted from the *B. trispora* strain grown YPD solid medium for 84 h in the dark and 60 h in the dark followed by 24 h under blue light. The mycelia were washed with ddH₂O and dried between paper towels and lyophilized. The freeze-dried mycelium powder was weighed and then suspended with 2 ml of ethyl acetate and extracted several times until the mycelium was colorless. The organic phase extracted was evaporated at 30 °C in a vacuum, dissolved in 500 µL ethyl acetate and subjected to HPLC. For HPLC analysis, carotenoids were identified and quantified using a column Ultimate XB-C18 end capped 5 µm, 4.6 × 150 mm reverse phase column (Welch Technology, China). The eluents used were acetonitrile: methanol: dichloromethane (7:2:2, v/v). Detection of β-carotene was made at 453 nm, which is the wavelength of maximum absorption of β-carotene. The concentrations of β-carotene were estimated at basis of an adequate standard curve using its standard compound.

The extraction and purification of mRNA

The mycelia of *B. trispora* grown in the dark and treated with blue light were harvested, frozen in liquid nitrogen, and ground. Total RNA was extracted using *TransZol/Up* RNA reagent (TransGen Biotech China) according to the manufacturer's instructions. The quality and integrity of total RNA was checked on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

High-throughput sequencing

For high-throughput sequencing, 3 µg total RNA obtained from the three biological replicates of *B. trispora* in the dark and blue light were used as input material for the RNA-seq sample preparations. Sequencing libraries were generated using the NEBNext Ultra II RNA Library Prep Kit for Illumina (#E7775) and then sequenced on a Novaseq platform (Illumina) by Shanghai Personal Biotechnology Cp. Ltd.

Data Analysis

The Illumina HiSeq 2000 system was used to sequence approximately 242 million 150 bp paired-end reads. These sequences were quality-filtered and mapped to the *B. trispora* F921 v1.0 reference genome, available from the JGI Genome Portal (https://mycocosm.jgi.doe.gov/Blatri_F921_1/Blatri_F921_1.home.html). The genes were annotated using *B. trispora* F921 v1.0 reference genome. For the differential expression analysis, using two-fold change cutoff, that is, \log_2 fold change ≥ 1 or ≤ -1 and an adjusted P-value ≤ 0.05 as thresholds. Functional enrichment analysis of differentially expressed genes, GO terms significantly enriched (that is, with P-values ≤ 0.05), and KEGG analysis were based on the integrated cloud platform of I-Sanger (<https://www.i-sanger.com/>).

Reverse transcription fluorescence quantitative PCR

The mycelia for real-time PCR were prepared same as mycelia for RNA-sequencing. RNA extraction was performed as previously reported. Total RNA was treated with DNaseI to remove gDNA. cDNA was subsequently synthesized using Takara PrimeSript first strand c-DNA synthesis kit (*EasyScript*[®] First-Strand cDNA Synthesis SuperMix, Beijing, China) according to the manufacturer's instructions. To validate the differentially expressed genes, 12 genes were randomly selected and the primers used were described in Table S1. Quantitative real-time PCRs were performed using SYBR green supermix (TransStart[®] Top Green qPCR SuperMix Takara, TransStart[®] Top Green qPCR SuperMix) on a LightCycler instrument (Roche). Reactions were performed in triplicates with a total volume of 20 µL, including 250nmol/L forward primer, 250nmol/L reverse primers and 2 µL cDNA template (10-fold diluted). The amplification program used in this study consisted of: 95°C for 3 minutes; 40 cycles of 95°C for 10 seconds and 60°C for 20 seconds; and melting curve of 60°C to 95°C at increments of 0.5°C for 10 seconds. The GAPDH gene was used as the endogenous control to normalize the total amount of cDNA present in each sample. Then, the results obtained from light stimulation mycelia were normalized to the results of mycelia grown in the dark. Data analysis from three biological for each gene was performed using the comparative CT method ($\Delta\Delta CT$).

Results

The response to blue light and transcription of key genes of carotenoid synthesis

The phenomenon of the photoresponse in *M. circinelloides* has been initially discovered several years ago and their gene involvement in the biological process has also been studied (Corrochano and Garre 2010). In order to study the effect of blue light on carotenoid synthesis of *B. trispora*, we examined the vegetative growth on solid media under dark condition and blue light irradiation. To our expected, although little difference could be seen in mycelial growth, the hypha appears white-yellowish color after 24 hours inoculation due to the production of small amounts of β -carotene in the dark, while it becomes deep yellow with the increasing blue light exposure time because of the rapid accumulation of β -carotene triggered by blue light (Fig. 1a). To confirm that color

differences are closely related to the level of carotenoid synthesis, we therefore determined the content of β -carotene in cells by high performance liquid chromatography. Compared to that in the hyphae kept in a dark environment, the yield of β -carotene reached to double-triple times after blue-light irradiation for 24 hours (Fig. 1b). In summary, blue light indeed contributes to accelerate carotenoid synthesis of *B. trispora*.

To further investigate the potential molecular mechanism on carotenogenesis after blue light stimulation in *B. trispora*, the transcription levels of *carRA* and *carB*, the key genes in the carotenoid synthesis pathway, were examined at different time point within 30 minutes after blue light irradiation. The result shows the transcription levels of the two genes are up-regulated about 15- and 40-fold respectively, which is consistent with the conspicuous increase in carotenoid production. It suggested that blue light regulates the transcription of the related genes in the carotenoid biosynthesis pathway of *B. trispora* directly or indirectly (data not shown) and could be attributed to up-regulation of the genes encoding carotenoid metabolic pathway enzymes

In order to study the time courses transcriptional response of these two genes after a prolonged exposure to blue light, and to select a optimum time point for subsequent RNA-seq assay, total RNA was isolated from mycelia irradiated with blue light for a period of up to 60 min and the induced transcriptional curves of *carB* and *carRA* were shown in Fig. 1c. The transcripts accumulation of both genes increased rapidly after blue light illumination. Interestingly, the maximum accumulation for *carB* reached to 80-fold within 10 minutes after irradiation and there existed two peaks in the transcription curve of *carRA* at time points of 8 minutes and 30 minutes. The transcription levels of both genes returned to near basal levels after 60 min. This result is also consistent with previous reports of *M. circinelloides*.

Analysis of genes differentially expressed under light and dark conditions

To investigate the role of blue light in the global transcriptome of *B. trispora*, we performed transcriptome analyses of mycelia using RNA-seq under dark and blue light conditions. The mycelia of *B. trispora* were collected when exposed to 10 minutes of blue light after 48 h of growth in the dark. Samples from three biological replicates of each condition were sequenced and resulted in approximately 242 million 150 bp paired-end reads, corresponding to 31 GBs of nucleotides (Table S2). There was a high Pearson correlation ($R^2 \geq 0.98$) between the three biological replicates of each condition. Differential expression analysis was performed between blue light and darkness conditions and 1864 modulated genes were identified, using a $p \leq 0.05$ as the threshold. Among these genes, 1124 were upregulated and 740 were downregulated, applying a two-fold change (that is, $|\log_2 \text{fold change}| \geq 1$) and an $FDR \leq 0.05$ as the threshold (Fig. 2a). To verify that our RNA-Seq data is reliable, *carRA*, *carB* and 10 other genes modulated by blue light were randomly chosen for RT-qPCR analysis. Of these genes, 8 were upregulated and 4 were downregulated (Table S3). The significant Pearson correlation ($R^2 = 0.9096$) of the \log_2 fold change of gene expression was demonstrated when comparing the data obtained by RNA sequencing and real-time PCR, which suggested that gene expression levels from the former were confirmed by the latter and the RNA-seq analysis is high in reliability (Fig. 2b).

Enrichment Analysis Of Go And Kegg

The functional categorization of the different expression genes was analyzed using the terms of GeneOntology (GO). The 1864 differentially expressed genes (DEGs) were partitioned into 41 functional group categories under three major categories: "cellular component", "biological process" and "molecular function" (Fig. 3a). These major

categories sorted into several subcategories and 5, 25, and 62 GO terms for the three categories respectively appeared enrichment based on $p \leq 0.05$. The most 20 enriched terms in these categories were shown in Fig. 3a. Within the biological process category, 14 terms, including “regulation of nucleobase-containing compound metabolic process” (GO:0019219), “regulation of transcription” (GO:0006355), “regulation of RNA metabolic process” (GO:0051252) and other processes involved in the regulation of gene expression (GO:1903506, GO:2001141, GO:0009889, GO:0010556, GO:0031326, GO:2000112, GO:0005975, GO:0010468, GO:0006351, GO:0097659 and GO:0032774) were the most representative. For the molecular function category, “DNA-binding transcription factor activity” (GO:0003700), “transcription regulator activity” (GO:0140110), “secondary active transmembrane transporter activity” (GO:0015291), “molecular transducer activity” (GO:0060089) and “sequence-specific DNA binding” (GO:0043565) were predominantly represented. And for the cellular components category, GO term “membrane” (GO:0016020) was significantly enriched (Fig. 3b).

To investigate metabolic pathways involved in the response to blue light, DEGs were subjected to KEGG pathways enrichment analysis (Fig. 4). However, since there was no pathway information available about this strain in the KEGG database, we analysis the metabolic pathways of DEGs using the reference genome from JGI. Up to 4640 DEGs could be annotated, and 2826 pathways were obtained. Most of the DEGs involved in the “Metabolism of Complex Lipids” (35 genes), “Metabolism of Complex Carbohydrates” (55 genes), “Carbohydrate Metabolism” (50 genes), “Amino Acid Metabolism” (47 genes), and “Metabolism of Cofactors and Vitamins” (22 genes). The top 20 enriched pathways were listed in Fig. 4. Considering $p \leq 0.05$ as the threshold, only six pathways were significantly enriched, including “Aminosugars metabolism,” “Fluorene degradation,” “starch and sucrose metabolism,” “Ascorbate and aldarate metabolism,” “Cyanoamino acid metabolism” and “Glycerolipid metabolism”. These enriched pathways provide us a direction to further investigate the metabolic processes and gene functions which participated in the blue light response in *B. trispora*.

The Identification Of Genes Involved In Blue Light Response

After a comprehensive overview of the transcriptome data, the top 20 upregulated genes with more than 2^7 fold increase in mRNA level and the top 10 downregulated genes with a $2^{3.5}$ to $2^{7.7}$ fold reduction in transcriptional level were selected to simplify the analysis (Table 1). The KOG description and KOG class about these genes were also listed in the table. The top 20 upregulated genes included four related to energy production and conversion (ID 529211, 542283, 373722 and 500322). A gene which encodes a deoxyribodipyrimidine photolyase (ID 383338) was predicted as the homologue of cryptochrome-DASH in *P. blakesleanus*. The 4-dihydrotrisporin dehydrogenase (ID 527621), which participate in trisporic acid synesis pathway is up-regulated by $2^{9.2}$. Genes that encode cytochrome P450 (ID 398086), E3 ubiquitin ligase (ID 497534), velvet factor (ID 530917), one protein containing FAS1 domain (ID 452672), one gluconate transport-inducing protein (ID 464910), one amino acid transporter (ID 420874) and one HMG box-containing protein (ID 481629) were also upregulated, as well as seven unknown proteins (ID 530917, 392850, 489762, 469733, 499226, 396672, and 525646). Among the downregulated genes, 7 proteins are associated with transport and metabolism, of which four involving in amino acid transport and metabolism (ID 416571, 566594, 430881 and 528068), two involving in lipid transport and metabolism (ID 417993 and 465246), and one involving in carbohydrate transport and metabolism (ID 457478). One protein participating in cell cycle control (ID 461298) and one putative protein phosphatase participating in posttranslational modification (ID 491548) were also annotated. Besides that, the functions of 11 proteins (ID 360254, 379721, 459644, 388849, 465314, 391569, 540410, 542974, 407061, 377374 and 484741) were still not

clarified. Nearly half of these 40 significantly DEGs with significant transcript levels encoded for unknown proteins, which provided us with many breakthroughs to study the mechanism of light response.

Table 1

Log2 fold change (FC) of the top 20 differentially expressed genes in *B. trispora* after blue light exposure.

	Protein ID	KOG description	KOG class	Annotation Description	Log ₂ FC
Up	398086	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies	Lipid transport and metabolism	cytochrome P-450 cyp509A1	12.379
	497534	Predicted E3 ubiquitin ligase	Posttranslational modification, protein turnover, chaperones	PUA-like domain-containing protein	12.004
	529211	NADH:flavin oxidoreductase/12-oxophytodienoate reductase	Energy production and conversion	hypothetical protein	11.038
	383338	Deoxyribodipyrimidine photolyase/cryptochrome	Replication, recombination and repair	DNA photolyase /Cryptochrome	10.87
	452672	Fasciclin and related adhesion glycoproteins	Cell wall/membrane/envelope biogenesis	FAS1 domain-containing protein	10.414
	530917	hypothetical protein	Unknown	Velvet factor-domain containing protein	9.834
	481629	HMG box-containing protein	General function prediction only	expressed protein	9.587
	527621	Predicted short chain-type dehydrogenase	General function prediction only	4-dihydrotrisporin dehydrogenase	9.245
	542283	Zinc-binding oxidoreductase	Energy production and conversion	hypothetical protein	9.004
	464910	Gluconate transport-inducing protein	Carbohydrate transport and metabolism	hypothetical protein	8.595
	392850	hypothetical protein	Unknown	hypothetical protein	7.786
	489762	hypothetical protein	Unknown	hypothetical protein	7.546
	469733	hypothetical protein	Unknown	hypothetical protein	7.193
	552594	GDSL lipase/esterase	hydrolase activity, acting on ester bonds	GDSL lipase/esterase	7.145
	499226	hypothetical protein	Unknown	expressed protein	7.107
	420874	Amino acid transporters	Amino acid transport and metabolism	Transmembrane amino acid transporter protein-domain containing protein	7.083

	Protein ID	KOG description	KOG class	Annotation Description	Log₂FC
	373722	Kynurenine 3-monooxygenase and related flavoprotein monooxygenases	Energy production and conversion	hypothetical protein	6.929
	500322	Mitochondrial carrier protein CGI-69	Energy production and conversion	Mitochondrial carrier domain-containing protein	6.893
	396672	hypothetical protein	Unknown	expressed protein	6.592
	525646	hypothetical protein	Unknown	expressed protein	6.535
Down	417993	Predicted lipase	Lipid transport and metabolism	lipase	-5.759
	360245	hypothetical protein	Unknown	hypothetical protein	-5.476
	379721	hypothetical protein	Unknown	hypothetical protein	-4.928
	416571	Amino acid transporters	Amino acid transport and metabolism	Transmembrane amino acid transporter protein-domain containing protein	-4.926
	566594	Amino acid transporters	Amino acid transport and metabolism	Amino acid permease-domain containing protein	-4.876
	459644	hypothetical protein	Unknown	expressed protein	-4.861
	465246	Predicted lipase	Lipid transport and metabolism	lipase	-4.67
	388849	hypothetical protein	Unknown	hypothetical protein	-4.59
	465314	hypothetical protein	Unknown	GPR1/FUN34/yaaH family-domain containing protein	-4.571
	391569	hypothetical protein	Unknown	hypothetical protein	-4.471
	457478	Permease of the major facilitator superfamily	Carbohydrate transport and metabolism	Major facilitator superfamily domain-containing protein	-4.437
	540410	hypothetical protein	Unknown	hypothetical protein	-4.432

Protein ID	KOG description	KOG class	Annotation Description	Log ₂ FC
430881	Amino acid transporters	Amino acid transport and metabolism	Transmembrane amino acid transporter protein-domain containing protein	-4.416
542974	hypothetical protein	Unknown	hypothetical protein	-4.358
461298	Putative transcriptional repressor regulating G2/M transition	Cell cycle control, cell division, chromosome partitioning	hypothetical protein	-4.355
407061	hypothetical protein	Unknown	expressed protein	-4.349
377374	hypothetical protein	Unknown	expressed protein	-4.31
491548	Protein phosphatase, regulatory subunit PPP1R3C/D	Posttranslational modification, protein turnover, chaperones	Putative phosphatase regulatory subunit-domain containing protein	-4.269
484741	hypothetical protein	Unknown	expressed protein	-4.239
528068	H ⁺ /oligopeptide symporter	Amino acid transport and metabolism	POT family-domain containing protein	-4.097

Transcriptional level analysis of genes related to carotenoid metabolic pathways

Light-induced carotenogenesis is a representative characterization of light responses in *B. trispora*. As the metabolic pathways shown (Fig. 5a), 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) is reducing to mevalonate by HMG-CoA reductase and the Mucorales use MVA pathway for the synthesis of the precursors of all terpenoids, including lycopene, β -carotene, ubiquinone and squalene, in which β -carotene is catabolized to trisporic acid C. Some genes of this pathway have been identified and the detailed quantifications of light-dependent mRNA accumulation of these genes in *B. trispora* were analyzed (Fig. 5b). The transcriptional levels of *carRA* and *carB* appear a significant increase, similar to the previous result shown in Fig. 2, whereas that of genes in the mevalonate pathway, the carotenoid catabolism and the two metabolic flux has no obvious change. The coding sequences of CarRA and CarB are separated by 611 base pairs and divergently transcribed, therefore we hypothesize that one or more unknown specific transcriptional regulators bind to the bidirectional promoter to regulate photo-carotenogenesis directly. It is worth mentioning that we identified four different GGPP synthetase genes (*carG*), of which only ID 402428 has been cloned and functional expressed. Transcriptome data indicate that there was no significant change for ID 402428 and ID 494297 in transcription level under blue light stimulation, while ID473880 and ID509961 with varying degrees of upregulation. The GGPP synthetases (GGPPS) in plants have been shown to play an important role in allocating metabolic fluxes and regulating diterpenoid synthesis. Generally, more than one GGPP synthetase were discovered in plant genomes with distinct the subcellular localization. Therefore, it is important to dissect the species-specific and spatio-temporal expression as well as the subcellular localization and molecular interaction of these GGPPS for understanding metabolic regulation and genetic engineering improvement of terpenoid metabolism.

Changes In Transcription Level Of Putative Transcription Factors

Transcription factors (TFs) are key DNA binding proteins involved in the regulation of genes expression of different signaling pathways, and the expression of stress-induced genes is mainly regulated by sequence-specific binding TFs. To identify TFs potentially involved in light response by analyzing differentially expressed mRNAs will enable us to unveil the light response mechanism, including the regulatory mechanisms of light-induced carotenogenesis. To date, 692 TFs were found in the genome of *B. trispora* (information based on the number of *B. trispora* transcription factors annotated in the genome portal <http://genome.jgi-psf.org/Blatri1/Blatri1.home.html>), with few have already been characterized and described. 142 TF genes belonging to 45 different superfamilies showed significant differential expression ($p \leq 0.05$), 62 of which were repressed by blue light (Table S4). The most expressed gene encoding C3HC4-type RING finger protein (ID Bt_497534) was almost 4000 times ($\log_2FC=12.004$) more heavily in the blue light condition compared to in the dark. A HMG-box protein (ID Bt_497534, $\log_2FC=9.586$) and another C3HC4-type RING finger protein (ID 184178, $\log_2FC=5.825$) rank second and third among the regulatory proteins. The HMG-box protein also has a SAM domain which plays a role in MAPK signaling pathway and probably helps the cell respond to external light stimuli. It is noteworthy that a GATA-type TF (ID Bt_511752) with a PAS domain, which was 16 times more heavily expressed, were predicted to be an homologous of the WC-1c protein in *M. circinelloides*. It suggested the white collar complex (WCC) in the classical light-responsive study is also present in *B. trispora* and the WC-2 protein of the complex in this strain needs to be further identified. The study of TFs interactions and their integration with metabolic network under a given condition will provide us new insights into the global gene expression regulation of light response.

Discussion

B. trispora is known to be the most effective native producer of carotenoids especially lycopene and β -carotene (Rodriguez-Saiz et al. 2004). Although the putative genes involved in this biosynthesis pathway have been identified for a long time, the lack of detailed genomic information and the unavailability of genetic manipulation towards the strain were the major obstacles to further study the function of the genes as well the metabolic pathway (Ge et al. 2021; Kuzina et al. 2008). Previous studies have also revealed that both pheromones (known as trisporic acid) and blue light irradiation could strongly activate carotenoid biosynthesis, but the exact mechanism still remains unclear (Modi 1977; Quiles-Rosillo et al. 2005). With the whole genome sequences of *B. trispora* available, it becomes possible to screen out the specific genes which were most likely to participated in the light response mechanism based on the extensive analysis of transcriptome sequencing results under two or more conditions.

The carotenoid synthesis pathway in *B. trispora* is a sequential reaction process originated from acetyl CoA, during which changes in gene transcription and in the enzymatic activity caused by post-translational modification may thus affect the synthesis of β -carotene (Breitenbach et al. 2012). The currently identified regulatory proteins involved in carotenoid synthesis, including BtCrgA and multi-WC-like proteins, are closely related to light regulation (Ge et al. 2021; Luo et al. 2020). CrgA in *B. trispora* is proved to be a negative regulatory protein in the process of light-induced carotenoid synthesis. Previous studies have shown that *crgA* gene is up-regulated after blue light irradiation compared to dark condition (Quiles-Rosillo et al. 2005). RNA sequencing analysis indicated that the transcription level of BtCrgA (ID 559549) was increased by 4.53 times, which is

consistent with previous reports (Quiles-Rosillo et al. 2005). In *M. circinelloides*, CrgA, played as an E3 ubiquitin ligase, inhibits photo-induced β -carotene synthesis by a proteolysis independent ubiquitylation, and McWC-1a directly regulates the transcription level of carotenoid synthesis genes through the deactivation of McWC-1b caused by the modification (Silva et al. 2008). Based on current data, it is still under investigation that how CrgA accepted and transmitted signals cooperatively with other light response factors. In addition, CrgA is a member of the undefined protein family which shares the character of two RING finger zinc-binding domains near the N-terminus followed by a LON domain. In general, proteins containing RING finger domains belong to the E3-ubiquitin ligase family, the role of which in plant photoresponse pathways has been extensively investigated (Morreale and Walden 2016). For example, the ring-finger E3 ubiquitin ligase COP1/SPA and PUB13 regulate the flowering and photomorphogenesis of *Arabidopsis* through direct or indirect effects on the stability of photoreceptors (Podolec and Ulm 2018; Ponnu 2020). Additionally, several E3 ubiquitin ligases named SINAT5, COP1, HOS1, ZTK, LKP2 and FKF1 are diversely involved in the photoperiodic rhythm regulation of flowering in *Arabidopsis* (Zoltowski and Imaizumi 2014). In *B. trispora*, three predicted E3 ubiquitin ligases besides CrgA have been identified (Table S4) which are all equipped with RING FINGER domain and N-terminal LON domain. The regulatory mechanism of E3 ubiquitin ligase participating in light response in plant will paving the way for clarifying the function of corresponding genes.

A detail examination on the differentially expressed genes by bioinformatics analysis, we found a locus of interest in the genome of *B. trispora*, Bt_383338 (predicted as deoxyribodipyrimidine photolyase/cryptochrome), the transcription level of which was increased by $2^{10.87}$ times. Moreover, we found that this protein was also a functional homolog of Cry-DASH in *P. blakesleeanus* based on multiple sequence alignment analysis. In *P. blakesleeanus*, Cry-DASH played the role of photocleavage synthase and the transcriptional activation depended on the formation of WCC complex. However, there is no experimental evidence till now that Cry-DASH as well as homologous protein functioned as photoreceptors in light signal transduction (Tagua et al. 2015). In *Fusarium Fujikuroi*, the mechanism that cryptochrome CryD, WcoA (homologous protein of WC-1) and VVD cooperatively regulated light-induced carotenoid synthesis was clearly clarified for the first time by means of the mature and stable genetic manipulation (Castrillo and Avalos 2015). Although *B. trispora* is phylogenetically close to *P. blakesleeanus* and the function of Cry-DASH is supposed to be conserved during the early stage of the evolution of fungi, we cannot rule out the possibility that Cry-DASH has acted as a photoreceptor in *B. trispora*. In addition to this, we also noticed in genome information that protein Bt_530917 in *B. trispora* was described as a velvet factor, which has been extensively studied in ascomycetes and basidiomycetes, but reported only in *P. blakesleeanus* among Zygomycete fungi (Tagua et al. 2020). VelB in *A. nidulans* regulated fungal development and secondary metabolism by forming complexes with other velvet proteins, which were influenced by environmental signaling factors such as light and temperature (Bayram and Braus 2012). Light on one hand inhibited the formation of fruited bodies during sexual reproductive process by reducing the number of VelB-VeA dimers in the nucleus, on the other hand, it also suppressed the formation of heterodimers of VelB and VosA, thus removing their inhibition to asexual development (Sarikaya Bayram et al. 2010). It has been proved that the sexual reproduction of *Mucorales* fungi was also inhibited under light conditions (Shakya and Idnurm 2017). Since most of these *Mucorales* species show heterothallism and zygospores were generated by close contact between positive and negative strains, it is necessary to hold a mating type locus (*sexP* and *sexM*) to regulate the production of pheromones between complementary mating partners during the stage of sexual reproduction. However, different to the fact that LaeA in *A. nidulans* inhibited the expression of *velB*, the transcription level of Bt_530917 was greatly increased. Since there was no evidence before that LaeA homologues exist in the order

Mucorales, we here identified the velvet protein suggesting that the light effect on sexual reproduction may be more complex.

Declarations

Authors' contributions Xin Ge and Qi Xin performed research, analyzed data, and wrote the paper. Ruiqing Li performed transcriptional analysis. Jingyi Zhao prepared and maintained the fungal culture. Xiaomeng Zhang and Yanan Zhang performed PCR and analyzed data. All authors reviewed and corrected the manuscript.

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Availability of data and material Please contact author for data requests.

Conflict of interest Xin Ge, Ruiqing Li, Xiaomeng Zhang, Jingyi Zhao, Yanan Zhang and Qi Xin declare that they have no conflict of interest.

Code availability Not applicable.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Figures

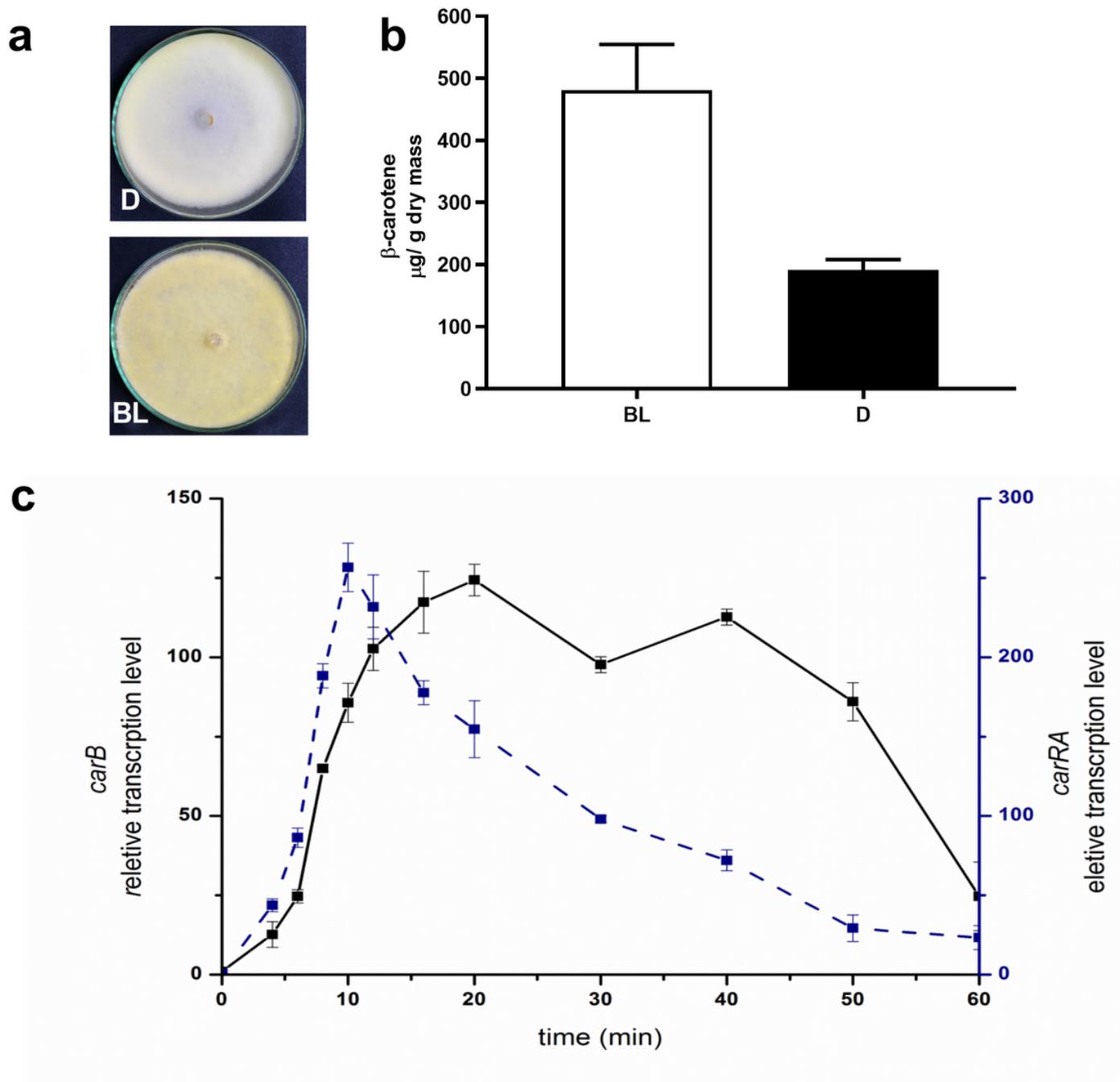


Figure 1

Light-induced accumulation of β -carotene contents and carotenogenic gene transcripts in *B. trispora*. Mycelia of the *B. trispora* grown for 60h in minimal medium in the dark were illuminated with blue light for 24h. (a) The phenotypes and (b) β -carotene contents in 24-hour-old cultures under blue light were dedicated. The symbol "BL" indicates cultures illuminated by blue light while the controls "D" were kept in the dark. (c) Time course curves of relative transcription levels for *carRA* (blue) and *carB* (black) in *B. trispora*. Data are the means of three independent experiments.

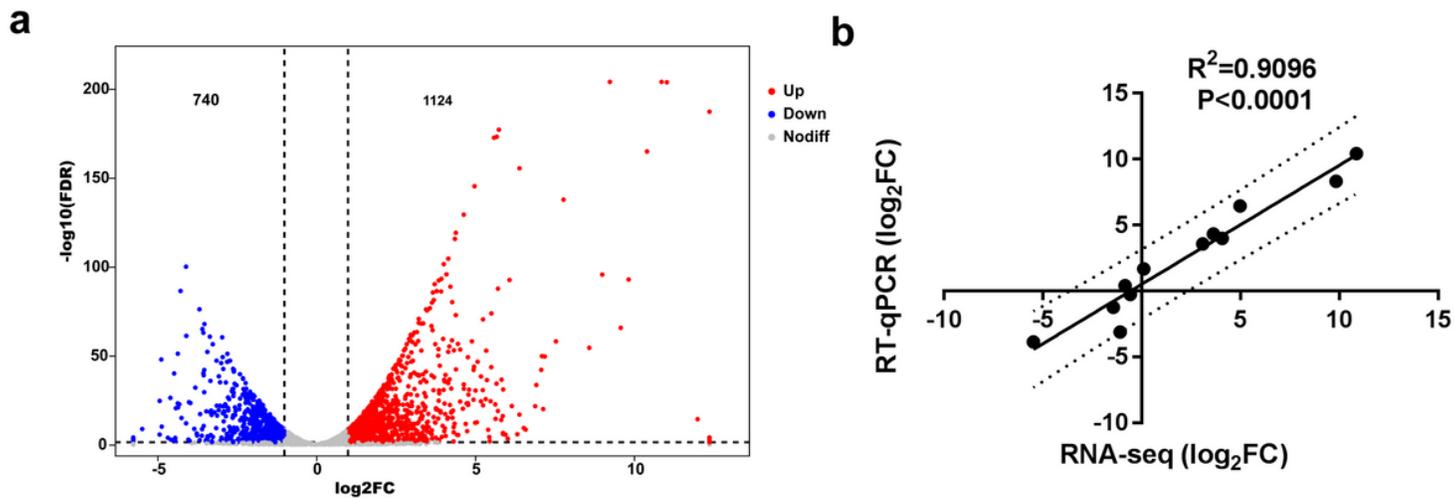
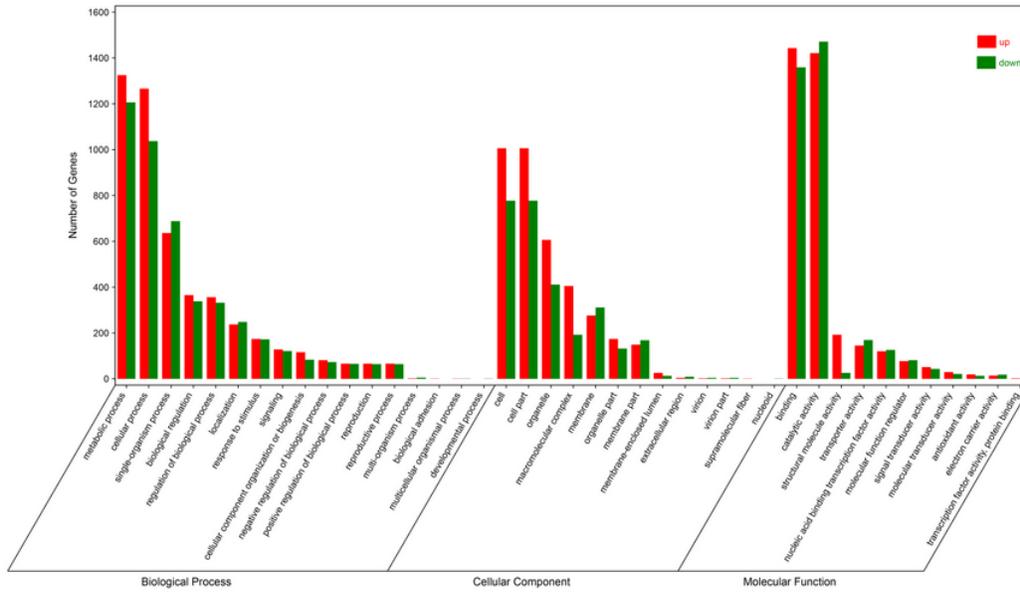
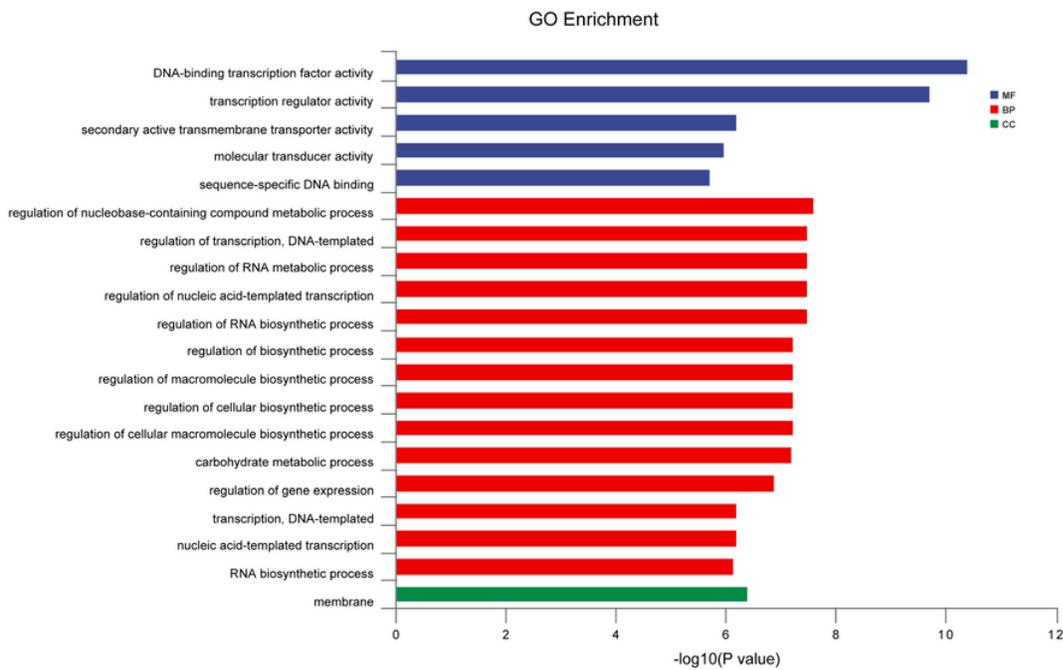


Figure 2

(a) Volcano plot representations of global expression data of the *B. trispora* genes after blue light exposure. (b) Correlation between RNAseq and quantitative real-time PCR (RT-qPCR). Comparison between the Log₂ fold change values obtained by RNA-seq and qRT-PCR for *carRA*, *carB* and ten other randomly selected genes, showing a high correlation between the two techniques ($R^2 = 0.9096$).

a**b****Figure 3**

The GO-term analysis of DEGs. A. These DEGs were classified into three main categories including cellular component, biological process and molecular function. B. the most 20 enriched GO subcategories.

KEGG Enrichment

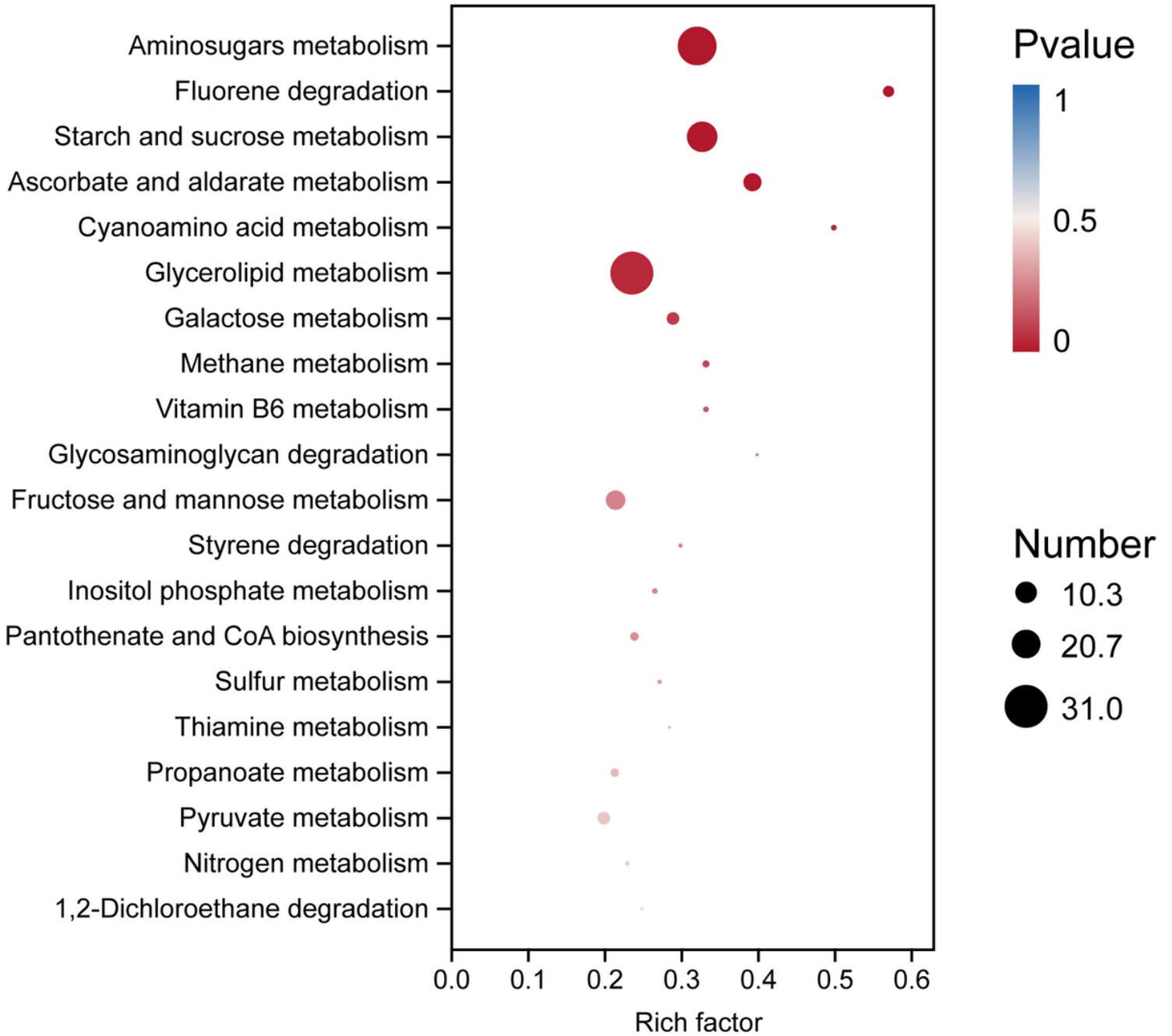


Figure 4

Top 20 KEGG pathways with the most significant enrichment correlated with blue light exposure.

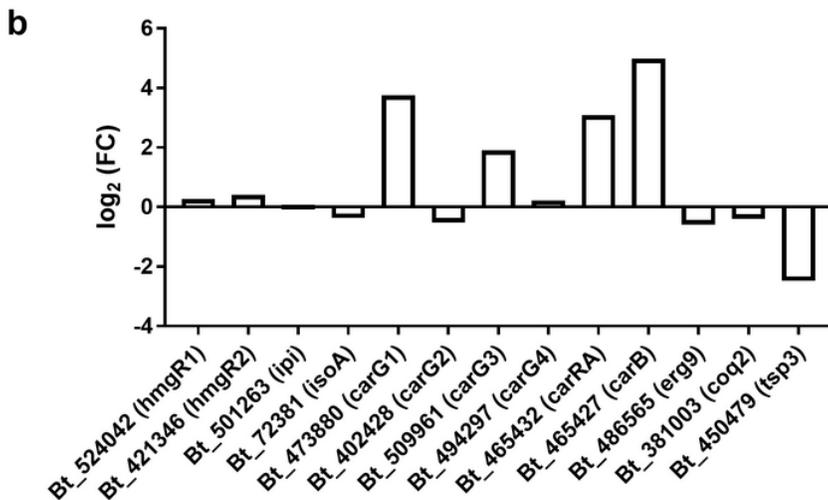
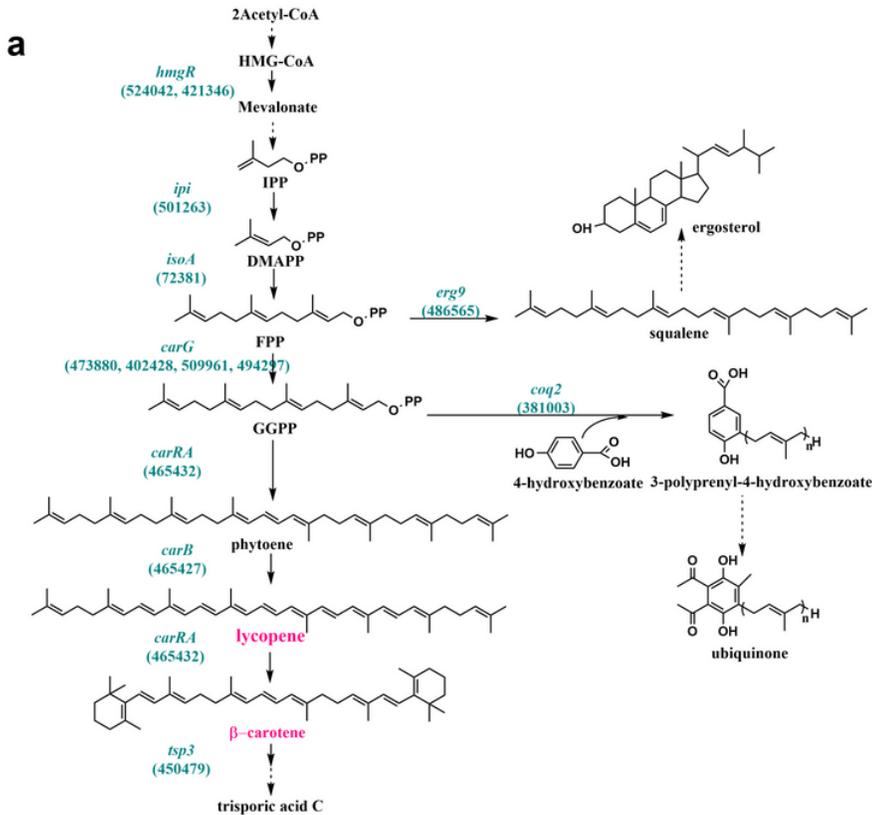


Figure 5

(a) Schematic diagram of the biosynthetic pathway and the branched metabolic pathway and genes encoding respective proteins involved in β-carotene metabolism. (b) The transcript levels of the related genes in (a) obtained from transcriptome data.

Supplementary Files

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