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Transcriptomic and Metabolomic Analyses Provide Insights into the Enhancement of Torulene and Torularhodin Production in *Rhodotorula glutinis* ZHK under Moderate Salt Conditions

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ABSTRACT: Carotenoids are a group of tetraterpene pigments widely used in the food, pharmaceutical, and cosmetic industries. Torulene, torularhodin, and β-carotene, three principal carotenoids synthesized by *Rhodotorula glutinis* ZHK, possess strong health-promoting properties such as antioxidant, provitamin A, and antitumor. Here, the effect of different salt conditions on carotenoids production of *R. glutinis*ZHK was investigated. The results showed that the total carotenoids were significantly enhanced in 0.5 M (3.91 mg/L) and 0.75 M (5.41 mg/L) NaCl treatments than that in 1.0 M (0.35 mg/L) and control (1.42 mg/L) after 120 h of cultivation. Of which, the increase in torulene and torularhodin production acts as the main contributor to the enhancement of total carotenoids. Transcriptome profiling revealed that salt stress efficiently promotes the gene expression of *crtI*, which could explain the molecular mechanisms of the enhanced torulene and torularhodin production under salt stress. Further experiments indicated that torulene and torularhodin play an important role in quenching excrescent reactive oxygen species induced by salt stress. Together, the present study reports an effective strategy for simultaneously improving torulene and torularhodin production in *R. glutinis* ZHK.

KEYWORDS: *carotenoids, Rhodotorula glutinis ZHK, salt stress, torularhodin, torulene*

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1. INTRODUCTION

Carotenoids are a group of more than 1100 naturally liposoluble pigment molecules distributed in plants, fungi, bacteria, algae, and some species of animals.[1] They present yellow orange, red, and purple colors and thus are widely used as natural colorants to enhance the acceptability of foods.[2] The humans cannot synthesize carotenoids, and these compounds therefore have to be supplemented in foods. Dietary intake of carotenoids is associated with a lower incidence of hypovitaminosis, cancer, cardiovascular diseases, diabetes, macular degeneration, as well as virus infections such as human immunodeficiency virus (HIV).[3] Since carotenoids are endowed with these health-promoting benefits, they already have broad applications in the pharmaceutical, food, feed, and cosmetic industries.

Rhodotorula glutinis ZHK is an oleaginous red yeast and primarily produces three kinds of carotenoids, including torulene, torularhodin, and β-carotene.[4] Torulene and torularhodin are two natural valuable carotenoids with relatively few studies as compared to β-carotene. The main producers of torulene and torularhodin contain oleaginous red yeasts of *Rhodotorula*, *Rhodosporidiobolus*, and *Sporobolomyces* genera.[5] Their molecular structures contain a β-ionone, a long polyene chain, and 13 double bonds, respectively (Figure 1), and thus exhibit strong antioxidative properties.[6] Furthermore, both torulene and torularhodin show strong provitamin A and antiprostate cancer activities.[7] Torularhodin attenuates D-galactose/$AlCl_3$-induced cognitive impairment and neuroinflammation[8] and D-galactose/H_2O_2-induced hepatic oxidative damage.[9] Torularhodin also has been considered as a potential protective agent against alcoholic liver diseases by decreasing aspartate transaminase, aspartate transaminase, and low-density lipoprotein levels as well as increasing high-density lipoprotein levels.[10] Torularhodin immobilization effectively enhances antimicrobial activity of the TiO_2 nanotube surface for coating of medical implants,[11] and thus, it has been used as a new natural antimicrobial agent in implanted medical products.[12] More importantly, toxicological experiments conducted on rats suggested that torulene and torularhodin produced by *R. glutinis* can be used as safe food additives with multiple biological activities and a colorant role.[13] Public interests in torulene and torularhodin have increased considerably in the past few years, mainly thanks to their health-promoting effects.[5] Therefore, further increasing the productivity of torulene and torularhodin will promote their industrial production and commercial applications.

Recently, the concerns on the negative effect of chemosynthetic pigments have gathered increasing interest in natural carotenoids. Microbial carotenoids are regarded as promising alternatives for chemosynthetic carotenoids used as food additives.[14] Because of its high synthesis efficiency, several yeast species assigned into the genus *Rhodotorula* have been considered as major sources of natural torulene and torularhodin.[5] *R. glutinis*, the representative species of this

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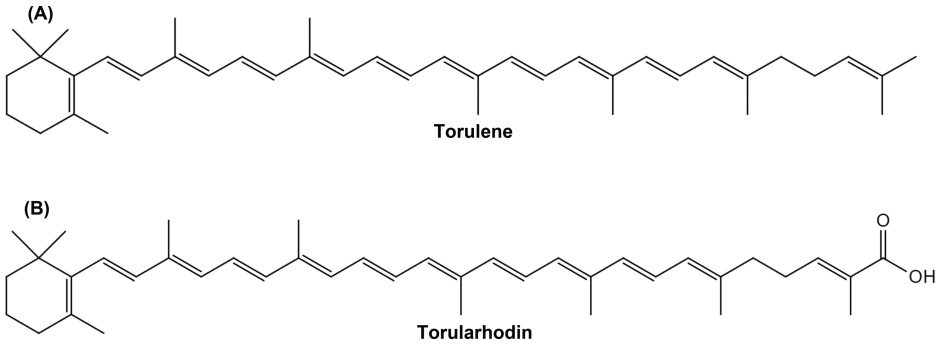

Figure 1. Chemical structures of torulene (A) and torularhodin (B).

genus, produces high yields of torulene and torularhodin.[15] Besides, the production of torulene and torularhodin primarily depends on the medium compositions and cultivation conditions, such as C/N, light, temperature, and metal ions.[15] Although a copious document presents the influence of environmental factors on the biosynthetic efficiency of torulene and torularhodin in *R. glutinis*, the specific role of salt stress has not been studied yet. Therefore, the main objective of this study is to investigate the effect of salt stress on torulene and torularhodin production of *R. glutinis* ZHK and determine the molecular mechanism by an integrated analysis of transcriptome and metabolome.

2. MATERIALS AND METHODS

2.1. Yeast Strain and Cultural Conditions. The *R. glutinis*ZHK was isolated from the Pearl River water (23°06′N, 113°17′E) in Guangzhou, China. The strain collection number of *R. glutinis* ZHK is recorded in the China Center for Type Culture Collection (Wuhan, China) as CCTCC M20211076. Cultures of *R. glutinis*ZHK were prepared in 500 mL Erlenmeyer containing 150 mL of yeast extract−peptone−dextrose (YPD) medium (yeast extract: 10 g/L, peptone: 20 g/L, dextrose: 20 g/L). Incubation was conducted at 25 °C on a rotary shaker at 180 rpm for 120 h. Growth kinetics were detected by the optical density (OD) value at 560 nm, and the samples of culture were withdrawn every 24 h based on previous work.[16] For salt treatments, *R. glutinis*ZHK were respectively cultivated in YPD medium with NaCl concentrations of 0.5, 0.75, and 1.0 M throughout 120 h of growth period. Carotenogenesis inhibition was gained by adding diphenylamine (DPA) (Aladdin, Shanghai, China) with a final concentration of 40 μM before inoculation. All treatments were conducted in batch culture in triplicates.

2.2. Determination of Intracellular Reactive Oxygen Species. Yeast cells were harvested through centrifugation at 5000*g* after 120 h of inoculation, washed thrice using sterile ultrapure water, and resuspended in 1 mL of phosphate-buffered saline (PBS, pH 7.0). The fluorescence method was used to assay the relative levels of reactive oxygen species (ROS) using oxidant-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) as described previously.[17] The fluorescence intensity was detected using an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) with the settings of emission at 530 nm and excitation at 490 nm.

2.3. Determination of Intracellular Superoxide Dismutase and Catalase. Yeast cells were harvested through centrifugation at 5000*g* after 120 h of inoculation, washed thrice using sterile ultrapure water, resuspended with 1 mL of phosphate-buffered saline (PBS, pH 7.0), and disrupted with an ultrasonic processor (power 200 W, 26 kHz) for 6.5 min. The suspension was centrifuged at 8000*g* for 5 min (4 °C), and the supernatant was transferred into a new EP tube. The water-soluble tetrazolium salt (WST-1) method was used to assay relative activities of superoxide dismutase (SOD) using the Superoxide Dismutase Assay Kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) according to operation specifications. The ammonium molybdate method was used to assay relative activities of catalase (CAT) using the catalase assay kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) according to operation specifications. SOD and CAT activities were evaluated using a Multiskan FC Microplate Reader (Thermo, Waltham) with the settings of optical density values of 450 and 405 nm, respectively.

2.4. Extraction and Quantification of Carotenoids. Cultures of different treatments were harvested by centrifugation at 5000*g* and washed thrice with ultrapure water for extraction of carotenoids. Carotenoids were extracted from freeze-drying of cells using the dimethyl sulfoxide (DMSO)−acetone (1:3, v/v) method as described in our previous study.[18] Extraction was repeated until the yeast cells become completely colorless. The extracted carotenoids were quantified by high-performance liquid chromatography (HPLC) on an Agilent 1200s system (Agilent, Waldbronn, Germany) equipped with a C18 reverse-phase column (5 μm, 250 × 4.6 mm^2) (all of the chromatograms and results of HPLC analysis are listed in Figures S1−S18 and Table S1, respectively). Gradient elution was used to conduct carotenoid quantification as described in our previous study.[18] Standards of torularhodin and torulene were purchased from Carote*Nature* (Münsingen, Switzerland); β-carotene was purchased from Sigma (St. Louis, MO).

2.5. Total RNA Extraction and Transcriptome Sequencing. Total RNA was extracted using Trizol reagent (Takara, Dalian, China) according to the operation specifications. The libraries for transcriptome sequencing were constructed using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, CA). Transcriptome sequencing was performed by an Illumina Novaseq. 6000 sequencer. Reference genome index of *R. glutinis*ZHK was built by the program Bowti2 (version 2.2.6) with default parameters.[4] Gene expression abundance quantification was performed using programs HTSeq (version 0.9.1) and DESeq (version 1.30.0) with default parameters.[19] These expressed genes with the $|\log_2$ (fold change)$| \geq 1$ and the adjusted p-value (p_{adj}) ≤ 0.05 were considered to be the differentially expressed genes (DEGs).[20] The raw data of RNA-seq are available at the National Center for Biotechnology Information Sequence Read Archive (SRA) database under BioProject PRJNA725640.

2.6. Metabolite Extraction and Metabolomic Profiling. Total metabolites were extracted from 60 mg of samples using the methods as described in our previous study.[21] Liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis was performed on a Vanquish UHPLC system (Thermo, MA) coupled to a Q-Exactive Plus Mass Spectrometer (Thermo, MA) with the electrospray ionization (ESI)-MSn technique. Mass spectrometric analysis was conducted in positive and negative modes with spray voltages of 3.5 and 2.5 kV, respectively. The metabolite identification was performed based on the databases of HMDB (http://www.hmdb.ca), Metlin (http://metlin.scripps.edu), MassBank (http://www.massbank.jp), LipidMaps (http://www.lipidmaps.org), and mzCloud (https://www.mzcloud.org). Metabolomic profiling was performed on software Analyst (AB Sciex, Darmstadt, Germany). Differentially accumulated metabolites (DAMs) were evaluated using the variable importance in

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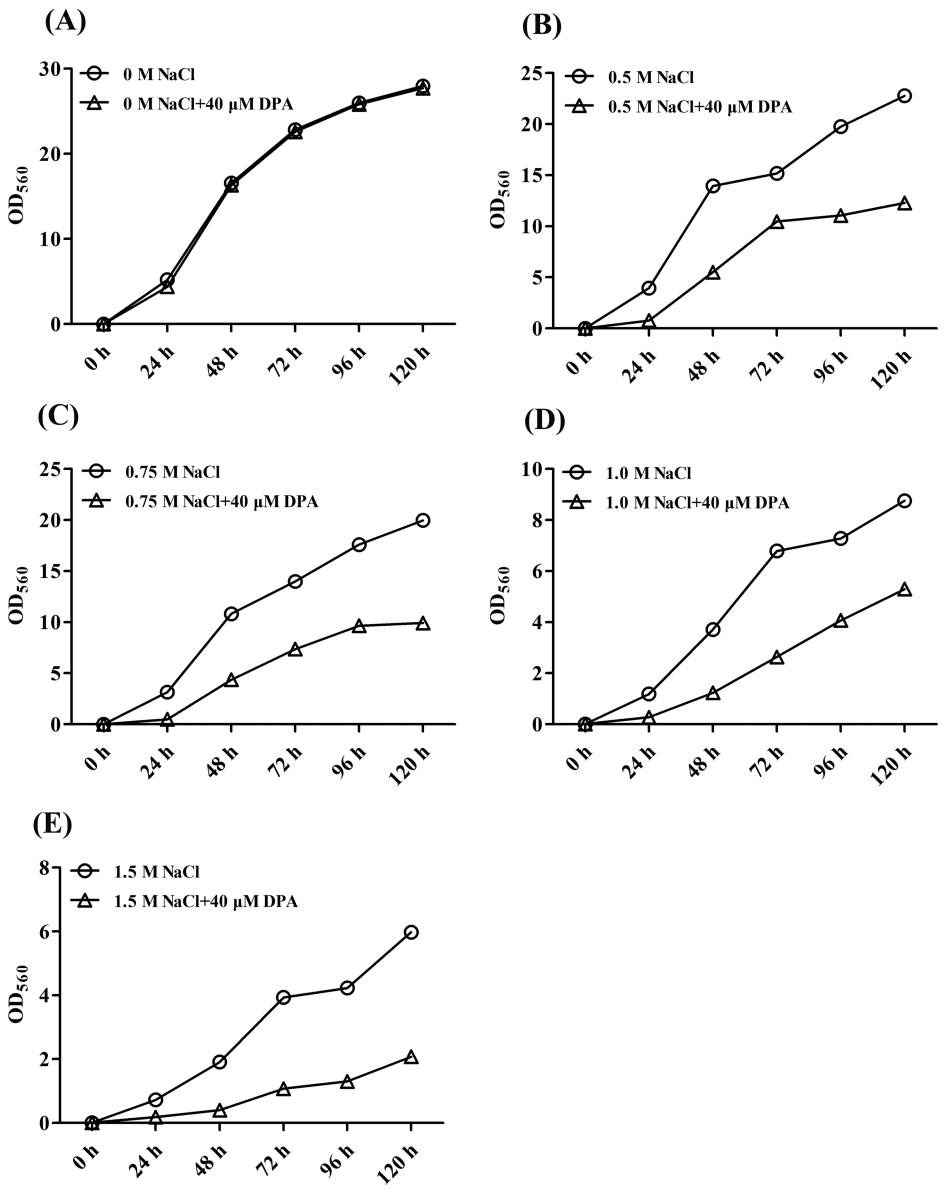

Figure 2. Effect of NaCl treatments on the growth of *R. glutinis* ZHK in the presence of 40 μM DPA (A–E). Growth kinetics was evaluated by optical density at 560 nm after incubation of 24, 48, 72, 96, and 120 h at 25 °C on a rotary at 180 rpm under NaCl concentrations of 0, 0.5, 0.75, 1.0, and 1.5 M, respectively. The data represent the means ± standard deviations of triplicate experiments.

projection (VIP) scores via the partial least squares-discriminant analysis (PLS-DA) model with the thresholds of VIP ≥ 1, fold change ≥ 1.5 or ≤ 0.667, and *p*-value ≤ 0.05.[22] Integration of metabolomic and transcriptomic analyses was conducted by Pearson's correlation coefficients as described previously.[23]

2.7. Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis. Quantitative real-time PCR (qPCR) was performed on an ABI 7500 FAST Real-Time PCR System (Applied Biosystems, CA) using the One-Step RT-PCR Mix Kit (Sangon, Shanghai, China) according to the manufacturer's protocols. Specific oligonucleotide primers were designed and synthesized for amplification of targeted genes. The relative expression of each gene was calculated by the comparative crossing point method (presented as $2^{-\Delta\Delta Ct}$) with three independent biological replicates. 26S rDNA was selected as the housekeeping gene.

2.8. Statistical Analysis. Statistical analysis was conducted using software SPSS 21.0 (SPSS Inc., Chicago, IL). All data were expressed as means ± standard error of triplicate independent cultures. The means of different treatments were compared by one-way analysis of variance (ANOVA) using Tukey's honestly significant difference test at *p*-value <0.05.

3. RESULTS AND DISCUSSION

3.1. Effect of Salt Stress on the Growth of *R. glutinis* ZHK. It was reported that salt stress induces osmotic stress, cation toxicity, and oxidative stress in microorganisms. In response to salt stress, microorganisms must adjust their biochemical processes to participate in the regulation of osmotic, ion and ROS homeostasis, as well as the control and repair of damage.[24] Our previous experiments found that *R. glutinis* ZHK can survive up to a NaCl concentration of 1.5 M and the relatively low concentration of NaCl makes the color of its fermentation broth become redder than control. To reveal the effect of different concentrations of NaCl on the growth, we incubated *R. glutinis* ZHK at final NaCl concentrations of 0, 0.5, 0.75, 1.0, and 1.5 M, respectively.

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To further estimate the role of carotenoids in salt tolerance in *R. glutinis* ZHK, the culture media were supplemented with carotenogenesis inhibitor DPA at 40 μM or not. As a result, the total carotenoid production was reduced from 1.42 mg/L (torulene, 0.19 mg/L; torularhodin, 0.52 mg/L; β-carotene, 0.71 mg/L) to 0.16 mg/L (torulene, 0.09 mg/L; torularhodin, 0.04 mg/L; β-carotene, 0.03 mg/L) as supplemented with 40 μM DPA. As shown in Figure 2, the results showed that NaCl treatment significantly inhibits the growth of *R. glutinis* ZHK. Moreover, supplementation of 40 μM DPA had almost no impact on the growth of *R. glutinis* ZHK (Figure 2A). Remarkably, under high salt conditions, supplementation of 40 μM DPA resulted in significant growth inhibition (Figure 2B–E). Our results preliminary suggested that the synthesis of intracellular carotenoids assists *R. glutinis* ZHK to alleviate the stress of high salt conditions. Similarly, under salt stress conditions, the oleaginous red yeast *Sporobolomyces pararoseus*NGR cultures with DPA showed significant growth inhibition as compared to the cultures without DPA.[18]

Indeed, the synthesis of carotenoids has been proven to be an adaptive mechanism against salt stress and other adverse environmental stress in microorganisms, algae, and plants. Different lines of observations support the protective roles of carotenoids against oxidative stress induced by adverse conditions.[25] For example, the oxidative stress tolerance of *Saccharomyces cerevisiae* is significantly enhanced after the introduction of the astaxanthin synthesis pathway.[26] Microorganisms have evolved effective antioxidant systems to cope with oxidative stress produced by basal metabolism. Microbial carotenoids may play a secondary protective role in preventing the cells from damage caused by excessive ROS produced in adverse conditions.[27] Besides, in plants, carotenoids can not only resist salt by quenching oxygen free radicals but also act as precursors of abscisic acid (ABA) and other phytohormones, thereby indirectly enhancing salt tolerance of plants.[28,29] However, the other functions of carotenoids produced by *R. glutinis* ZHK in response to salt stress are less clear, and further research is still needed.

3.2. Effect of Salt Stress on the Intracellular ROS, SOD, and CAT. Intracellular ROS levels are naturally generated during mitochondrial aerobic metabolism and maintain dynamic balance as cultured in normal conditions. However, upon exposure to salt stress, ROS homeostasis will be broken and, at the same time, the first antioxidant mechanism will be activated, such as antioxidant enzymes SOD and CAT.[30] In addition, increasing observations suggested that carotenoids also play important roles in coping with the generation of ROS. To access the relationship between carotenoid synthesis and antioxidant responses under salt stress, the *R. glutinis* ZHK strain was incubated with 0.75 M NaCl or without NaCl treatment for 120 h in the presence or absence of 40 μM DPA. After that, the changes of intracellular ROS, SOD, and CAT levels between different treatments were assayed. As shown in Figure 3, as compared to the control, 0.75 M NaCl treatment significantly increases the intracellular ROS, SOD, and CAT levels. Supplementation of 40 μM DPA resulted in the intracellular ROS, SOD, and CAT levels have a further increase than that without DPA treatment upon exposure to 0.75 M NaCl treatment. The ROS, SOD, or CAT levels are negatively correlated with carotenoid synthesis. These results indicated that carotenoids might play an important role in mitigating the antioxidant responses and

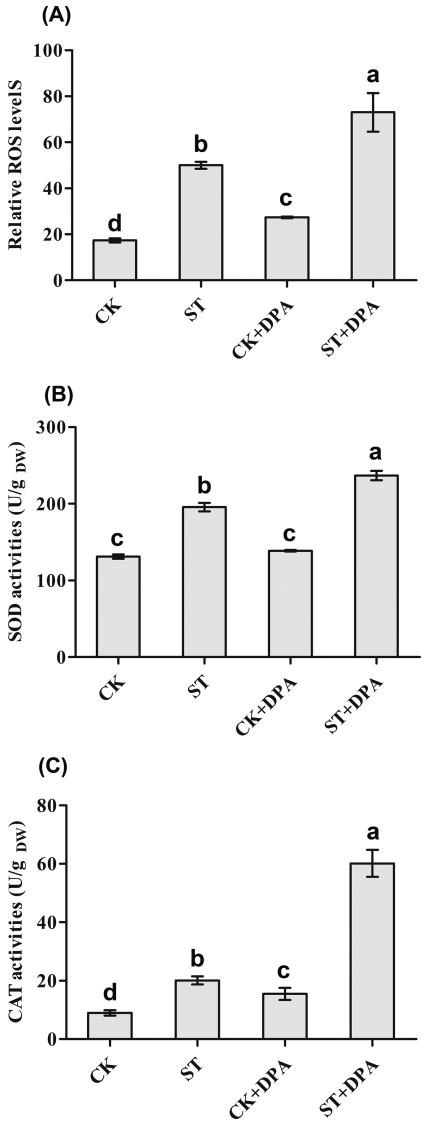

Figure 3. Effect of salt stress on intracellular ROS, SOD, and CAT levels of *R. glutinis*ZHK in the presence of 40 μM DPA (A–C). Determination of intracellular ROS, SOD, and CAT levels was performed after incubation of 120 h at 25 °C on a rotary shaker at 180 rpm under a NaCl concentration of 0.75 M (CK, 0 M NaCl + 0 M DPA; ST, 0.75 M NaCl + 0 M DPA; CK + DPA, 0 M NaCl + 40 μM DPA; ST + DPA, 0.75 M NaCl + 40 μM DPA). The data represent the means ± standard deviations of triplicate experiments. The different letters (a–d) indicate significant differences between treatments.

preventing the strains from oxidative damage induced by salt stress via quenching ROS.

This phenomenon was similar to several previous studies on plants, algae, and photosynthetic bacteria. For example, increasing the total carotenoid content through genetic manipulation can enhance salt tolerance of transgenic cultured cells of *Arabidopsis*,[31] sweetpotato,[32] and tobacco.[33] Furthermore, moderate salt stress can increase the carotenoid content in green alga *Dunaliella salina*[34] and photosynthetic bacteria *Rhodopseudomonas* sp.[35] Therefore, we proposed that carotenoid synthesis can enhance salt tolerance, and salt stress can also induce carotenoid synthesis. In addition, the chemical structure of a carotenoid decides its antioxidant capacity.[25]

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Upon exposure to salt stress, microorganisms probably prefer to increase the production and percentage of carotenoids with strong antioxidant capacity for better enhancing salt tolerance.

3.3. Effect of Salt Stress on the Carotenoid Contents.

*R. glutinis*ZHK mainly produces torulene, torularhodin, and β-carotene. In this study, the effect of salt stress on carotenoid contents (w/v; v, the volume of culture medium) of *R. glutinis* ZHK after 120 h of cultivation was evaluated by HPLC. As shown in Figure 4A, as compared to control (1.428 mg/L), the total carotenoids were significantly increased in 0.5 M (3.911 mg/L) and 0.75 M (5.412 mg/L) NaCl treatments and significantly decreased in 1.0 M (0.35 mg/L) NaCl treatment. As shown in Figure 4B, as compared to control (0.190 mg/mL), the torulene production of 0.5 M (1.096 mg/mL) and 0.75 M (2.284 mg/mL) NaCl treatments has a significant increase (476.84 and 1102.11%, respectively). Besides, there were no obvious change in torulene production between 1.0 M NaCl treatment (0.193 mg/mL) and control (0.190 mg/mL). Moreover, as compared to control (0.528 mg/mL), the torularhodin production of 0.5 M (1.445 mg/mL) and 0.75 M (1.421 mg/mL) NaCl treatments has a significant increase (173.67 and 169.13%, respectively). However, under 1 M NaCl treatment, the yield of torularhodin has been reduced to 0.015 mg/mL, which is remarkably lower than that of the control (0.528 mg/mL). As compared to other reported results, the torulene and torularhodin production in *R. glutinis*ZHK under moderate salt conditions is relatively high.[5,36] Furthermore, as compared to control (0.710 mg/mL), the β-carotene production of 0.5 M (1.371 mg/mL) and 0.75 M (1.707 mg/mL) NaCl treatments also has a significant increase (93.10 and 140.42%, respectively). Likewise, the yield of β-carotene also significantly decreased with treatment of 1 M NaCl (0.138 mg/mL) as compared to that of the control (0.710 mg/mL). Moreover, as shown in Figure 4C, the relative proportion of torulene in total carotenoids under 0.5 M (28.02%), 0.75 M (42.21%), and 1 M (55.87%) NaCl treatment also has a remarkable increase than the control (19.04%). However, the percentage of torularhodin gradually decreases when exposed to salt stress (0.5 M, 36.94; 0.75 M, 26.25; 1.0 M, 4.25%) as compared to the control (36.98%). Similarly, the relative proportion of β-carotene is also significantly decreased under 0.5 M (35.04%), 0.75 M (31.55%) and 1.0 M (39.88%) NaCl treatments as compared to the control (49.69%). Although the NaCl treatments of 0.5 and 0.75 M increase the production of β-carotene, they distinctly reduce its relative proportion in total carotenoids. Furthermore, upon exposure to salt stress, the yield of β-carotene is also relatively low as compared to other reports.[15] Therefore, salt stress treatment is not suitable for obtaining a high yield of β-carotene in *R. glutinis* ZHK. In summary, these results indicated that moderate salt stress (0.5 and 0.75 M) can significantly increase carotenoid production of *R. glutinis*ZHK, especially torulene and torularhodin.

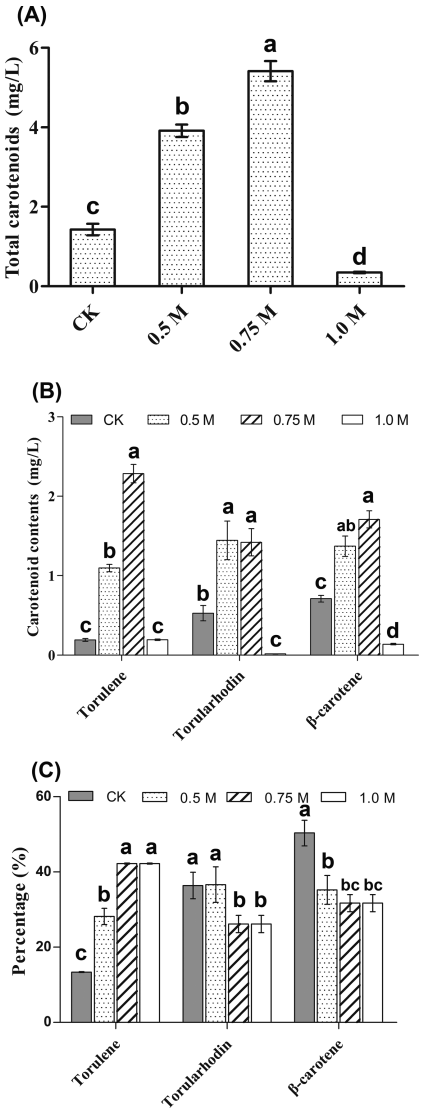

Figure 4. Effect of NaCl treatments on carotenoid contents of *R. glutinis* ZHK after 120 h of cultivation (A–C). Identification and quantitation of carotenoids were performed by HPLC after incubation of 120 h at 25 °C on a rotary shaker at 180 rpm under NaCl concentrations of 0, 0.5, 0.75, and 1.0 M, respectively. The data represent the means ± standard deviations of triplicate experiments. The different letters (a–d) indicate significant differences between treatments.

Previous studies have presented that the synthetic efficiency of torulene and torularhodin of *R. glutinis* is primarily affected by the factors of media components and cultivation conditions.[15] These factors mainly include the type of carbon sources, temperature, metal ions, and light. For instance, several studies reported that using glucose or concentrated grape juice as a carbon source can obtain relatively high yields of torulene and torularhodin in *R. glutinis*.[37,38] Meanwhile, high C/N ratios and nitrogen-limiting culture conditions are further conducive to this increase in torulene and torularhodin production. Moreover, a higher temperature (30–35 °C) is considered as an effective measure to improve the production and proportion of torulene and torularhodin in *R. glutinis*.[39] In addition, previous study mentioned that the torulene and torularhodin production of *R. glutinis* was significantly increased after adding 1.0 mM $NiSO_4$ to the medium as compared to the control.[40] The medium supplemented with 0.7 mM $Al_2(SO_4)_3$ leads to a specific enhancement in torulene production of *R. glutinis*.[41] Furthermore, the white light irradiated at 3500 lx can intensify the synthesis of torulene and torularhodin of *R. glutinis* from 79 and 292 to 142 and 322 $\mu g/g_{d.w.}$, respectively.[42] More importantly, the synthesis efficiency of torulene and torularhodin also depends to a large extent on the characteristics of the strain itself. Red yeasts

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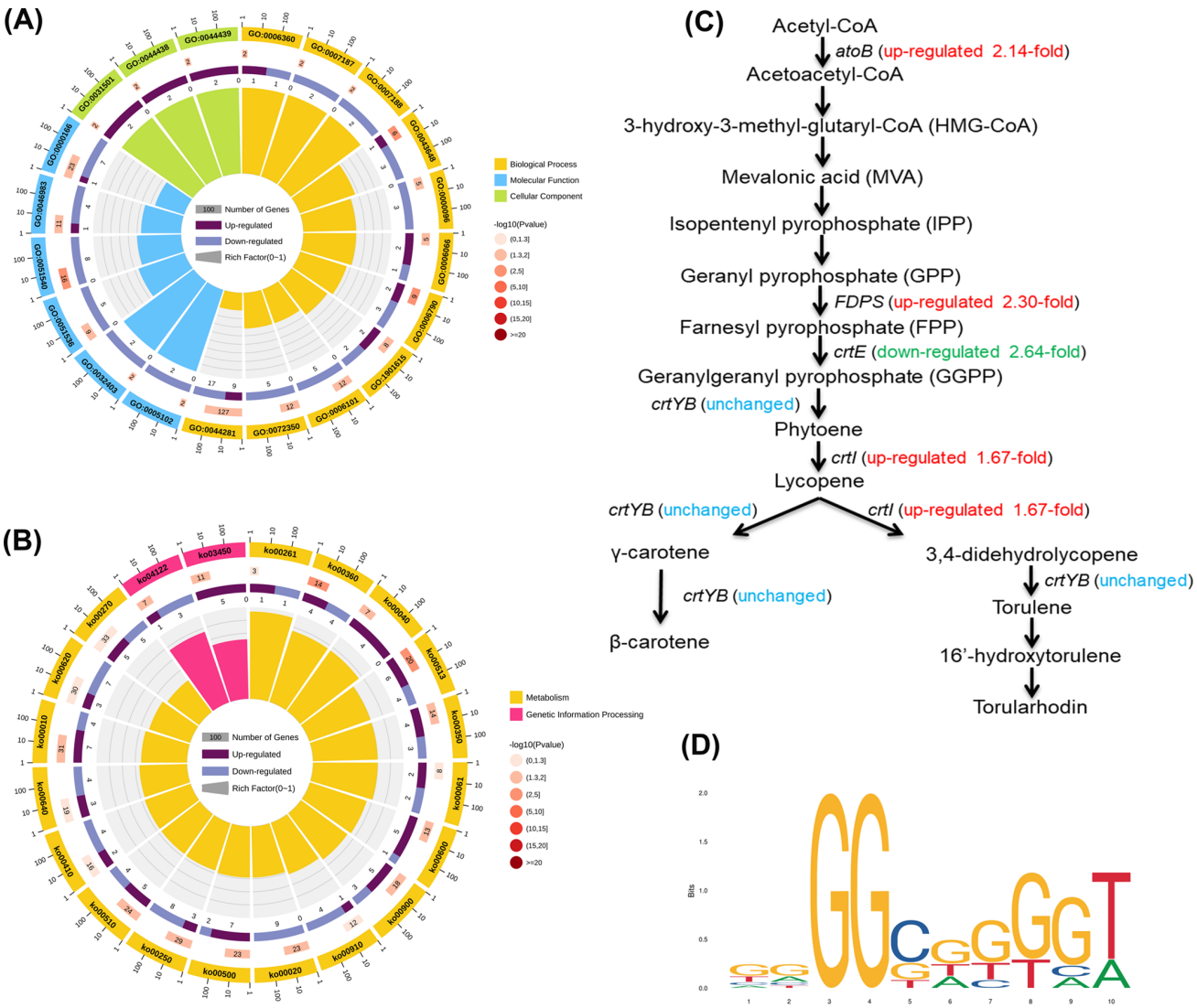

Figure 5. Transcriptome profiling of *R. glutinis* ZHK in response to salt stress. (A) Circos plots of GO enrichment analysis of DEGs. (B) Circos plots of KEGG enrichment analysis of DEGs. From the outer circle to the inner, the top 20 enriched GO terms or KEGG pathways (ring 1), numbers of background genes in the genome (ring 2), numbers of upregulated and downregulated genes (ring 3), and rich factor of DEGs in corresponding GO terms or KEGG pathways (ring 4) are represented. (C) Proposed carotenoids synthesis pathway and its related DEGs shown in italics at corresponding arrows.

of *Rhodotorula*, *Rhodosporidiobolus*, and *Sporobolomyces* genera are recognized as the most important producers of torulene and torularhodin.[5] Although the types of carotenoids they produce are roughly the same, there are still some differences in the yield and percentage of individual carotenoid fractions. Some of the environmental factors mentioned above that affect the synthesis efficiency of torulene and torularhodin also presented similar roles in other red yeasts of *Rhodosporidiobolus* and *Sporobolomyces* genera, especially the adverse conditions that cause oxidative stress.[18]

In the present study, we have proved that moderate salt exposure is an efficient technique to simultaneously improve torulene and torularhodin production of *R. glutinis*. Since torulene and torularhodin possess stronger antioxidant capacity than *β*-carotene.[6,43] As compared to *β*-carotene, torulene and torularhodin can better improve the salt tolerance of *R. glutinis* and thus might be prioritized to scavenging oxygen free radicals induced by salt stress. This seems to be the reason why the pathway of torulene and torularhodin production is favored over *β*-carotene production in response to salt conditions. It is worth noting that, as compared to 0.5 M (1.445 mg/L) NaCl treatment, although the production of torulene and *β*-carotene increased significantly under the treatment of 0.75 M, the yield of torularhodin has hardly changed (1.421 mg/L). It is proposed that the improvement of NaCl concentration from 0.5 to 0.75 M induces more ROS production, which further stimulates carotenogenesis for quenching these excrescent ROS. Among them, the enhanced production of torulene and *β*-carotene is more than the consumption, while the increased torularhodin might be almost the same as the consumption. Probably thanks to torularhodin that is endowed with a stronger antioxidant capacity than torulene and *β*-carotene,[44] which allows it prior to participating in ROS scavenging. In view of the fact that torulene and torularhodin have a variety of important biological activities and the absence of effective strategies to

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realize high yields, moderate salt treatment could be considered as a feasible and low-cost approach to simultaneously enhancing the synthesis efficiency of torulene and torularhodin of *R. glutinis* on an industrial scale.

3.4. Differentially Expressed Genes in Response to Salt Stress. To identify the differentially expressed genes (DEGs) in *R. glutinis* ZHK under salt stress of 0.75 M NaCl, we constructed six cDNA libraries for transcriptome sequencing using an Illumina NovaSeq 6000 sequencer. As a result, we obtained 41.73 Gb high-quality clean data after sequencing six cDNA libraries. DEGs were identified with the thresholds of $|\log_2 (\text{fold change})| \geq 1$ and $p_{adj} \leq 0.05$. As compared to the control, a total of 1829 genes were differentially expressed under salt stress, with 840 upregulated and 989 downregulated. These DEGs may play key roles in responding to salt stress and stimulating carotenogenesis, especially torulene and torularhodin.

To further understand the biological functions of these DEGs and the related biological processes they are involved in, a series of GO and KEGG enrichment analyses were performed, respectively. Regarding the GO analysis, DEGs under salt stress were enriched into 30 terms in the GO categories of the cellular component (CC), biological process (BP), and metabolic functions (MF). As shown in Figure 5A, within the CC category, the top three significantly enriched GO terms were mannosyltransferase complex (GO:0031501), microbody part (GO:0044438), and peroxisomal part (GO:0044439). Within the BP category, the top three significantly enriched GO terms were the sulfur compound metabolic process (GO:0006790), dicarboxylic acid metabolic process (GO:0043648), and organic hydroxy compound metabolic process (GO:1901615). Within the MF category, the top three significantly enriched GO terms were metal cluster binding (GO:0051540), iron-sulfur cluster binding (GO:0051536), and receptor binding (GO:0005102). Regarding the KEGG analysis, DEGs under salt stress were enriched in a total of 328 pathways. As shown in Figure 5B, the top 20 significantly enriched pathways were mainly associated with the pattern of "Metabolism" and "Genetic Information Processing" in KEGG level-1 and "Amino acid metabolism", "Biosynthesis of other secondary metabolites", "Carbohydrate metabolism", "Energy metabolism", "Folding, sorting, and degradation", "Glycan biosynthesis and metabolism", "Lipid metabolism", "Metabolism of other amino acids", "Metabolism of terpenoids and polyketides", and "Replication and repair" in KEGG level-2. Among them, the pathways related to salt tolerance are mainly assigned into amino acid metabolism and carbohydrate metabolism such as "Phenylalanine metabolism (ko00360)", "Tyrosine metabolism (ko00350)", "Alanine, aspartate and glutamate metabolism (ko00250)", "Cysteine and methionine metabolism (ko00270)", "Citrate cycle (TCA cycle) (ko00020)", "Starch and sucrose metabolism (ko00500)", "Glycolysis/Gluconeogenesis (ko00010)", and "Pentose and glucuronate interconversions (ko00040)". Similarly, the important roles of amino acid metabolism and carbohydrate metabolism in the process of adaptation to salt stress have been proved in tomato.[45] Moreover, salt-stress-responsive genes were significantly enriched into the categories of amino acid metabolism and carbohydrate metabolism in grapevine rootstock.[46] Under high salt conditions, amino acids could act as an osmoprotectant to re-establish osmotic homeostasis,[47] while carbohydrates might provide energy for the salt tolerance process or serve as precursors of other osmoprotective and antioxidant substances.[48]

In the present study, salt stress significantly enhances torulene and torularhodin production of *R. glutinis* ZHK. The changes of carotenoid contents are probably related to the expression of carotenogenic genes. Previous studies concluded that the synthetic pathway of carotenoids in *R. glutinis* includes three stages:[15] (1) mevalonate pathway (from acetyl-CoA to mevalonate-phrophosphate), (2) isoprene biosynthesis (from mevalonate-phrophosphate to geranylgeranyl pyrophosphate), and (3) carotenogenic pathway (from geranylgeranyl pyrophosphate to torulene, torularhodin, and β-carotene). Among them, geranylgeranyl pyrophosphate (GGPP) synthase CrtE catalyzes the conversion of isopentenyl pyrophosphate (IPP) and farnesyl pyrophosphate (FPP) to GGPP; bifunctional phytoene synthase/lycopene cyclase CrtYB condenses two molecules of GGPP to form phytoene; phytoene dehydrogenase CrtI catalyzes the bioconversion from phytoene to lycopene (fourth step product) and 3,4-didehydrolycopene (fifth step product). Later, the CrtYB cyclizes lycopene and 3,4-didehydrolycopene to form β-carotene and torulene, respectively. Torulene is then converted into torularhodin via intermediate 16′-hydroxytorulene.[49] Although the conversion of torulene to torularhodin involves hydroxylation and oxidation has been proposed, the related carotene hydroxylase and monooxygenase involved in this conversion still remain unclear.

Transcriptomic analysis showed that carotenogenic genes *atoB* (2.17-fold), farnesyl diphosphate synthase (*FDPS*) (2.30-fold), *crtE* (−2.64-fold), *crtI* (1.67-fold), and *crtYB* (−1.01-fold) were respectively upregulated, upregulated, downregulated, upregulated, and unchanged under salt stress (Figure 5C). In general, the upregulated expression of *atoB* and *FDPS* genes enhanced the metabolic flux of carotenoid synthesis. Additionally, the up-regulation of gene *crtI* might further promote the production of 3,4-didehydrolycopene, which could thus result in the enhancement of torulene and torularhodin production of *R. glutinis* ZHK. These upregulated genes *atoB*, *FDPS*, and *crtI* are probably caused by excessive ROS induced by salt stress. Furthermore, promoter analysis revealed that carotenogenic genes *atoB*, *FDPS*, and *crtI* harbor a common sequence motif (GCCCCKCCCCC) of transcription factor *SP1* in their promoters using the MEME online program (version 5.3.3) (Figure 5D).[50] Therefore, these carotenogenic genes may be regulated by the same transcription factor *SP1*, which may be the reason why these three genes are synchronously upregulated under salt stress. In addition, we found some upregulated candidate genes putatively encoding hydroxylase (*R. glutinis_G04456*, *R. glutinis_G04508*, and *R. glutinis_G01719*) and monooxygenase (*R. glutinis_G05690*, *R. glutinis_G00553*, and *R. glutinis_G05814*) by transcriptome analysis, which may be involved in the bioconversion process of torulene to torularhodin. These upregulated candidate genes might promote the bioconversion of torulene to torularhodin and therefore lead to an increase in the yield of torularhodin under salt stress. However, further function verification of these candidate genes is still needed. In short, our findings provide important clues for further improving the torulene and torularhodin productivity of *R. glutinis* ZHK through genetic engineering.

3.5. Differentially Accumulated Metabolites in Response to Salt Stress. To assess the overview of metabolic changes in response to salt stress, metabolomic profiling was

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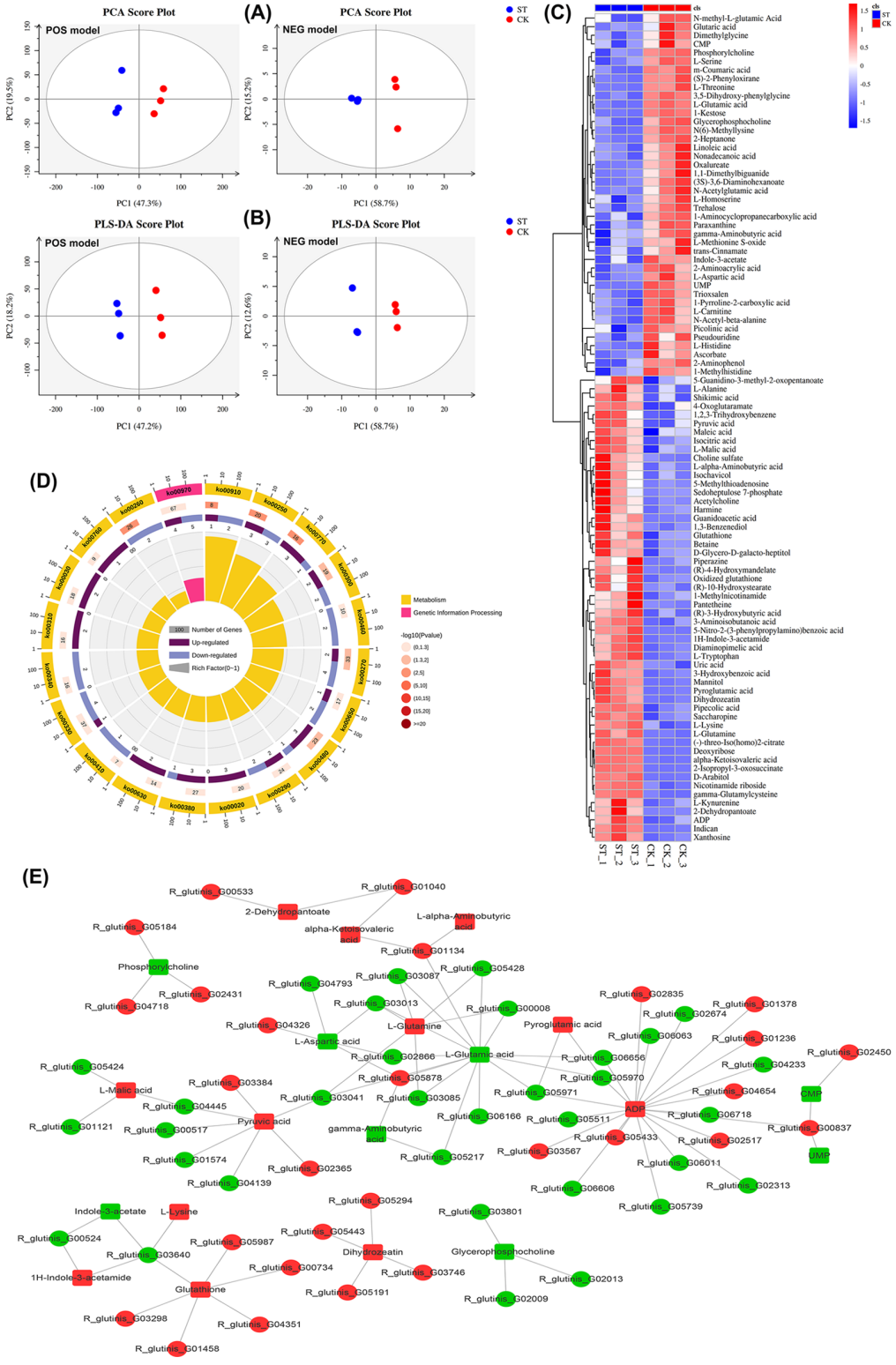

Figure 6. Metabolome profiling of *R. glutinis* ZHK in response to salt stress. (A) PCA plots between salt treatment (ST) and control (CK) groups. (B) PLS-DA plots between salt treatment (ST) and control (CK) groups. POS and NEG represent the positive and negative ion modes of mass spectrometry, respectively. (C) Hierarchical cluster and heatmap displaying the abundances levels of DAMs in all samples. (D) Circos plots of KEGG enrichment analysis of DAMs. From the outer circle to the inner, the top 20 enriched KEGG pathways (ring 1), numbers of background genes in the genome (ring 2), numbers of upregulated and downregulated genes (ring 3), and rich factor of DEGs in corresponding KEGG pathways (ring 4) are represented, respectively. (E) Integrated analysis of the transcriptome and metabolome. The correlation network was plotted using the related DEGs and DAMs with a threshold of Pearson correlation coefficient >0.99, *p*-value <0.01, and connectedness > 2.

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conducted using LC−MS/MS. Principal component analysis (PCA) showed an obvious tendency of separation among the groups of ST (salt treatment) and CK (Figure 6A). Moreover, PLS-DA models indicated that the ST and CK groups were separated from each other (Figure 6B). These permutation tests suggested its good model validation and accuracy and thus could be used for subsequent analysis. As a result, a total of 497 metabolites were annotated between all samples based on MS2 spectra database. Of which, 96 differentially accumulated metabolites (DAMs) were identified with the thresholds of VIP $\geq$ 1, fold change $\geq$1.5 or $\leq$ 0.667, and $p \leq 0.05$, including 54 upregulated and 42 downregulated metabolites (Figure 6C). To better understand the biological functions of these DAMs and the related biological process they are involved in, the KEGG enrichment analysis were conducted. As a result, DAMs under salt stress were enriched into a total of 34 KEGG pathways. As shown in Figure 6D, the top 20 significantly enriched pathways were mainly assigned into the pattern of metabolism and genetic information processing in KEGG level-1 and amino acid metabolism, carbohydrate metabolism, metabolism of other amino acids, metabolism of cofactors and vitamins, energy metabolism, and translation in KEGG level-2. Remarkably, the most significantly enriched KEGG pathways of DAMs in metabolome are highly consistent with the transcriptome, which are mainly assigned to amino acid metabolism and carbohydrate metabolism. These pathways are therefore important for the response to salt stress in *R. glutinis* ZHK.

Furthermore, to obtain an in-depth analysis of changes in *R. glutinis* ZHK under salt stress, an integrative analysis of the transcriptome and metabolome was conducted. As expected, the pathways involved in amino acid and carbohydrate metabolism were significantly enriched in the results of integrated analysis. Meanwhile, the network analysis was performed using the significantly enriched pathways with the Pearson correlation coefficient >0.99 and *p*-value <0.01. As shown in Figure 6E, network analysis indicated that there are 71 DEGs (29 upregulated and 42 downregulated) and 29 DAMs (17 upregulated and 12 downregulated) that are closely correlated in response to salt stress. Of which, the DEGs *R. glutinis_G05971*, *R. glutinis_G03041*, *R. glutinis_G05970*, *R. glutinis_G06656*, and *R. glutinis_G03640* as well as the DAMs adenosine 5′-diphosphate (ADP), L-glutamic acid, pyruvic acid, glutathione, dihydrozeatin, and L-glutamine might play a central regulatory role in response of salt stress. Moreover, in the present study, torulene, torularhodin, β-carotene, and their precursor substances were not detected due to the technical bottleneck of the current metabolomic databases. Furthermore, the putative carotenogenic genes were not significantly differentially expressed in response to salt stress, although the carotenoid contents have increased significantly. Consequently, the enhancement of carotenoid production might be act as the second defense line of *R. glutinis* ZHK against salt stress. These results present herein also provided insights into the tolerance mechanisms of *R. glutinis* ZHK in response to salt stress. More importantly, our findings lay a theoretical foundation for improving salt tolerance by genetic manipulation to further enhance the synthesis efficiency of torulene and torularhodin in *R. glutinis* ZHK.

3.6. Validation of RNA-Seq Data by qPCR. In this study, a total of three carotenogenic genes and seven DEGs were selected for qPCR. The results showed that the qPCR results were highly consistent with the transcriptome profiles. This result suggested that the RNA-seq data were reliable in this study.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c04028.

All of the chromatograms of HPLC analysis (Figures S1−S18) and effect of different concentrations of NaCl on carotenoid contents in *R. glutinis* ZHK (Table S1) (PDF)

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Author Contributions

C.L. conceived the study ideas, designed the experiments, analyzed the data, and wrote and revised this manuscript. Z.L., Y.X., Y.S., and D.Q. provided technical support in experimental preparation and data analysis. P.C. and G.Y. provided funding

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and supervised the overall written process of the paper. All authors have approved the final manuscript.

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Notes

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