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Tolerance response and metabolism of acetic acid by biodetoxification fungus *Amorphotheca resinae* ZN1

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ABSTRACT

Removal of acetic acid from pretreated lignocellulose biomass is an important step for the consequent fermentation on production of cellulosic ethanol and biobased chemicals. This study elucidates the biological metabolism and tolerance response of acetic acid by a widely used biodetoxification fungus *Amorphotheca resinae* ZN1. Acetic acid is consumed as a prior substrate to glucose and xylose by *A. resinae* ZN1, and the consumption is highly accelerated by solid state culture. Acetic acid is metabolized through the tricarboxylic acid (TCA) cycle when glucose exists in the medium, while through the two cycles of both the TCA cycle and glyoxylate cycle when there is no sugar in the medium. The tolerance response of *A. resinae* ZN1 to acetic acid includes various biological processes such as activation of ions transport, increase in amino acids uptake and biosynthesis, as well as induction of ergosterol biosynthesis and ATP generation. The study provided important basis for the future biodetoxification strain modification for enhanced acetic acid removal.

1. Introduction

Pretreatment is the essential step to overcome the biorecalcitrance of lignocellulose biomass for releasing fermentable sugars in the consequent enzymatic hydrolysis step (Yang and Wyman, 2008). During this harsh process, various inhibitor components are generated including furan aldehydes, phenolic compounds, and weak organic acids (Palmqvist and Hahn-Hägerdal, 2000). Acetic acid is derived from the de-acetylation of hemicellulose and may accumulate as high as 2–3% of the dry biomass weight. Cellulase activity is reduced by 10% at the existence of 1 g/L acetic acid (Kothari and Lee, 2011), which leads to the cell death of *Saccharomyces cerevisiae* at pH 3.0 (Ludovico et al., 2001). Cell growth and ethanol generation of *Pichia stipites* are completely ceased by 3.5 g/L acetic acid (Bellido et al., 2011). Xylose utilization of the engineered *S. cerevisiae* (Helle et al., 2003) and *Zymomonas mobilis* (Yang et al., 2014) is also significantly suppressed by the low level of acetic acid. It is crucially important to efficiently remove acetic acid from the pretreated lignocellulose feedstock for achieving the high fermentation yield of ethanol.

Biological metabolism of acetic acid by microorganisms is the most promising way by its removal with less water usage and low energy input (Dong and Bao, 2010; Parawira and Tekere, 2011). Acetic acid assimilation has been demonstrated in various yeasts, fungi, and bacteria including *S. cerevisiae* (Schneider, 1996), *Zygosaccharomyces bailii* (Rodrigues et al., 2012), *Schizosaccharomyces pombe* (Tsai et al., 1989), *Issatchenkia orienalis* (Zhang et al., 2009), *Issatchenkia occidentalis* (Fonseca et al., 2011), *Amorphotheca resinae* ZN1 (Zhang et al., 2010), *Acinetobacter baylyi* ADP1 (Kannisto et al., 2015), *Escherichia coli* (Lakshmanaswamy et al., 2011; Wolfe, 2005), *Bacillus subtilis* (Grundy et al., 1994), and *Corynebacterium glutamicum* (Wendisch et al., 2000). The biodetoxification fungus *A. resinae* ZN1 is able to ultimately degrade various inhibitor compounds including high level of acetic acid from pretreated lignocellulose feedstock and has been applied for production of ethanol (Liu et al., 2017), lactic acid (Yi et al., 2016), gluconic acid (Zhang et al., 2016), and microbial lipid (Wang et al., 2016) with high product yield and zero waste water generation. The uniqueness of *A. resinae* ZN1 include the high metabolism rate of acetic acid, consuming acetic acid prior to fermentable sugars, and metabolizing acetic acid in the solid state culture. Understanding the acetic acid metabolism behaviors of *A. resinae* ZN1 at the metabolic and genetic levels will help for improved detoxification efficiency on acetic acid.

This study examined the acetic acid metabolism behaviors and the tolerance response (acetic acid stress response of the global

Abbreviations: RNA-Seq, high-throughput mRNA sequencing; qRT-PCR, real-time quantitative polymerase chain reaction; GO, gene ontology; TCA cycle, tricarboxylic acid cycle; ACS, acetyl-CoA synthetase; ACH, acetyl-CoA hydrolases; CS, citrate synthase; IDH, isocitrate dehydrogenase; LSC, succinyl-CoA ligase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; ICL, isocitrate lyase; MS, malate synthetase; ATP, adenosine triphosphate

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transcriptome) of *A. resinae* ZN1 by high-throughput mRNA sequencing (RNA-Seq) and real-time quantitative PCR (qRT-PCR) under acetic acid stress. The acetic acid metabolism pathway was proposed and the important genes responsible for acetic acid tolerance in *A. resinae* ZN1 were identified as the gene library for developing high acetic acid tolerant strains.

2. Materials and methods

2.1. Raw materials and reagents

Corn stover was harvested in fall 2012 from Dancheng, Henan, China. After collection, the corn stover was milled coarsely and washed to remove field dirt, stones and metals, then dried to constant weight. The dried corn stover was milled again into fine materials and sealed in plastic bags for future pretreatment. The prepared corn stover was dry acid pretreated (He et al., 2016; Zhang et al., 2011). Briefly, 1200 g of dry corn stover and 600 g of 5% (w/w) diluted sulfuric acid were co-currently fed into a 20 L pretreatment reactor under the mild helical agitation at 175 °C for 5 min.

Ceramic fiber and basalt fiber were purchased from Tianlun Fiber Co., Yangzhou, China. The two materials were washed to remove dirt, then dried to constant weight for future use. Acetic acid was purchased from Sinopharm Chemical Reagent Co., Shanghai, China. Glucose, xylose and other chemical reagents used in this study were obtained from Shanghai Lingfeng Reagents Co., Shanghai, China.

2.2. Strains and culture

The biodetoxification strain *A. resinae* ZN1 was isolated from the pretreated corn stover (Zhang et al., 2010) and stored in China General Microbiological Culture Collection Center, Beijing, China under the registration number of CGMCC 7452. The culture media used in this study include: (1) Potato dextrose agar (PDA): potato extract juice, 200 g/L; glucose, 20 g/L; agar, 15 g/L. (2) Seed culture medium: KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 1 g/L; $MgSO_4{\cdot}7H_2O$, 1 g/L; $CaCl_2$, 0.5 g/L; yeast extract, 1 g/L; glucose, 20 g/L. (3) Pre-culture medium: KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 1 g/L; $MgSO_4{\cdot}7H_2O$, 1 g/L; $CaCl_2$, 0.5 g/L; yeast extract, 1 g/L; glucose, 5 g/L. (4) Inorganic salt medium: KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 1 g/L; $MgSO_4{\cdot}7H_2O$, 1 g/L; $CaCl_2$, 0.5 g/L. (5) Fermentation medium: KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 1 g/L; $MgSO_4{\cdot}7H_2O$, 1 g/L; $CaCl_2$, 0.5 g/L; yeast extract, 1 g/L; and different sugars including 5 g/L glucose, 20 g/L glucose, 5 g/L xylose, 20 g/L xylose, a mixture of 5 g/L glucose and 15 g/L xylose, a mixture of 10 g/L glucose and 10 g/L xylose, and a mixture of 15 g/L glucose and 5 g/L xylose, respectively.

A. resinae ZN1 spores were initially cultured on PDA slant at 28 °C for 4 days. Then the spores were washed with the sterile deionized water and 20 mL of the washed spore suspension (4–5 × 10^6 spores/mL) was inoculated into 200 mL seed culture medium for 4 days at 28 °C for preparation of seeds. The cell mass was harvested, washed with sterile deionized water twice to remove remaining glucose, re-suspended in 200 mL sterile deionized water, and then dispersed and filtered to prepare the seed inoculum used for no sugar addition. The seed inoculum used for sugar addition was prepared by directly dispersing and filtering the 4 days cultured cell mass. For acetic acid metabolism by *A. resinae* ZN1 in submerged liquid culture, 5 mL of seed inoculum was inoculated into 250 mL Erlenmeyer flasks containing 50 mL of liquid medium supplied with 4 g/L of acetic acid and different glucose and xylose. For acetic acid metabolism by *A. resinae* ZN1 on the solid carrier, seed inoculum was inoculated into the liquid medium containing 4 g/L of acetic acid and 5 g/L of glucose with a 10% (v/v) inoculum size. Then, the inoculated mixture was added into the sterilized dry solid material in 250 mL Erlenmeyer flasks to make the liquid weight percentage ranging at 50%, 60%, 70%, 80%, 85%, 90% and 95% (w/w) respectively. The inoculated cultures without solid carrier materials were used as the liquid culture controls. All of the inoculated flasks were cultured statically in the incubator at 28 °C, and the whole flask of the culture samples were withdrew after culture for 192 h for the liquid culture or 24 h for the solid state culture to analyze the acetic acid, glucose and xylose consumption.

2.3. HPLC analysis

Acetic acid, glucose and xylose were analyzed by HPLC equipped with a refractive index detector RID-10A (Shimadzu, Kyoto, Japan) and Bio-rad Aminex HPX-87H column (Bio-rad, Hercules, CA, USA). The column temperature was controlled at 65 °C and 5 mM H_2SO_4 solution was used as the flow phase with a flow rate of 0.6 mL/min.

2.4. Samples collection

When glucose was added, *A. resinae* ZN1 seeds were inoculated into the pre-culture medium with 5 g/L glucose, pre-cultured at 28 °C for two days. Then 2 g/L of acetic acid was added and the initial pH of the culture medium was neutralized to pH 5.0 using 5 M NaOH. The cultures without acetic acid addition were served as the controls. After culture for 24 h, the cell mass was collected by centrifuging at 4 °C and 13,400 g, for 10 min, then washed for three times by sterile deionized water and quickly frozen in liquid nitrogen before stored at -80 °C.

When no sugar was added, *A. resinae* ZN1 seeds were inoculated into the pre-culture medium with 5 g/L glucose, pre-cultured at 28 °C for two days. The whole flask of the cell hyphae was harvested by centrifuging at 4 °C and 2300 g, for 5 min, washed by sterile deionized water to remove the remaining glucose, then transferred to the same volume of fresh inorganic salt medium containing 2 g/L of acetic acid (pH 5.0 controlled by 5 M NaOH) or fermentation medium containing only 5 g/L of glucose, respectively. The cells grown in 5 g/L of glucose were served as the controls. The cell mass was collected after culture for 24 h.

2.5. RNA extraction and RNA-Seq assay

Total RNA was extracted according to the protocol of Trizol reagent (RNAiso Plus, Takara, Otsu, Japan), and the RNA quality was checked by the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA).

RNA-Seq was performed by Novel Bioinformatics Co., Ltd, Shanghai, China. Briefly, after purification of RNA by Dynabeads mRNA Purification Kit (Life Technologies, USA), the cDNA library was prepared using Ion Total RNA-Seq Kit v2.0 (Life Technologies, USA) according to the manufacturer's protocol. The template-positive Ion PI™ Ion Sphere™ Particles were prepared according to Ion PI™ Template OT2 200 Kit v2.0 (Life Technologies, USA), enriched and loaded on the 1 P1v2 Proton Chip (Life Technologies, USA), and then sequenced on Proton Sequencers according to Ion PI Sequencing 200 Kit v2.0 (Life Technologies, USA).

The clean reads were obtained after the raw data filtering, then were mapped to the genome of *A. resinae* ZN1 (GenBank accession no. JZSE00000000) (Wang et al., 2015) using the MapSplice program (v 2.1.6). The gene expression level was normalized by Reads Per Kilobases per Million mapped Reads (RPKM) quantification and Upper-quartile correction. Fold change (FC) of the differentially expressed gene (DEG) was obtained based on the normalized gene expression level between the acetic acid treatment and control samples. P-Value was calculated by DEG algorithm package using negative binomial distribution. False discovery rate (FDR) analysis was performed by Benjamini-Hochberg (BH) algorithm utilizing multiple hypothesis testing to adjust P-Value and reduce the false positive rate of the result. The differentially expressed genes were screened using DEseq algorithm with the criteria of FC $\geq$ 2.0 or $\leq$ 0.5 and the FDR $<$ 0.05.

The main function of the differential expression genes was analyzed according to the Gene Ontology (GO) (http://geneontology.org/),

which is the key functional classification of NCBI (http://www.ncbi.nlm.nih.gov/) (Ashburner et al., 2000). Fisher's exact test and χ^2 test were used to classify the GO category and calculate the P-value of the GO terms. The significant GO term was defined as P-value < 0.05.

The pathway of the differential expression genes was identified according to the pathway annotations using KEGG (http://www.genome.jp/kegg/) (Draghici et al., 2007). Fisher's exact test was used to select the significant pathway. The significant enrichment pathway was defined as P-value < 0.05.

2.6. qRT-PCR assay

cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan). The qRT-PCR amplification was prepared with SYBR Green Realtime PCR Master Mix kit (Toyobo, Osaka, Japan) and performed on a BioRad CFX 96 system (BioRad, Hercules, CA, USA) with the procedure as follows: 95 °C for 1 min, 40 cycles at 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s, and a final melting curve step by heating from 65 to 95 °C with a speed of 0.5 °C per 5 s. Primers used are shown in Tables S1 and S2. β-actin gene *ARZ_9569_T1* was selected as an internal control to normalize the difference of the data. The formula $2^{-\Delta\Delta Ct}$ method was used to quantify the expression levels of these tested genes. The significantly up-regulated or down-regulated genes were defined by the criteria of FC ≥ 2.0 or ≤ 0.5, respectively. Two and three biological replicates were run for the RNA-Seq and qRT-PCR analysis, respectively.

3. Results and discussion

3.1. Acetic acid metabolism performance by A. resinae ZN1

Acetic acid metabolism by *A. resinae* ZN1 was conducted in the submerged liquid culture with varying glucose and xylose (Fig. 1, Table S3). When acetic acid was the sole carbon source, 85.5% of the initial 4 g/L acetic acid was consumed within 192 h culture. When 5 g/L glucose and 5 g/L xylose was added to the medium, 75.2% and 90.5% of acetic acid was consumed while 24.2% of glucose and 25.0% of xylose was consumed, respectively. When the sugar concentration increased to 20 g/L glucose and 20 g/L xylose, acetic acid consumption was 56.3% and 98.1% with no glucose consumption and only 1.7% xylose consumption, respectively. When the 20 g/L of the mixed glucose and xylose was added, 78.8%–93.7% of acetic acid was consumed while the maximum sugar consumption was only 10.2%. These results clearly showed that acetic acid was consumed prior to glucose and xylose by *A. resinae* ZN1. The unique acetic acid metabolic performance of *A. resinae* ZN1 was similar to that of the two furan aldehydes of furfural and 5-hydroxymethylfurfural, which were also demonstrated to be prior to glucose as the substrates for *A. resinae* ZN1 (Wang et al., 2015). This property preserved glucose and xylose in the period of biodetoxification for the consequent fermentation use. The main reason is probably due to the inhibition of acetic acid on uptake or metabolism of glucose and xylose. Further RNA-Seq global transcriptome profiling analysis reveals that transcription of multiple genes responsible for sugar uptake and metabolism are significantly inhibited by acetic acid, including the *SNF3*, *RGT2*, *RGT2* and *HXT3* genes involving glucose and xylsoe uptake, as well as the *GND1*, *PDC1*, *GDB1*, *PMI40*, *SOR1*, *ATH1* and *IMA1* genes involving glycolysis and sugars metabolism (File S1). On the other hand, 90.4% of glucose, 66.0% of xylose, 9.3% of glucose, 19.9% of xylose were consumed when cultured in the media containing only 5 g/L of glucose, 5 g/L of xylose, 20 g/L of glucose and 20 g/L of xylose respectively, comparing to 24.2%, 25.0%, 0.0% and 1.7% with 4 g/L of acetic acid addition (Table S3), further indicating that the sugar consumption by *A. resinae* ZN1 was inhibited by the presence of acetic acid in the culture medium.

Acetic acid metabolism by *A. resinae* ZN1 was further examined in the solid state culture by absorbing acetic acid solution onto different solid carrier materials (native corn stover, pretreated corn stover, ceramic fiber and basalt fiber) up to 80% (w/w) of the total weight (Fig. 2, Table S3). Acetic acid consumption rate increased by approximately 20, 23, 17 and 10 folds, respectively, comparing to that of the

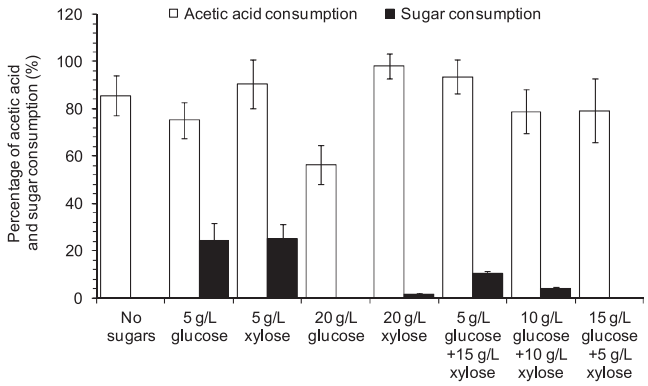

Fig. 1. Acetic acid and sugar consumptions by *A. resinae* ZN1 in submerged liquid culture containing different glucose and xylose. 4 g/L of acetic acid was added to the medium and cultured for 192 h. Conditions: 10% (v/v) inoculum size, 28 °C, in static state culture. Mean values are presented with error bars representing two standard deviations.

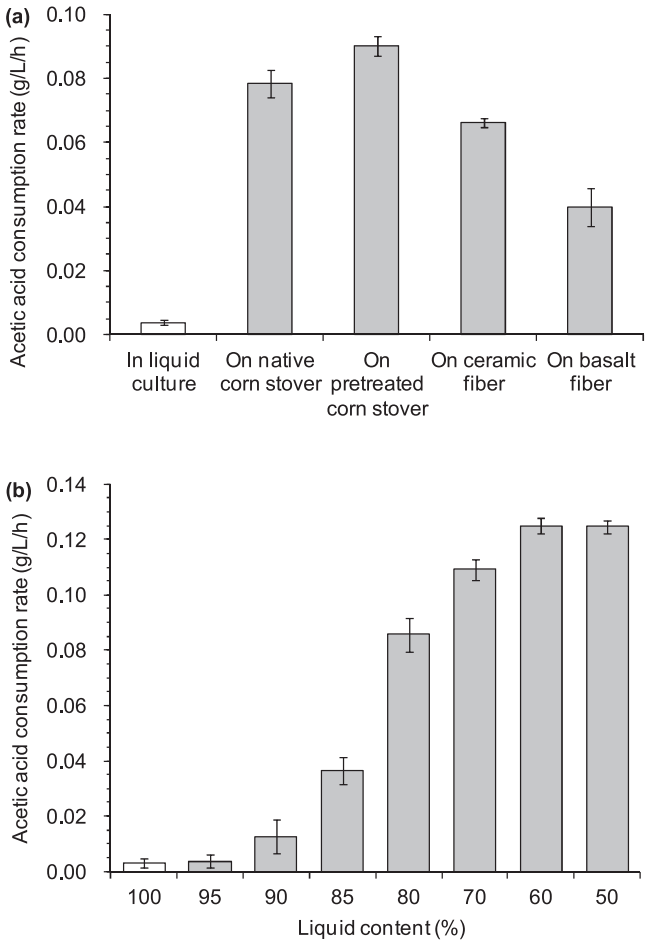

Fig. 2. Acetic acid consumption by *A. resinae* ZN1 (a) on the solid carrier including native corn stover, pretreated corn stover, ceramic fiber and basalt fiber with 80% of liquid loading; (b) on the pretreated corn stover with different liquid contents of 50–95% (w/w). 4 g/L of acetic acid was added to the culture system and cultured for 24 h. Conditions: 10% (v/v) inoculum size, 28 °C, in static state culture. Mean values are presented with error bars representing two standard deviations.

submerged liquid culture (Fig. 2a). The acetic acid consumption on the pretreated corn stover carrier was accelerated with decreasing liquid loading at the range of 50%–95% (w/w) (Fig. 2b). The results indicate that acetic acid consumption by *A. resinae* ZN1 preferred the solid state culture with higher solid content to the submerged liquid culture. As to the same ratio of spores to acetic acid between the solid state and liquid cultures, the improved acetic acid consumption rate in the solid state culture might be greatly related to the special growth characteristic of *A. resinae* ZN1 as a fungus, whose mycelium could well adhere to and creep along the solid carrier materials to make a larger air contact area and thus get more oxygen, which in turn sped up the acetic acid consumption.

3.2. Acetic acid metabolism pathway in *A. resinae ZN1*

Acetic acid metabolism pathway and tolerance response in *A. resinae* ZN1 were investigated by genome-wide RNA-Seq analysis (NCBI Sequence Read Archive, SRX3052496-SRX3052499). Fifteen differentially expressed genes were selected for qRT-PCR validation of the RNA-Seq data, and a high consistency was obtained (Fig. S1). Totally 1761 genes were differentially expressed in response to acetic acid, of which 955 genes were significantly up-regulated and 806 genes were significantly down-regulated (Fig. 3a, File S1). GO enrichment analysis indicates that acetic acid affected various biological processes including

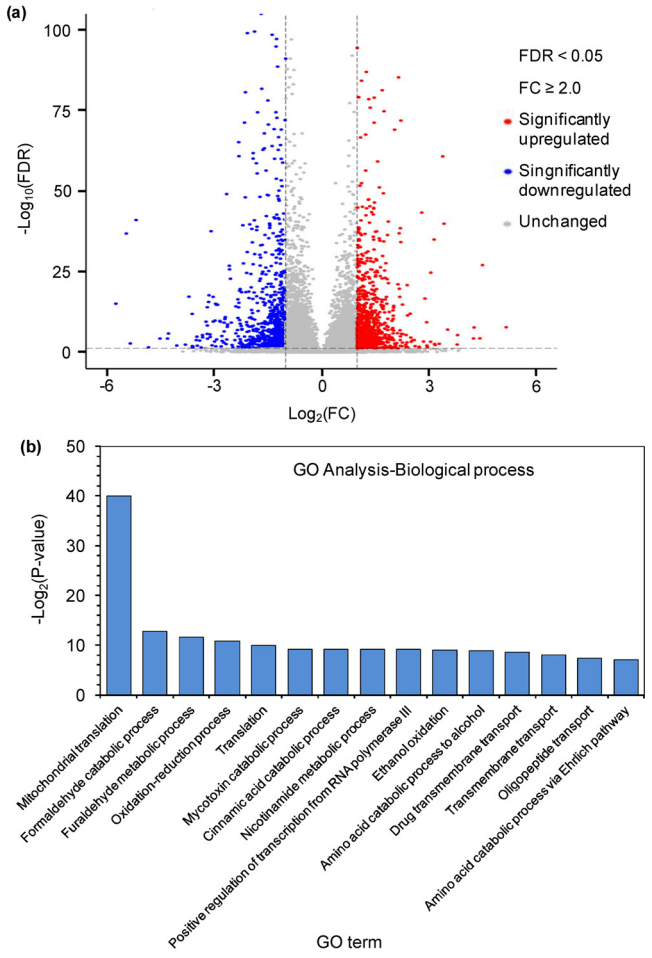

Fig. 3. Global genome expression response of *A. resinae* ZN1 in response to 2 g/L of acetic acid when cultured in medium with the presence of 5 g/L glucose. (a) Volcano plot of the transcriptional differences in *A. resinae* ZN1; (b) The top fifteen significant GO terms of the differentially expressed genes on biological process. Cell mass of *A. resinae* ZN1 was collected after treatment for 24 h by acetic acid.

translation process, metabolic process, oxidation-reduction process, transcription regulation process and transport process (Fig. 3b, File S2).

When glucose exists in the liquid medium, the *ACS* gene encoding acetyl-CoA synthetase and *ACH* gene encoding acetyl-CoA hydrolase were up-regulated by more than twofold in response to acetic acid (Table 1, File S1). The CIT1 gene encoding citrate synthase for the further acetyl-CoA metabolism in the TCA cycle was up-regulated by 2.17-fold. Other significantly up-regulated genes in the TCA cycle in response to acetic acid also included IDP1 gene encoding isocitrate dehydrogenase, LSC1 gene encoding succinyl-CoA ligase, *SHH* gene encoding paralogous succinate dehydrogenase, *FUM* gene encoding fumarase and MDH1 gene encoding mitochondrial malate dehydrogenase. On the other hand, the genes in glyoxylate cycle were not significantly changed or down-regulated (Table 1, File S1). To provide more evidence for the acetic acid metabolism pathway in *A. resinae* ZN1 when cultured in the liquid medium with different sugars, twenty-five genes involving acetic acid metabolism (six *ACS*, eleven TCA cycle unique genes of three *CS1*, six *IDH* and two MDH1, eight glyoxylate cycle unique genes of two *CS2*, four *ICL*, one *MS* and one MDH2) were screened and selected from the genome of *A. resinae* ZN1 (GenBank accession no. JZSE00000000) (Wang et al., 2015). Then, the transcription levels of these selected genes in response to acetic acid were analyzed by qRT-PCR using cells grown in the presence of varying glucose and xylose (Table S4). The results still show that only the *ACS* genes and the TCA cycle genes were up-regulated by more than twofold when cultured in the presence of glucose and xylose. These results indicate that *A. resinae* ZN1 assimilates acetic acid through acetyl-CoA formation into the TCA cycle when glucose and xylose exist in the medium.

When no glucose or xylose exists in the liquid medium, the transcription levels of the twenty-five genes in response to acetic acid were also analyzed using qRT-PCR (Fig. 4 and Table S4). The results show that the four *ACS* genes, one *CS1*, two *IDH*, one *MDH1* of the TCA cycle, and one *CS2*, two *ICL*, one *MS* and one *MDH2* of the glyoxylate cycle genes were all up-regulated by more than twofold, indicating that the two cycles of both the TCA cycle and glyoxylate cycle are involved in the acetic acid metabolism when glucose and xylose are absent in the liquid medium. Besides, transcription level of the specific gene was greatly different between cells grown in the presence and absence of sugar, such as *ARZ_1358_T1* and *ARZ_11892_T1* showed induction only in the absence of sugar, whereas the *ARZ_12088_T1* showed induction only in the presence of glucose. This difference is probably due to the effect of glucose and xylose on transcription of the specific gene.

Acetic acid metabolism pathway in *A. resinae* ZN1 is proposed in Fig. 5. Briefly, acetic acid is first converted into acetyl-CoA by ACS in cytoplasm and ACH in mitochondrial, then actyle-CoA is assimilated into the TCA cycle when acetic acid is co-cultured with glucose. When there is no sugar in the culture medium, acetyl-CoA is assimilated into the two cycles of both the TCA cycle and glyoxylate cycle. In contrast to *A. resinae* ZN1, the glyoxylate cycle is active for acetic acid metabolism in *C. glutamicum* regardless of the presence or absence of glucose (Wendisch et al., 2000). This difference between *A. resinae* ZN1 and *C. glutamicum* is probably due to the different transcription regulation. In *C. glutamicum*, the transcription of the glyoxylate cycle unique *ICL* and *MS* genes is increased in response to acetate in cells grown in both the absence and presence of glucose (Wendisch et al., 1997). In *A. resinae* ZN1, however, transcription of the glyoxylate cycle genes is repressed in the presence of glucose, but derepressed in the absence of glucose (Tables 1, S4 and Fig. 4). This physiological feature contributes to the acetate metabolism and tolerance in *A. resinae* ZN1 by providing additional energy source from the respiratory metabolism of acetic acid.

3.3. Tolerance response of *A. resinae ZN1* to acetic acid

Various biological processes including transport, metabolism, translation, and transcription regulation were affected by acetic acid

Table 1
Important genes involved in tolerance to acetic acid for *A. resinae* ZN1 based on RNA-Seq global transcriptome profiling analysis. (For interpretation of the references to colour in this Table legend, the reader is referred to the web version of this article.)

Functional Category	Gene ID	Gene Symbol	Gene Description	Fold Change	FDR
Monocarboxylate transport	*ARZ_16547_T1*	ESBP6	Protein with similarity to monocarboxylate permeases	2.80	0.02
	ARZ_15018_T1	MCH4	Protein with similarity to mammalian monocarboxylate permeases	0.36	0.03
	ARZ_8616_T1	MCH4		0.20	0.00
Polyamine transport	*ARZ_118_T1*	TPO1	Polyamine transporter involved in uptake of polyamines at alkaline pH and excretion at acidic pH	0.12	0.00
	ARZ_12711_T1			0.50	0.00
	ARZ_6742_T1			0.13	0.00
	ARZ_9045_T1			0.27	0.00
	ARZ_8569_T1	TPO2	Polyamine transporter of the MFS, transcription regulated by Haa1p	4.69	0.00
	ARZ_15897_T1	TPO3	Polyamine transporter of the MFS, transcription regulated by Haa1p	10.57	0.00
Ion transport	*ARZ_10034_T1*	PMA1	Plasma membrane H^+-ATPase involved in pumping protons out of the cell	2.08	0.00
	ARZ_14218_T1	TRK2	Component of the Trk1p-Trk2p potassium transport system	2.68	0.00
	ARZ_17860_T1			2.39	0.00
	ARZ_15781_T1	FSF1	Putative protein, predicted to play a role in iron homeostasis	2.55	0.00
	ARZ_6868_T1	VHC1	Vacuolar membrane transporter for K^+ and Cl^- cotransport into the vacuole	0.50	0.00
	ARZ_15489_T1	VCX1	Vacuolar membrane Ca^{2+}/H^+ and K^+/H^+ antiporter	0.43	0.00
	ARZ_13259_T1	SMF1	Divalent metal ion transporter	0.41	0.00
	ARZ_4510_T1	FTR1	Iron permease involved in the transport of iron across the plasma	0.48	0.03
Amino acid transport	*ARZ_12366_T1*	GAP1	General amino acid permease	2.22	0.00
	ARZ_4606_T1			2.55	0.00
	ARZ_10977_T1	VBA5	Plasma membrane MFS protein involved in amino acid uptake	2.76	0.00
	ARZ_2775_T1			2.66	0.00
	ARZ_9337_T1			2.59	0.00
	ARZ_11439_T1	AQR1	Plasma membrane transporter involved in amino acids excretion	0.38	0.04
	ARZ_379_T1	DIP5	Dicarboxylic amino acid permease	0.42	0.03
	ARZ_14818_T1	VBA1	Permease of basic amino acids in the vacuolar membrane	2.07	0.00
Amino acid transport	*ARZ_6119_T1*	VBA1	Permease of basic amino acids in the vacuolar membrane	2.52	0.00
	ARZ_12147_T1			0.30	0.00
	ARZ_1863_T1			0.21	0.00
	ARZ_12229_T1	VBA2	Permease of basic amino acids in the vacuolar membrane	2.55	0.00
	ARZ_12228_T1			0.37	0.00
Peptide transport	*ARZ_11114_T1*	OPT1	Proton-coupled oligopeptide transporter of the plasma membrane	2.53	0.00
	ARZ_15002_T1			2.09	0.01
	ARZ_16609_T1			2.07	0.00
	ARZ_7674_T1			2.01	0.02
	ARZ_16100_T1	PTR2	Integral membrane peptide transporter	2.06	0.00
	ARZ_9652_T1			2.59	0.00
	ARZ_6488_T1	DAL5	Allantoate permease, also transports dipeptides	2.75	0.00
Pyridoxine transport	*ARZ_11727_T1*	TPN1	Plasma membrane pyridoxine (vitamin B6) transporter	2.13	0.00
	ARZ_12536_T1			2.04	0.00
	ARZ_2485_T1			2.16	0.03
Riboflavin transport	*ARZ_11459_T1*	MCH5	Plasma membrane riboflavin transporter	0.13	0.00
	ARZ_1724_T1			0.37	0.00
	ARZ_7406_T1			0.23	0.00
Drug transport	*ARZ_260_T1*	FLR1	Plasma membrane MFS transporter involved in efflux of multidrug	0.41	0.00
	ARZ_3556_T1			0.22	0.00
	ARZ_4049_T1			0.25	0.00
	ARZ_13449_T1			0.32	0.00
	ARZ_12937_T1	QDR2	Plasma membrane MFS transporter involved in multidrug transport	0.34	0.00
	ARZ_18182_T1			0.16	0.00
	ARZ_16455_T1	SNQ2	Plasma membrane ABC transporter involved in multidrug resistance	0.40	0.00
	ARZ_198_T1			0.49	0.00
	ARZ_2780_T1			0.32	0.00
	ARZ_2033_T1	YOR1	Plasma membrane ABC transporter involved in multidrug transport	0.25	0.00

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Table 1 *(continued)*

Drug transport	*ARZ_9332_T1*	PDR18	Putative ABC transporter involved in drug resistance	0.26	0.00
Choline, ethanolamine, and carnitine tranport	*ARZ_3440_T1*	HNM1	Plasma membrane transporter for choline, ethanolamine, and carnitine	3.74	0.00
	ARZ_5476_T1			2.03	0.02
	ARZ_14908_T1			0.40	0.00
	ARZ_35_T1			0.38	0.03
	ARZ_6665_T1			0.30	0.03
Glycerol transport	*ARZ_18343_T1*	STL1	Plasma membrane glycerol proton symporter	2.79	0.00
	ARZ_15611_T1			0.38	0.00
	ARZ_16871_T1			0.33	0.00
	ARZ_2743_T1			0.24	0.00
	ARZ_3393_T1			0.41	0.00
Acetate metabolism	*ARZ_12088_T1*	ACS1	Acetyl-coA synthetase isoform	2.24	0.02
	ARZ_1260_T1	ACH1	Acetyl-CoA hydrolase with CoA transferase activity, particularly for	2.95	0.00
	ARZ_4922_T1		CoASH transfer from succinyl-CoA to acetate	3.29	0.00
	ARZ_17756_T1	CIT1	Citrate synthase in the TCA cycle	2.17	0.00
	ARZ_445_T1	IDP1	Isocitrate dehydrogenase in the TCA cycle	3.26	0.00
	ARZ_5863_T1			3.00	0.00
	ARZ_15744_T1	LSC1	Succinyl-CoA ligase in the TCA cycle	3.33	0.00
	ARZ_5502_T1	SHH3	Mitochondrial inner membrane protein similar to paralogous SDH3	3.08	0.00
	ARZ_11779_T1			2.15	0.00
	ARZ_3623_T1	SHH4	Mitochondrial inner membrane protein paralog to SDH4	2.63	0.00
	ARZ_11189_T1	FUM1	Fumarase in the TCA cycle	2.12	0.00
	ARZ_16701_T1	MDH1	Mitochondrial malate dehydrogenase in the TCA cycle	2.26	0.00
	ARZ_14970_T1	ICL1	Isocitrate lyase in the glyoxylate cycle	0.26	0.00
Amino acid biosynthesis	*ARZ_11510_T1*	MET1	S-adenosyl-L-methionine uroporphyrinogen III transmethylase required for	2.10	0.00
	ARZ_7457_T1		methionine biosynthesis	2.10	0.00
	ARZ_17686_T1	MDE1	Protein of in methionine salvage pathway	2.32	0.00
	ARZ_7428_T1	LYS4	Homoaconitase for lysine biosynthesis	2.22	0.00
Amino acid biosynthesis	*ARZ_11139_T1*	LYS2	Alpha aminoadipate reductase for lysine biosynthesis	3.59	0.00
	ARZ_14298_T1	LYS20	Homocitrate synthase isozyme for lysine biosynthesis	0.30	0.00
	ARZ_1215_T1	RNP1	Imidazoleglycerol-phosphate dehydratase for histidine biosynthesis	2.02	0.00
	ARZ_3144_T1	HIS5	Histidinol-phosphate aminotransferase for histidine biosynthesis	2.32	0.00
	ARZ_12063_T1	PRO3	Delta 1-pyrroline-5-carboxylate reductase for proline biosynthesis	2.03	0.00
	ARZ_17307_T1			2.40	0.00
	ARZ_14505_T1	UPS3	Putative cysteine synthase	2.17	0.00
	ARZ_5372_T1			2.05	0.00
	ARZ_17964_T1	TYR1	Prephenate dehydrogenase for tyrosine biosynthesis	2.42	0.00
	ARZ_14023_T1	GLY1	Threonine aldolase for glycine biosynthesis	2.28	0.00
	ARZ_836_T1	BAT1	Aminotransferase for BCAA biosynthesis	2.38	0.00
	ARZ_13710_T1	CAR1	Arginase involved in arginine degradation to ornithine	2.22	0.00
	ARZ_15123_T1	CAR2	L-ornithine transaminase involved in ornithine degradation	0.32	0.00
Membrane composition biosynthesis	*ARZ_17918_T1*	ERG5	C-22 sterol desaturase involved in ergosterol biosynthesis	2.69	0.00
	ARZ_9753_T1			2.67	0.00
	ARZ_6854_T1	ERG26	C-3 sterol dehydrogenase involved in ergosterol biosynthesis	2.17	0.00
	ARZ_5044_T1	ERG6	Delta(24)-sterol C-methyltransferase involved in ergosterol biosynthesis	0.41	0.00
	ARZ_3336_T1	CYB5	Cytochrome b5 involved in the sterol and lipid biosynthesis	2.09	0.00
	ARZ_9032_T1	ATG26	UDP-glucose:sterol glucosyltransferase	0.25	0.00
	ARZ_6066_T1	TGL1	Steryl ester hydrolase	0.46	0.00
	ARZ_14703_T1	SPO14	Phospholipase D	0.40	0.00
	ARZ_17437_T1			0.28	0.00
	ARZ_18139_T1			2.26	0.00
	ARZ_6149_T1	PSD2	Phosphatidylserine decarboxylase	0.20	0.00
	ARZ_6703_T1	PGC1	Phosphatidyl Glycerol phospholipase C	0.40	0.00
ATP biosynthesis	*ARZ_8849_T1*	RIB4	Lumazine synthase for riboflavin synthesis	2.19	0.00
	ARZ_3351_T1	FMN1	Riboflavin kinase, produces riboflavin monophosphate	2.14	0.00

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Table 1 *(continued)*

ATP biosynthesis	*ARZ_13420_T1*	FMN1	Riboflavin kinase, produces riboflavin monophosphate	2.31	0.00
	ARZ_5502_T1	SHH3	Mitochondrial inner membrane protein similar to paralogous SDH3	3.08	0.00
	ARZ_11779_T1			2.15	0.00
	ARZ_3623_T1	SHH4	Mitochondrial inner membrane protein paralog to SDH4	2.63	0.00
	ARZ_9948_T1	RIP1	Ubiquinol-cytochrome-c reductase	2.19	0.00
	ARZ_9112_T1	CYT1	Cytochrome c1	2.33	0.00
	ARZ_6747_T1	QCR8	Subunit 8 of ubiquinol cytochrome-c reductase	2.51	0.00
	ARZ_1321_T1	QCR7	Subunit 7 of ubiquinol cytochrome-c reductase	2.39	0.00
	ARZ_11078_T1	MZM1	Protein required for assembly of ubiquinol cytochrome-c reductase	2.48	0.00
	ARZ_17887_T1			2.46	0.00
	ARZ_13779_T1	CBP3	Protein required for assembly of ubiquinol cytochrome-c reductase	2.49	0.00
	ARZ_12641_T1	COA1	Protein required for assembly of cytochrome c oxidase complex	3.08	0.00
	ARZ_4687_T1			2.81	0.00
	ARZ_2998_T1	PET191	Protein required for assembly of cytochrome c oxidase	2.88	0.00
	ARZ_6067_T1			3.39	0.00
	ARZ_8355_T1	COX12	Subunit VIb of cytochrome c oxidase	2.12	0.00
	ARZ_10374_T1	AFG1	Protein that may act as a chaperone for cytochrome c oxidase subunits	2.53	0.00
	ARZ_8287_T1			2.76	0.00
	ARZ_4242_T1	OYE3	Heme A:farnesyltransferase required for cytochrome c oxidase activity	2.06	0.00
	ARZ_16480_T1	ATP10	Assembly factor for the F0 sector of mitochondrial F1F0 ATP synthase	2.92	0.00
	ARZ_223_T1			2.15	0.00
	ARZ_9366_T1	ATP23	Protein involved in assembly of the F0 sector of the F1F0 ATP synthase	2.21	0.00
	ARZ_17219_T1	ATP12	Assembly factor for the F1 sector of mitochondrial F1F0 ATP synthase	2.02	0.00
	ARZ_4410_T1	COQ3	O-methyltransferase involved in ubiquinone (Coenzyme Q) biosynthesis	2.42	0.00
	ARZ_4_T1	MVD1	Protein involved in ubiquinone biosynthesis	2.63	0.00
Transcription and regulation	*ARZ_9195_T1*	DAP1	Heme-binding protein for regulation of cytochrome P450 protein Erg11p	2.17	0.00
	ARZ_3904_T1			2.06	0.00
	ARZ_6606_T1	MET32	Transcriptional regulation factor of the methionine biosynthetic genes	3.13	0.00
Transcription and regulation	*ARZ_10766_T1*	LEU3	Zinc-knuckle transcription factor of BCAA biosynthesis genes	0.48	0.03
	ARZ_14968_T1			0.31	0.01
	ARZ_11248_T1	CRZ1	Transcription factor, activates transcription of stress response genes	2.28	0.00

All the genes listed in this table were differentially expressed (Fold change ≥ 2.0 or ≤ 0.5 and the FDR < 0.05) under the acetic acid stress. Red and green marks indicate expression of relative gene was up-regulated or down-regulated with a more than twofold change respectively.

(Table 1, File S2). For transport processes, the *TPO2* and *TPO3* genes encoding the plasma membrane polyamine transporters responsible for exporting the intracellular acetate anions (Mira et al., 2010) were up-regulated by 4.69 and 10.57-folds, respectively (Table 1, File S1). The *PMA1* gene encoding plasma membrane H^+-ATPase for pumping internal H^+ out of cell and the *TRK2* gene encoding Trk1p-Trk2p potassium transport system for K^+ uptake and control of the plasma membrane potential (Madrid et al., 1998) were also significantly up-regulated. The *TPO1* gene encoding polyamine transporter for excretion of polyamines at acidic pH, the *VHC1* and *VCX1* genes encoding vacuolar membrane transporter responsible for transporting K^+ from cytoplasm to vacuole were significantly down-regulated. The results indicate these transport genes contribute to acetic acid tolerance for *A. resinae* ZN1 through exporting proton and acetate anions, repairing the plasma membrane potential damage, and maintaining the cytosolic polyamine and K^+ level.

Multiple genes responsible for amino acid uptake and biosynthesis were differentially expressed in response to acetic acid (Table 1, File S1). The *GAP1* and *VBA5* genes involving cytoplasmic amino acids uptake were significantly up-regulated, while the *AQR1* and *DIP5* genes involving cytoplasmic amino acids efflux were significantly down-regulated. The genes involving amino acid biosynthesis were also significantly up-regulated, including *MET1* and *MDE1* for methionine synthesis, *LYS4* and *LYS2* for lysine synthesis, *RNP1* and *HIS5* for histidine synthesis, *PRO3* for proline synthesis, *UPS3* for cysteine synthesis, *TYR1* for tyrosine synthesis, *GLY1* for glycine synthesis and *BAT1* for branched-chain amino acid synthesis. This response was consistent with that of *S. cerevisiae* and *E. coli*, in which the biosynthesis of varying amino acids was induced under acetic acid stress (Kirkpatrick et al., 2001; Mira et al., 2010).

For genes responsible for modification of the membrane components, the *ERG5*, *ERG26*, and *CYB5* genes encoding enzymes for ergosterol biosynthesis were significantly up-regulated in response to acetic acid (Table 1, File S1). While the *SPO14* gene encoding phospholipase D, *PSD2* gene encoding phosphatidylserine decarboxylase and *PGC1* gene encoding phosphatidyl glycerol phospholipase C involving catabolism of phosphatidylcholine, phosphatidylserine and phosphatidylglycerol respectively were significantly down-regulated. The important link between lipid composition of plasma membrane and acetic acid tolerance was also observed in *S. cerevisiae* and *Z. bailii*, while instead of sterols, glycerophospholipids and sphingolipids were identified to be the critical lipid classes in response to acetic acid in the two strains (Lindberg et al., 2013).

Multiple genes involving ATP biosynthesis were significantly up-regulated in response to acetic acid, including *RIB4* and *FMN1* for NADH-ubiquinone oxidoreductase biosynthesis, *SHH3* and *SHH4* for succinate-ubiquinone oxidoreductase biosynthesis, *RIP1*, *CYT1*, *QCR8*, *QCR7*, *MZM1* and *CBP3* for ubiquinol cytochrome-c reductase biosynthesis, *COA1*, *PET191*, *COX12*, *AFG1* and *OYE3* for cytochrome c oxidase biosynthesis, *ATP10*, *ATP23* and *ATP12* for F1F0 ATP synthase biosynthesis, as well as *COQ3* and *MVD1* for ubiquinone biosynthesis (Table 1, File S1). The results suggest that the increased ATP

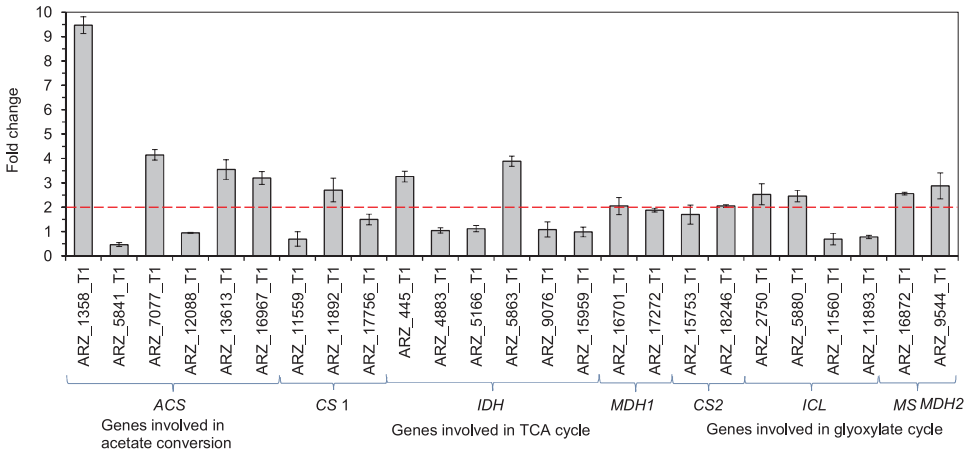

Fig. 4. Transcription level analysis of the twenty-five selected relevant genes in *A. resinae* ZN1 in response to 2 g/L of acetic acid when cultured in medium without sugar. Three biological and technical replicates were run for the qRT-PCR analysis.

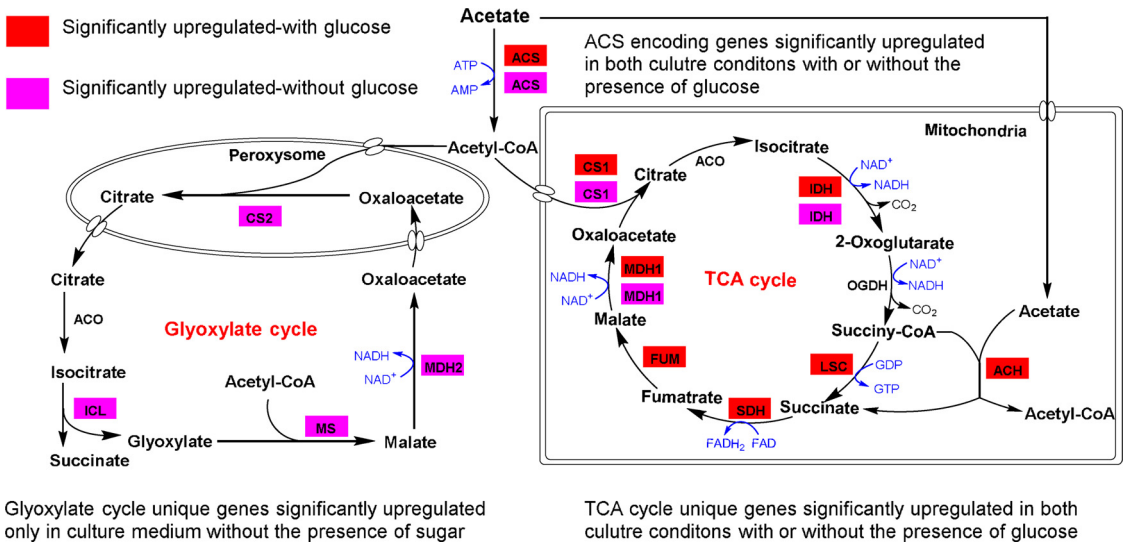

Fig. 5. Predicted acetic acid metabolism pathway in *A. resinae* ZN1 based on the RNA-Seq and qRT-PCR analysis. Acetic acid is first converted to acetyl-CoA, then is assimilated through the TCA cycle when glucose exists in the medium. When there is no sugar in the culture medium, acetyl-CoA is assimilated through the two cycles of both the TCA cycle and glyoxylate cycle. Transcription levels of the specific set of genes are shown in Tables 1 and S4. The red and pink marks indicate the relevant encoding genes were significantly up-regulated by more than twofold in the presence and absence of sugar respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

biosynthesis is associated with acetic acid tolerance by providing energy for *A. resinae* ZN1 to resist acetic acid stress. This was totally different with the response of *S. cerevisiae*, in which the genes involving energy generation and electron transport chain were down-regulated in response to acetic acid (Li and Yuan, 2010).

These results reveal that the tolerance response of *A. resinae* ZN1 to acetic acid includes the reduction of intracellular proton and acetate accumulation by activating ion transport genes, the reduced acetic acid diffusion into the cells by increasing the biosynthesis of ergosterol, as well as the increased cell damage repair by activating the Trk1p-Trk2p transport system, amino acids uptake and biosynthesis, and ATP biosynthesis.

4. Conclusions

Acetic acid metabolism pathway and tolerance response by *A. resinae* ZN1 were analyzed by microbial culture and RNA-Seq sequencing in response to acetic acid stress. Acetic acid is a prior substrate for *A. resinae* ZN1 to glucose and xylose, and the metabolism is significantly accelerated by solid state culture. *A. resinae* ZN1 assimilates acetic acid through acetyl-CoA into the TCA cycle when glucose exists in the medium, while into the two cycles of both the TCA cycle and glyoxylate cycle when there is no sugar in the medium. The acetic acid tolerance response in *A. resinae* ZN1 includes genes involving transport of protons, acetate anions and potassium ions, amino acids uptake and biosynthesis, as well as biosynthesis of ergosterol and ATP. The results provided the important gene resources for development of high acetic acid tolerant strains to efficiently produce biofuel and biochemical in lignocellulose biorefinery.

Contributors

XCG and JB designed the experiment; JB and QQG conceived the study; XCG conducted the experiment; QQG analyzed the methods; XCG, QQG and JB wrote the manuscript. All authors read and approved the manuscript.

Ethical approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Appendix A. Supplementary data

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