Decreased expression of LRA4, a key gene involved in rhamnose metabolism, caused up-regulated expression of the genes in this pathway and autophagy in *Pichia pastoris*

Jian Jiao†, Shuai Wang†, Hui Tian, Xinxin Xu, Yuhong Zhang, Bo Liu* and Wei Zhang*

Abstract

In a previous study, we developed *Pichia pastoris* GS115m, an engineered strain with decreased expression of one key gene, LRA4, in rhamnose metabolism. *P. pastoris* GS115m/LacB was subsequently constructed via introducing a β-galactosidase gene, LacB, under the control of rhamnose-inducible *P* _LRA3_ into *P. pastoris* GS115m. *P. pastoris* GS115m/LacB greatly improved recombinant protein production relative to the parental strain (*P. pastoris* GS115/LacB). In the present study, transcriptomes of *P. pastoris* GS115m/LacB and *P. pastoris* GS115/LacB grown in YPR medium were analyzed. *P. pastoris* GS115m/LacB was found to suffer from the mild carbon source starvation. To attenuate the starvation stress, *P. pastoris* GS115m/LacB attempted to enhance rhamnose metabolism by elevating the transcription levels of rhamnose-utilization genes LRA1-3 and RhaR. The transcription level of LacB under the control of *P* _LRA3_ thereby increased, resulting in the improved production of recombinant protein in *P. pastoris* GS115m/LacB. It was also revealed that *P. pastoris* GS115m/LacB cells coped with carbon starvation mostly via autophagy.

Keywords: *Pichia pastoris*, Rhamnose metabolism, Transcriptome, Autophagy

Introduction

Because of its toxic and flammable properties, the use of methanol is dangerous during recombinant protein production. Hence, safe compound-inducible promoters are needed as alternatives to the methanol-inducible promoter *P* _AOX1_ in *Pichia pastoris* expression systems (Vogl and Glieder 2013). Several strong and inducible promoters, such as *P* _PHO89_ or *P* _THI411_, have been discovered to date (Ahn et al. 2009; Stadlmayr et al. 2010). Extensive efforts have been made in our laboratory to identify some other alternatives to *P* _AOX1_, and we have discovered several rhamnose utilization related genes (four enzyme-coding genes, LRA1–4, and one regulator-coding gene, RhaR).

Simultaneously, the promoters of LRA3 and LRA4, *P* _LRA3_ and *P* _LRA4_, were identified as two strong rhamnose-inducible promoters (Jiao et al. 2019; Liu et al. 2016). Subsequently, a *Pichia* expression system based on *P* _LRA3_ designated as the *P* _LRA3_ system, was developed using rhamnose as the inducer. However, the *P* _LRA3_ system did not produce recombinant proteins as efficiently as the *P* _AOX1_ system. To enhance recombinant protein production in the *P* _LRA3_ system, the engineered strain *P. pastoris* GS115m with decreased rhamnose metabolism flux was constructed via replacing the strong rhamnose-inducible promoter *P* _LRA4_ by another weak rhamnose-inducible promoter *P* _LRA2_. *P. pastoris* GS115m presented several different profiles compared to the parental strain *P. pastoris* GS115 as follows: (i) lower rhamnose utilization rate due to decreased expression of LRA4, (ii) reduced cell biomass and growth rate, and (iii) improved recombinant protein production (Yan et al. 2018). Additionally, the
engineered strain exhibited flocculation and rapid sedimentation at high cell densities (OD_{600} > 6) when it was grown in rhamnose-containing media, particularly YPR medium (Yan et al. 2018).

There are several possible explanations for the different physiological profiles of the engineered strain from the parental strain. Theoretically, down-regulating the expression of LRA4, which encodes a rate-limited enzyme involved in rhamnose metabolism, should reduce the utilization efficiency of rhamnose in the engineered strain cultured in rhamnose-containing media (e.g., YPR). In turn, this may result in mild carbon starvation stress due to insufficient rhamnose utilization. Subsequently, numerous physiological profiles including cell viability, autophagy and cell apoptosis, which were reported to be subject to carbon starvation stress (Oda et al. 2015; Weidberg et al. 2011; Wang et al. 2018b), would be altered in the engineered strain to adapt to the mild carbon starvation stress. To verify these speculations and shed light on the related responses, in this study we investigated the differences in transcriptomes and several physiological profiles between the engineered strain *P. pastoris* GS115m/*LacB* and the parental strain *P. pastoris* GS115/*LacB* during growth on rhamnose. According to the evidence, we elucidated the molecular mechanism for the improved production of recombinant protein in *P. pastoris* GS115m/*LacB*. Simultaneously, it was disclosed that *P. pastoris* GS115m/*LacB* coped with the mild carbon source starvation mostly via elevating autophagy level. The results would help to understand the survival mechanism responsible for starvation stress including but not limited to carbon source starvation and simultaneously provide a novel strategy for engineering strain to improve produce of target products.

**Materials and methods**

**Strains and medium**

*Pichia pastoris* GS115m was developed from *P. pastoris* GS115 by replacing the strong rhamnose-inducible promoter P_{LRA4} with the weak rhamnose-inducible promoter P_{LRA2}. In the *P. pastoris* GS115m/*LacB* and *P. pastoris* GS115m/*LacB* strains, the β-galactosidase coding gene *LacB* is under the control of the rhamnose-inducible promoter P_{LRA3}. All strains were described in detail in a previous study (Yan et al. 2018).

The YPD medium contained 1% yeast extract, 2% peptone, and 2% dextrose. The YPR medium contained 1% yeast extract, 2% peptone, and 2% rhamnose. The MR medium contained 1.34% yeast nitrogen base, 4 × 10^{-5} M biotin, and 2% rhamnose. To prepare solid medium, agar was supplemented into the above media to a final concentration of 2%.

**Production determination of recombinant protein**

Cultivation of *P. pastoris* GS115m/*LacB* or *P. pastoris* GS115m/*LacB* in YPR medium and analysis of β-galactosidase activities in the culture supernatants were carried out as previously described (Yan et al. 2018). The wet cell weight (WCW) per milliliter of culture was simultaneously determined at intervals. The β-galactosidase productivity per mg of WCW was assayed based on β-galactosidase activities in the culture supernatants, specific activity of β-galactosidase (575 U/mg), and WCW.

**Total RNA preparation for RNA-seq and real-time polymerase chain reaction (PCR)**

Each strain (*P. pastoris* GS115m/*LacB* and *P. pastoris* GS115m/*LacB*) was inoculated into YPR medium and grown until an OD_{600} of ~2 or ~6 was reached. The cells were collected using centrifugation (12,000 g) at 4 °C for 4 min and stored at −80 °C before total RNA extraction. Total RNA extraction and trace DNA removal were performed according to previously described methods (Liu et al. 2016). RNA concentration was determined with a Qubit® 2.0 fluorometer (Life Technologies, CA, USA), and the quality was checked using an Agilent RNA 6000 Nano kit combined with an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

**RNA sequencing and RNA-seq data analysis**

An RNA-seq library was constructed using the NEBNext Ultra™ RNA Library Prep kit for Illumina® (New England Biolabs, Ipswich, MA, USA) according to the manufacturer’s instructions. RNA library quality confirmation and RNA sequencing were carried out according to the process described by Wang et al. (2018a).

Clean reads were obtained from the raw data by removing low-quality bases (< Q20) using Trim_galore® (Bolger et al. 2014). The mapping of clean reads onto the reference genome of *P. pastoris* GS115 was performed using TopHat (v2.0.12) (Trapnell et al. 2009). The strand-specific and unique mapped reads were analyzed using HTSeq (v0.6.1) (Anders et al. 2015). Fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) was used to assay the abundance of each gene (Trapnell et al. 2010). Differentially expressed genes (DEGs) were identified using DEseq 2 with an FDR-adjusted *p* value of < 0.05 and the fold-change ≥ 2.

**Real-time PCR**

Real-time PCR assays were performed using previously described methods (Yan et al. 2018). The primers used for real-time PCR are listed in Additional file 1: Table S1. The relative expression level of each test gene in *P. pastoris* GS115m/*LacB* was determined by real-time PCR.
GS115/LacB was assigned as 1, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene. Each gene was analyzed in triplicate, and the data are represented as the means ± (SD) standard deviations.

**Cell viability assay**
Cells of *P. pastoris* GS115/LacB (WT) and *P. pastoris* GS115m/LacB (MT) were grown in liquid YPR medium to an OD_{600} of ~6. Cultures were diluted to an OD_{600} of ~1 and then washed. After serial dilution (1: 10), 5 μL of samples was spotted on YPR media and 10 μL of samples were spotted on MR media, respectively. The colonies were observed after incubation at 28 °C for 72 h.

**Cell apoptosis analysis**
The Annexin V-FITC/PI Cell Apoptosis Detection Kit (TransGen, Beijing, China) was used for Annexin V staining. Cultures of *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB were diluted to an OD_{600} of ~0.5 after grown in YPR medium to an OD_{600} of ~6. The cultures were washed twice with PBS. Subsequently, the cells were incubated in 100 μL of Annexin V binding buffer containing 5 μL of Annexin V-FITC and 5 μL of PI, followed by incubation at room temperature for 15 min. Later, the cells were washed once in 200 μL of Annexin V binding buffer and resuspended in 100 μL of Annexin V binding buffer. Cells were immediately visualized by laser scanning confocal microscope at a 488 nm excitation wavelength, and fluorescence intensities from ~20,000 cells were determined by flow cytometry (BD LSRFortessa).

**Detection of reactive oxygen species (ROS)**
Intracellular levels of ROS were measured with ROS Assay Kit (BioDee, Beijing, China). *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB were grown in YPR medium to an OD_{600} of ~6. The cells were diluted to OD_{600} ~0.5. Collected and washed the cells twice with PBS incubated 30 min at 30 °C with 30 μM of dihydrodorhamidine 123 (DH2R123). After centrifugation (800g, 5 min, 4 °C), the cells were washed with 1 mL of PBS and resuspended in 100 μL of PBS, and then were observed using laser scanning confocal microscope at a 488 nm excitation wavelength and an emission wavelength shifting from green (~525 nm), and fluorescence intensities from ~20,000 cells were monitored by flow cytometry.

**Results**
**Recombinant protein production in *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB**
In a previous study, growth rate and maximal biomass were lower for *P. pastoris* GS115m/LacB grown in YPR than for *P. pastoris* GS115/LacB. However, the amount of recombinant protein, β-galactosidase encoded by LacB, in the supernatants of *P. pastoris* GS115m/LacB cultures was higher than that of *P. pastoris* GS115/LacB (Yan et al. 2018). This indicated that *P. pastoris* GS115m/LacB produced the target protein more efficiently. To verify this result, the production of recombinant protein in the two strains grown in YPR medium was investigated based on protein production vs wet cell weight (WCW). The results revealed that *Pichia* cells efficiently produced target proteins from 12 to 36 h, and the recombinant protein production at 72 h was more than twofold higher in *P. pastoris* GS115m/LacB cell than in *P. pastoris* GS115/LacB (Fig. 1). The underlying mechanisms for the improved production of recombinant protein, which
could provide some important information for strain engineering, were further investigated.

**Global transcriptional profiles of *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB**

*Pichia pastoris* GS115/LacB and *P. pastoris* GS115m/LacB were cultured in YPR medium until an OD<sub>600</sub> of ~2 or ~6, respectively. The cells from two biological replicates of *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB were collected, and total RNA from these cells was prepared for transcriptome analysis using RNA-seq. The RNA-seq data were deposited in the CNGBdb (https://db.cngb.org) with Accession Number CNP0000622 and CNP0000710. The overall expression levels in the two biological replicates of each group were highly similar to each other (R² beyond 0.98) (Additional file 2: Fig. S1). At an OD<sub>600</sub> of ~2, a total of 749 DEGs including 281 down-regulated and 468 up-regulated genes were identified among 5,041 genes, including an exogenous gene, *LacB*. The large number of DEGs indicated that the decrease in rhamnose metabolic flux exerted wide-ranging effects on the transcriptomes of *Pichia* cells and thereby led to various changes in physiological profiles.

To further examine gene expression profiles, real-time PCR was performed to investigate the relative expression of four up-regulated DEGs (*PAS_chr4_0550, PAS_chr1-1_0356, PAS_chr3_0798*, and *PAS_chr4_0146*), four down-regulated DEGs (*PAS_chr3_0095, PAS_chr3_0403, PAS_chr4_0799*, and *PAS_chr3_0257*), one non-DEG (*PAS_chr3_0229*), and two genes of interest (*LRA4* and *LacB*). The trends in expression of the target genes were consistent between real-time PCR and RNA-seq despite the presence of minor differences in the expression levels of certain genes between the two methods (Fig. 2). The genes involved in rhamnose metabolism included five genes such as four enzyme-coding genes (*LRA1–4*) and a regulator-coding gene (*RhaR*). As expected, the expression levels of all the genes except for *LRA4* in *P. pastoris* GS115m/LacB were differentially up-regulated more than twofold compared with *P. pastoris*.

**Improved expression of LRA3 in *P. pastoris* GS115m/LacB**

Highly transcribed genes usually play crucial roles in organism survival, and their expression changes under different conditions. According to the FPKM value of each gene, the 25 most highly expressed genes in *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB were identified at an OD<sub>600</sub> of ~2 (Additional file 3: Table S2) and an OD<sub>600</sub> of ~6 (Additional file 4: Table S3), respectively. Theoretically, these genes are expected to play important roles in the survival of *Pichia* cells using rhamnose as the sole carbon source.

Notably, *LRA3* was also one of the 25 most highly expressed genes in both strains grown to OD<sub>600</sub>~2 and ~6, which suggested that *LRA3* expression was intensively induced in the presence of rhamnose. At OD<sub>600</sub> of ~2 and ~6, the *LRA3* transcription level was ranked 21st and 14th in *P. pastoris* GS115/LacB, and while it was ranked 2nd and 6th in *P. pastoris* GS115m/LacB, respectively. These results showed that *LRA3* expression level was significantly elevated in *P. pastoris* GS115m/LacB with rhamnose induction. In addition, it was surprising that the *LRA3* transcription level was even higher than that of GAPDH in *P. pastoris* GS115m/LacB.

**Transcription profiles of the genes related to rhamnose metabolism during incubation**

Rhamnose was the main carbon source for cell survival when *Pichia* cells were grown in YPR medium, and the rhamnose utilization rate was therefore a key factor affecting energy production, biomass biogenesis, and physiological profiles in *Pichia* cells. Down-regulating the expression of key rate-determining step enzymes such as *LRA4* would decrease rhamnose utilization efficiency, resulting in insufficient supply of energy and carbon matrices for primary and secondary metabolism, growth, and propagation. To adapt to these conditions, the strain should up-regulate the expression of rhamnose-utilization genes to accelerate rhamnose metabolism. This expectation was borne out by transcriptome analysis.

The genes involved in rhamnose metabolism included five genes such as four enzyme-coding genes (*LRA1–4*) and a regulator-coding gene (*RhaR*). As expected, the expression levels of all the genes except for *LRA4* in *P. pastoris* GS115m/LacB were differentially up-regulated more than twofold compared with *P. pastoris*.
GS115/LacB at an OD_{600} of ~2 (Fig. 3). Simultaneously, LRA4 maintained its low expression as it was under the control of the weak promoter P_{LRA2} (Fig. 3). These results indicated that the low production rate of the key rhamnose metabolism-related enzyme LRA4 led to inadequate production of energy and carbon matrices required for normal growth, and P. pastoris GS115m/LacB therefore enhanced the expression of rhamnose metabolism-related genes to increase rhamnose utilization to provide more energy and biomass for cell growth.

To further investigate the expression profiles of these genes during incubation, the genes were examined when the strains were grown to a high cell density (OD_{600} ~6). Relative expression of all genes, except for LRA4, decreased compared with that at an OD_{600} of ~2 (Fig. 3). When grown to OD_{600} ~12, the expression levels of these genes in P. pastoris GS115m/LacB were almost equal to those of P. pastoris GS115m/LacB (with the exception of LRA4), which was reported previously (Yan et al. 2018). This could be explained that the decreasing concentration of residual rhamnose in the medium made a declining induction to the related gene expression. Overall, the relative expression of genes other than LRA4 was dynamic; it was closely associated with the concentration of residual rhamnose in the medium and decreased with the consumption of rhamnose.

**LacB expression profiles during incubation**

Recombinant protein production, an important index for an expression system, was closely and positively dependent on the transcription activity of its promoter. In P. pastoris GS115m/LacB, LacB expression was controlled under P_{LRA3} and thereby the production of the recombinant protein, β-galactosidase encoded by LacB, was largely subject to the transcriptional activity of P_{LRA3}. As mentioned above, LRA3 was one of the most highly transcribed genes, and LRA3 expression was greatly enhanced in P. pastoris GS115m/LacB (Additional file 3: Table S2). Under the control of the same promoter, P_{LRA3}, the trend in LacB expression was consistent with that of LRA3 (Fig. 4). The production of recombinant protein in P. pastoris GS115m/LacB improved with the increase of P_{LRA3} transcription activity.

**Declined cell viability in P. pastoris GS115m/LacB grown on rhamnose**

Generally, rhamnose metabolism was down-regulated due to the decreased expression of the rate-limiting enzyme LRA4. Low rhamnose metabolism was accompanied by low energy supply and reduced sources of carbon-based biomass components. This resulted in decreased growth rate and declined cell biomass in P. pastoris GS115m/LacB grown in YPR medium, which was confirmed by the results of our previous study (Yan et al. 2018). Additionally, it was reported that cell viability was also affected by carbon starvation such as glucose shortage (Oda et al. 2015).

To confirm whether cell viability of P. pastoris GS115m/LacB altered, cell growth assay was performed. Differences in the number and size of cell colonies indicated the various profiles of cell viability and generation time of the tested strains, respectively. Small colonies as well as decreased number of cell colonies were observed in P. pastoris GS115m/LacB compared with P. pastoris GS115/LacB when rhamnose as the carbon source (YPR
and MR) (Fig. 5), which indicated prolonged propagation and declined viability in *P. pastoris* GS115m/LacB cells. This led to a lower biomass of *P. pastoris* GS115m/LacB grown in YPR and MR, which was described in another study (Yan et al. 2018).

**Increased autophagy level in *P. pastoris* GS115m/LacB during growth on rhamnose**

Autophagy is a principal catabolic pathway for degrading cellular components including organelles and dysfunctional proteins. Some nonessential cellular components can be degraded via autophagy to synthesize critical components (Devenish and Prescott 2015; Olsvik et al. 2019). Autophagy occurs at low levels under normal conditions (Huang et al. 2015) and increases under adverse conditions such as nutrient deficiency, hypoxia, and oxidative stress (Onodera and Ohsumi 2005; Scherz-Shouval and Elazar 2011; Shpilka et al. 2015; Weidberg et al. 2011). Similarly, autophagy might be induced by the insufficient carbon metabolism due to the decreased rhamnose utilization.

To investigate that autophagy could be triggered by the mild carbon starvation, the autophagy in *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB cells was monitored using autophagosomes staining. Intensive autophagy signals were detected in *P. pastoris* GS115m/LacB cells compared with *P. pastoris* GS115/LacB cells. Obviously, the carbon starvation arose from the decrease of rhamnose metabolism indeed caused autophagy in *P. pastoris* GS115m/LacB (Fig. 6). We assumed that *P. pastoris* GS115m/LacB cells recycled non-essential components to synthesize essential components for cell survival and reduce cell apoptosis via autophagy under carbon starvation.

**Undetectable effect on cell apoptosis due to decreased LRA4 expression**

Autophagy is interconnected with apoptosis because both of them might be triggered by same signals. Autophagy happened in *P. pastoris* GS115m/LacB, and apoptosis was thereby concerned. In order to understand whether apoptosis underwent in *P. pastoris* GS115m/LacB, apoptosis profiles in cells of *P. pastoris* GS115m/LacB and *P. pastoris* GS115/LacB was analyzed by flow cytometry using the Annexin V-FITC/PI apoptosis detection kit. Relatively low apoptosis level occurred in both kinds of cells, and no differences were observed in them (Fig. 7). It indicated that apoptosis did not obviously occur in cells of *P. pastoris* GS115m/LacB and *P. pastoris* GS115/LacB although the intensive autophagy occurred in *P. pastoris* GS115m/LacB. The results showed that the decreased rhamnose metabolism only led to a mild carbon source starvation, which was different from the carbon source starvation due to depletion of carbon source, and brought to a slight alteration of physiological state of *P. pastoris* GS115m/LacB instead of death such as apoptosis.

**Low level of reactive oxygen species (ROS) in *P. pastoris* GS115m/LacB**

ROS, which at a high level can induce apoptosis (Sullivan and Chandel 2014), was elevated in production when cells survived nutrient starvation such as inadequate supply of glucose (Wang et al. 2018b). Apoptosis did not occur in *P. pastoris* GS115m/LacB, indicating a low level of ROS in *P. pastoris* GS115m/LacB cells. The low levels of ROS in both kinds of cells were further confirmed by flow cytometry after DHR123 staining (Fig. 8).

---

**Fig. 5** The phenotypes of *P. pastoris* GS115/LacB and *P. pastoris* GS115m/LacB grown on YPR and MR. The two strains were grown in liquid YPR medium to an OD600 of ~6, and the cultures were diluted to an OD600 of ~1, and then serially diluted. After dilution with 10^3 and 10^4 times, 5 μL and 10 μL of the diluted cultures were spotted onto YPR and MR, respectively. The colonies were recorded after incubation at 28 °C for 72 h.
Rational metabolic engineering has been adopted to enhance the production of target products (Maervoet et al. 2016; Song and Lee 2015), such as deleting the bypass pathway or/and strengthening the precursor synthetic pathway (Fan et al. 2016). In the $P_{LRA3}$ system, recombinant proteins were the primary products, and production of recombinant proteins was directly related to the transcriptional activity of $P_{LRA3}$. $P_{LRA3}$ activity was positively correlated to two factors, rhamnose concentration and rhamnose induction duration. We considered that the increase in the two factors could be
realized via decreased metabolism, so the engineering strain was developed by down-regulating the expression of one of key rhamnose utilization related genes, LRA4. As expected, the engineering strains presented the expected profile, increasing the production of target proteins. However, the actual mechanism on the improved production was not elucidated in detail.

In this study, \( P_{LRA3} \) activity in \( P. \) pastoris GS115m/LacB as well as \( P. \) pastoris GS115/LacB was disclosed by determining the transcriptional level of \( LRA3 \) in the transcriptome. \( P_{LRA3} \) was found to be one of the strongest promoters in the presence of rhamnose, and this indicated that recombinant proteins would be highly expressed under the control of \( P_{LRA3} \). Simultaneously, it was noted that \( P_{LRA3} \) activity was lower than that of \( P_{GAP} \) in the parental strain while higher in the engineering strain, suggesting an improved transcriptional level of \( P_{LRA3} \) in the engineering strain. Transcriptional level of recombinant protein gene, LacB, under the control of \( P_{LRA3} \) was thereby enhanced in the engineering strain, leading to an improved production of LacB. Totally, the elevated \( P_{LRA3} \) activity directly contributed to the improved production of recombinant proteins.

Rhamnose utilization efficiency in the engineering strain was confirmed to decrease in our previous study. The decreased rhamnose metabolism caused carbon starvation to some extent, and the host would alter some physiological profiles to adapt this kind of stress although this stress was different from the carbon starvation stress arose from glucose depletion. To present, numerous studies have been carried out to investigate carbon starvation due to carbon depletion (Adachi et al. 2017; Marshall and Vierstra 2018; Schwarz et al. 2017), and few reporters have focused specifically on low metabolic flux.

Down-regulated expression of LRA4 exposed \( P. \) pastoris GS115m/LacB to a slight carbon starvation stress. As a rescue strategy, \( P. \) pastoris GS115m/LacB induced the transcription of rhamnose-utilization genes to increase rhamnose metabolism to attenuate the starvation. In addition, serious carbon starvation usually brought to some changes of physiological profiles, such as ROS, autophagy, and even apoptosis. Autophagy can block apoptosis to maintain cell survival or induce apoptosis to result in cell death, relying on the nutrient situation. The mild starvation due to insufficient utilization of rhamnose seemed no serious damages to the \( P. \) pastoris GS115m/LacB cells because only obvious autophagy instead of apoptosis occurred. \( P. \) pastoris GS115m/LacB reused some unessential components via autophagy to keep cell viability for survival.

Comprehensively, these findings provided insight into the adaptation mechanisms of microbes under insufficient carbon utilization and some strategies for engineering strain.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13568-020-00971-2.

**Additional file 1: Table S1.** Primers used for real-time PCR.

**Additional file 2: Figure S1.** Comparison of gene expression between two biological replicates in RNA-seq analysis.

**Additional file 3: Table S2.** The 25 most highly expressed genes in \( P. \) pastoris GS115/LacB and \( P. \) pastoris GS115m/LacB (OD \( 600 \sim 2 \)).
Acknowledgements
Not applicable.

Authors’ contributions
BL and WZ designed the study. JJ, SW, HT, XX and YZ performed experiments; JJ, SW, BL and WZ drafted the manuscript. All authors reviewed the results and participated in the writing of the manuscript. All authors read and approved the final manuscript.

Funding
This study was funded by the National Natural Science Foundation of China (Grant number: 31671802), National Transgenic Major Program (2019ZX08010-004) and Central Public-interest Scientific Institution Basal Research Fund (No. Y2019XX19).

Availability of data and materials
The RNA-seq data were deposited in the CNGBdb (https://db.cngb.org) with accession number CNP0000622 and CNP0000710. The authors declare that all data supporting the findings of this study are available from the corresponding authors upon request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that have no competing interests.

Received: 6 November 2019 Accepted: 11 February 2020
Published online: 25 February 2020

References
Adachi A, Koizumi M, Ohsumi Y (2017) Autophagy induction under carbon starvation conditions is negatively regulated by carbon catabolite repression. J Biol Chem 292(48):19905–19918
Ahn J, Hong J, Park M, Lee H, Lee E, Kim C, Lee J, Choi ES, Jung JK, Lee H (2009) Phosphate-responsive promoter of a Pichia pastoris sodium phosphate symporter. Appl Environ Microb 75(11):3526–3534
Anders S, Pyl PT, Huber W (2015) HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics 31(2):166–169
Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics 30(15):2114–2120
Devenish RJ, Prescott M (2015) Autophagy: starvation relieves transcriptional repression of ATG genes. Curr Biol 25(6):R238–R240
Fan Y, Hu T, Wei L, Bai L, Hua Q (2016) Effects of modulation of pentose-phosphate pathway on biosynthesis of ansamycins in Actinosynnema pretiosum. J Biotechnol 230:3–10
Huang HH, Kawamata T, Hirata R, Tsugawa H, Nakayama Y, Ohsumi Y, Fukusaki E (2015) Bulk RNA degradation by nitrogen starvation-induced autophagy in yeast. EMBO J 34(2):154–168
Jiao J, Wang S, Liang ML, Zhang YH, Xu XX, Zhang W, Liu B (2019) Basal transcription profiles of the rhamnose-inducible promoter P_rha and the development of efficient P_rha-based systems for markerless gene deletion and a mutant library in Pichia pastoris. Curr Genet 65(3):785–798
Liu B, Zhang YW, Zhang X, Yan CL, Zhang YH, Xu XX, Zhang W (2016) Discovery of a rhamnose utilization pathway and rhamnose-inducible promoters in Pichia pastoris. Sci Rep. 6:27352
Maenovet VET, De Maeseneire SL, Arci FG, Beauprez J, Soetaert WK, De Mey M (2016) High yield 1,3-propanediol production by rational engineering of the 3-hydroxypropionaldehyde bottleneck in Citrobacter wuilliamii. Microb Cell Fact 15:23
Marshall RS, Vierstra RD (2018) Proteasome storage granules protect proteasomes from autophagic degradation upon carbon starvation. Elife 7:e34532
Oda A, Takamata N, Hirata Y, Miyoshi T, Suzuki Y, Sugano S, Ohta K (2015) Dynamic transition of transcription and chromatin landscape during fitness yeast adaptation to glucose starvation. Genes Cells 20(5):392–407
Olsvik H, Svennving S, Abudu YP, Brech A, Stenmark H, Johansen T, Meijling J (2019) Endosomal microautophagy is an integrated part of the autophagic response to amino acid starvation. Autophagy 15(1):182–183
Onodera J, Ohsumi Y (2005) Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. J Biol Chem 280(36):31582–31586
Schierz-Shouval R, Elazar Z (2011) Regulation of autophagy by ROS: physiology and pathology. Trends Biochem Sci 36(1):30–38
Schwarz V, Andosch A, Geretschläger A, Affenzeller M, Lutz-Meindl U (2017) Carbon starvation induces lipid degradation via autophagy in the model alga Micrasterias. J Plant Physiol 208:115–127
Shpilek T, Welter E, Borovsky N, Amar N, Shimron F, Peleg Y, Elazar Z (2015) Fatty acid synthase is preferentially degraded by autophagy upon nitrogen starvation in yeast. Proc Natl Acad Sci USA 112(5):1434–1439
Song CW, Lee SY (2015) Combining rational metabolic engineering and flux optimization strategies for efficient production of fumaric acid. Appl Microbiol Biotechnol 99(20):8455–8464
Stadlmayr G, Mecklenbrauker A, Rothmuller M, Maurer M, Sauer M, Mattoon J, Gasser D, Gasser B (2010) Identification and characterisation of novel Pichia pastoris promoters for heterologous protein production. J Biotechnol 150(4):519–529
Sullivan LB, Chandel NS (2014) Mitochondrial reactive oxygen species and cancer. Cancer Metab 2:17
Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25(9):1105–1111
Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28(5):511–515
Vogl T, Glieder A (2013) Regulation of Pichia pastoris promoters and its consequences for protein production. New Biotechnol 30(4):385–404
Wang GY, Li DL, Miao ZG, Zhang SS, Liang W, Liu L (2018a) Comparative transcriptome analysis reveals multiple functions for Mfh1p1 in lipid biosynthesis in the oleaginous yeast Yarrowia lipolytica. Biochim Biophys Acta Mol Cell Biol Lipids 1863(1):81–90
Wang L, Minchin RF, Butcher NJ (2018b) Aramid N-acetyltransferase 1 protects against reactive oxygen species during glucose starvation: role in the regulation of p53 stability. PLoS ONE 13(3):e0193560
Weidberg H, Shvets E, Elazar Z (2011) Biogenesis and cargo selectivity of autophagosomes. Annu Rev Biochem 80:125–156
Yan CL, Xu XX, Zhang X, Zhang YW, Zhang YH, Zhang ZF, Zhang W, Liu B (2018) Decreased rhamnose metabolic flux improved production of target proteins and cell flocculation in Pichia pastoris. Front Microbiol 9:1771

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.