Functional Gene Identification and Corresponding Tolerant Mechanism of High Furfural-Tolerant Zymomonas mobilis Strain F211

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Furfural is a major inhibitor in lignocellulose hydrolysate for Zymomonas mobilis. A mutant F211 strain with high furfural tolerance was obtained from our previous study. Thus, its key tolerance mechanism was studied in the present study. The function of mutated genes in F211 was identified by functional complementation experiments, revealing that the improved furfural tolerance was resulted from the C493T mutation of the ZCP4_0270 gene promoting cell flocculation and the mutation (G1075A)/downregulation of ZCP4_0970. Comparative transcriptome analysis revealed 139 differentially expressed genes between F211 and the control, CP4, in response to furfural stress. In addition, the reliability of the RNA-Seq data was also confirmed. The potential tolerance mechanism was further demonstrated by functional identification of tolerance genes as follows: (I) some upregulated or downregulated genes increase the levels of NAD(P)H, which is involved in the reduction of furfural to less toxic furfuryl alcohol, thus accelerating the detoxification of furfural; (II) the mutated ZCP4_0270 and upregulated cellulose synthetase gene (ZCP4_0241 and ZCP4_0242) increased flocculation to resist furfural stress; (III) upregulated molecular chaperone genes promote protein synthesis and repair stress-damaged proteins; and (IV) transporter genes ZCP4_1623–1,625 and ZCP4_1702–1703 were downregulated, saving energy for cell growth. The furfural-tolerant mechanism and corresponding functional genes were revealed, which provides a theoretical basis for developing robust chassis strains for synthetic biology efforts.

Keywords: Zymomonas mobilis, furfural, tolerance, RNA-Seq, functional investigation

INTRODUCTION

Lignocellulosic biomass is an abundant renewable and sustainable resource used to produce biofuels and high-value chemicals (Yang et al., 2020). A major hindrance in converting lignocellulose to ethanol is the recalcitrance of biomass degradation, and pretreatment processes are thus required to release fermentable sugars (Moreno et al., 2015). Furan aldehyde (furfural and hydroxymethyl furfural), phenolic chlorine compounds, and organic acids are the major
Zymomonas mobilis, a facultative anaerobic gram-negative bacterium that naturally produces ethanol, has excellent characteristics, such as a high specific rate of sugar uptake and high theoretical ethanol yield (Wang et al., 2018). It is also a promising chassis strain for the production of high value-added products from lignocellulosic biomass (He et al., 2014). Zymomonas mobilis detoxifies phenolic aldehydes to less toxic alcohols and exhibits stronger tolerance to phenolic acids than Saccharomyces cerevisiae (Gu et al., 2015). Nevertheless, aldehydes, especially furfural, tend to be more toxic to Z. mobilis than organic acids; therefore, the development of high furfural-tolerant Z. mobilis is crucial for the utilization of lignocellulose (Franden et al., 2009).

High furfural-tolerant Z. mobilis strains have been developed by directed evolution. For instance, ZMF3-3 (Shui et al., 2015) obtained by adaptive laboratory evolution (ALE) and ZM4-MF2 (Tan et al., 2015) developed from transcriptional engineering was able to tolerate 3 g/L furfural. In our previous study, the strain F211 (Huang et al., 2018), obtained through error-prone PCR-based whole genome shuffling, could tolerate up to 3.5 g/L of furfural. A rational design was also used to obtain furfural-tolerant strains. Overexpression of the ZMO1771 gene encoding alcohol dehydrogenase improved furfural tolerance by accelerating the reduction of furfural (Wang et al., 2017). Co-expression of ZMO1771 and udhA genes, encoding a transhydrogenase catalyzing the interconversion of NADH and NADPH, can maintain the balance between NAD(P)H/NADP+, resulting in a more effective furfural tolerance (Wang et al., 2017).

Under furfural stress, the growth and survival of Z. mobilis were inhibited, and the specific production rate of ethanol was correspondingly reduced (Franden et al., 2009; Nouri et al., 2020). In addition, furfural also inhibits the synthesis of various proteins, causes DNA damage, and leads to the differential expression of genes involved in membrane and cell wall biogenesis, transcriptional regulators, and energy metabolism (He et al., 2012; Yang et al., 2020). Zymomonas mobilis may respond to stress in the following ways: (1) conversion of furfural to furfuryl alcohol reduces toxicity (Wang et al., 2017); (2) the genes related to macromolecule synthesis are upregulated to resist furfural stress (Miller et al., 2009; Yang et al., 2020); and (3) transcription factors regulate the expression of multiple genes in response to stress (He et al., 2012; Nouri et al., 2020). However, the defined roles of these corresponding genes are not yet known.

In summary, there is still a lack of comprehensive understanding of furfural tolerance. The high furfural tolerance mutant strain F211 was obtained in an earlier study, and single nucleotide polymorphisms (SNPs) between F211 and control strain CP4 were identified by genome resequencing of F211 (Huang et al., 2018); in this study, functional identification of the mutations and RNA-Seq was therefore performed to investigate the genotypic changes associated with furfural tolerance and reveal the molecular mechanism responsible for the improved furfural tolerance in F211.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

The wild-type strain Z. mobilis CP4 was purchased from the China Center for Type Culture Collection (CCTCC; Accession NO.CCC10232). F211 is a strain of Z. mobilis with high furfural tolerance obtained in the early stage of our laboratory (Huang et al., 2018). Zymomonas mobilis was cultured in Rich Medium (10 g/L yeast extract, 2 g/L KH₂PO₄, 20 g/L glucose, and different concentration of furfural as required) at 30°C without shaking.

Construction of Deletion and Overexpression Strains

Nine deletion strains (Supplementary Table S1) were constructed by homologous recombination as described in our previous study with some modification (Wang et al., 2013; Huang et al., 2018). The left and right homologous arm DNA fragments of the target gene were ligated to flank both sides of a chloramphenicol resistance gene, generating the homologous recombinant fragments (Section 1.1 of Supplementary Materials and Methods). The homologous recombinant fragments were ligated into the SphI and SacII sites of pUC19 to yield the plasmid pT-gene (Supplementary Table S1) by using the Gibson assembly (GA; Gibson et al., 2009). The target plasmids were then transformed into CP4 competent cells by electroporation (Ma et al., 2012). The target gene was replaced with the chloramphenicol resistance gene by homologous recombination using its native RecA recombinase (Supplementary Figure S1; Savvides et al., 2000; Huang et al., 2018). Transformants were screened on RM agar plates with 50 μg/ml chloramphenicol. Positive single colonies were confirmed by PCR using the appropriate geneGF/geneGR, geneGF/T-CM-R, and T-CM-F/geneGR (Supplementary Table S2) primer pairs (Supplementary Materials and Methods).

Overexpression vectors were constructed by ligating the target wild-type gene or its mutated gene into multiple clone sites of pEZ15Asp (pE)/pHW20a (pH) vector. The target genes were amplified from the genomes of CP4 and F211 using the primers gene OF/OR (Supplementary Table S3), and the Pₐₛₚ promoter (Yang et al., 2019) was amplified from the genome of CP4 using the primers pgap-F/pgap-R (Supplementary Table S3). Then, the two fragments were fused by overlap-extension PCR using the primers pgap-F/gene-OR. The fusion fragment was inserted into the Psrl and EcoRI sites of pE/pH to yield the plasmid pE-gene/pH-gene by using the aforementioned GA (Gibson et al., 2009). Positive clones were further verified by DNA sequencing. The correctly sequenced recombinant plasmids were transformed into CP4 competent cells by electroporation.
The target plasmids were confirmed by PCR using the appropriate pEZ-F/pEZ-R (Supplementary Table S3) primer pairs.

**Furfural Tolerance Assay**

Strain was precultured in RM at 30°C to the late exponential stage, and the cultures were concentrated to an OD$_{600}$ of 20 by centrifugation. The concentrated cells were inoculated into 100 ml RM medium containing 3.5 g/L furfural with an initial OD$_{600}$ of 0.2, and the OD$_{600}$ value was measured every 6h to detect the growth of the cells for the evaluation of furfural tolerance. The degradation rate of furfural was calculated by the following formula:

\[
\text{The degradation rate of furfural} = \frac{C_{\text{initial}} - C_{\text{final}}}{T}
\]

where “$T$” indicated the fermentation time, and “$C_{\text{initial}}$” and “$C_{\text{final}}$” indicated “initial medium furfural concentration” and “final medium furfural concentration,” respectively. All experiments were performed in triplicates, and statistical differences were analyzed using a one-way analysis of variance (ANOVA). *$p<0.05$ was considered statistically significant, and **$p<0.01$ was considered extremely significant.

**Flocculation Measurement**

Strains were cultured in RM medium containing 3 g/L furfural for 36h, and 10 μl of the culture was taken to observe the flocculation under a light microscope (Olympus BX51, Japan). Five milliliter culture of each strain was collected and was shaken vigorously for 15 s and then rested for 5 min. Suspension of 1 ml was sampled from the upper section to measure OD$_{600}$ and quantify the non-flocculating cells (Cf), and 1.2 units of cellulase (Novozymes, Denmark) was added to the remaining culture, which was incubated at 50°C for complete de-flocculation of the bacterial flocs to measure the concentration of total cells (Ct). The flocculation efficiency of Z. mobilis was calculated based on the formula:

\[
\text{R}\% = \left(1 - \frac{\text{Cf}}{\text{Ct}}\right) \times 100 \text{ (Xia et al., 2018).}
\]

**RNA-Seq Transcriptomic Analysis**

Strain F211 and CP4 were cultured statically in RM medium with 3 g/L furfural. The cultures were collected by centrifugation when the OD$_{600}$ reached 0.8–1.4, and the cells were washed with PBS buffer (pH = 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$·12H$_2$O, and 1.4 mM KH$_2$PO$_4$). The cells were centrifuged and freeze in liquid nitrogen for 1h. The samples were submitted to Novogene (China, Beijing) for RNA-Seq.

The integrity of the extracted RNA was tested using the Agilent Bioanalyzer 2,100 system (Agilent Technologies, CA, United States). cDNA library preparation and sequencing were conducted by the Novogene Technology Company in Beijing, China. The clean reads were obtained by sequencing and mapped to the Z. mobilis CP4 genome sequence (GenBank: CP006818.1) using Bowtie2-2.2.3. Differentially expressed genes (DEGs) between the two samples were identified using the DESeq package (1.20.0), and |log2(FoldChange)| > 1 and q-value < 0.005 were used as the threshold to identify significant differences in gene expression analysis of Gene Ontology (GO), which assigns genes into functional categories, was performed using the GO R packages. KOBAS software could perform KEGG enrichment analysis on differentially expressed genes to determine the metabolic pathways of genes.

**Real-Time Quantitative Reverse Transcription PCR Analysis**

The expression levels of 22 DEGs representing different functional categories were assessed by real-time quantitative reverse transcription PCR (qRT-PCR) to validate the reliability of the RNA-Seq data. The culture conditions of CP4 and F211 were the same as the RNA-Seq experiment. When the strains concentration reached OD$_{600}$ = 0.8–1.4, about 10$^4$ cells were collected from each strain, and the cells are treated in liquid nitrogen for 5 min. Subsequently, the bacterial cells were ground in a mortar pre-cooled with liquid nitrogen, and the powder was transferred to 100 μl TE buffer (containing 400 μg/ml lysozyme), mixed, and placed at room temperature for 4 min to dissolve the cell wall. RNA was extracted with TRizol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions.

DNase (RQ1 RNase-Free DNase, Promega, United States) was added to the RNA samples to remove genomic DNA. The PCR reaction with primer pair 16SrRNA-F/16SrRNA-R was performed on the total RNA in order to check for genomic DNA contamination. No product was observed, demonstrating that the sample had no genomic DNA contaminants and was suitable for qRT-PCR assay. Subsequently, cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) with the purified total RNA of CP4 or F211 as a template following the manufacturer's instructions. Real-time qRT-PCR experiments were performed using CFX96 Real-Time System (Bio-Rad). Each reaction contained 2 μl of diluted (1/10) cDNA, Taq SYBR Green qPCR Premix (Yugong Biolabs Inc., Jiangsu, China) and the corresponding primer pairs (Supplementary Table S4). The 16S rRNA gene was used as a reference to normalize the qRT-PCR data. The expression level of each gene was calculated according to the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

**RESULTS AND DISCUSSION**

**Functional Identification of Mutated Genes in F211**

Eleven SNPs (single nucleotide polymorphisms) were found in high furfural-tolerant F211 compared with the sequence (GenBank No. CP006818) of CP4 from Agricultural Research...
Service Culture Collection (NRRL No. B-14023; Huang et al., 2018). The initial strain CP4 (CICC 10132) of F211 and the reference CP4 may have different SNPs. Therefore, in this study, except for the three synonymous mutated genes of ZCP4_0096, ZCP4_0593, and ZCP4_1887, seven missense mutated genes and one nonsense mutated gene of F211, along with the starting strain, CP4, were PCR-amplified and sequenced. F211 and CP4 shared consistent sequences of ZCP4_1431, ZCP4_1_616, ZCP4_0244, and ZCP4_0525. The ZCP4_1919, ZCP4_1419, and ZCP4_0270 genes in F211 carried missense mutations in G335A, C85T, and C493T, resulting in a substitution of V119I, ZCP4_0270 encodes a bifunctional protein with diguanylate cyclase (DGC)/phosphodiesterase (PDE), which synthesizes/degrades bis-(39–59)-cyclic guanosine monophosphate (c-di-GMP). The deletion strain Δ0270 (pH) and overexpression strain Δ0270 (pH-W0270) did not show significant growth differences compared to those of the control strains CP4 (pH) and Δ0270 (pE-W0270) (Figures 1C,D), indicating that deletion or overexpression of the native ZCP4_0270 gene did not affect furfural tolerance. The specific growth rate, degradation rate of furfural in the medium (0–48 h), and ethanol production of the mutated gene overexpression strain Δ0270 (pH-M0270) were 62, 8.3, and 38.5% higher than those of the control strain Δ0270 (pE-M0270) (Figures 1C,D), respectively, indicating that the C493T mutation of the ZCP4_0270 gene can promote the degradation of furfural, thereby improving furfural tolerance. Flocculated yeast showed a higher tolerance to furfural than free yeast (Westman, 2014). The increased c-di-GMP, which is regulated by PDEs, can promote the flocculation of Z. mobilis (Tan et al., 2014). Therefore, the mutated ZCP4_0270 may improve furfural tolerance by promoting flocculation, and thus, the flocculation of F211 and Δ0270 (pH-M0270) was investigated. Flocculation was observed in both strains (Figures 2A,C), and the flocculation rates of F211 and Δ0270 (pH-M0270) were 600 and 470% higher than that of CP4, respectively (Figure 2B). The flocculation rate of the wild-type ZCP4_0270 gene overexpressing strain Δ0270 (pH-W0270) and the engineered strains of ZCP4_0970 and ZCP4_1419 were similar to those of the control CP4 (Figure 2B). These results show that the mutated ZCP4_0270 gene in F211 causes flocculation, resulting in an improved furfural tolerance.

**Transcriptome Analysis of F211 and CP4 Under Furfural Stress**

The bifunctional DGCs/PDEs encoded by the ZCP4_0270 gene can regulate the levels of the second messenger c-di-GMP, which coordinates diverse aspects of bacterial growth and behavior, including motility, virulence, biofilm formation, and cell cycle progression (Jenal et al., 2017). Therefore, enhanced tolerance by the mutated ZCP4_0270 also resulted in a change in the physiological, biochemical, and expression levels of some genes. Consequently, RNA-Seq was performed to analyze the transcriptome differences between F211 and CP4 under furfural stress and to explore the potential furfural tolerance mechanism.

A total of 139 DEGs were identified in F211 compared with the control, with 77 and 62 genes being upregulated and downregulated, respectively. The expression levels of the three missense genes in F211 did not change significantly, indicating

### Table 1: Mutations identified in the F211 strain.

| Gene   | Function                  | Mutation | Effect  |
|--------|---------------------------|----------|---------|
| ZCP4_0270 | Diguanylate cyclase/ phosphodiesterase | C493T | G166S   |
| ZCP4_0970 | Type I secretion outer membrane protein, ToC family | G1075A | Q359X   |
| ZCP4_1419 | Surface lipoprotein | C85T | G29S    |
| ZCP4_1919 | A C-terminal OMP/outer membrane protein domain | G335A | V119I   |

*Termination codon.*
that its enhanced tolerance was due to the changed protein function caused by the mutated ZCP4_0270 and ZCP4_0970 genes, rather than the changed transcript abundance. DEGs were subjected to GO enrichment analysis. The five GO clusters with the highest enrichment were as follows: “localization,” “transport,” “establishment of localization,” “cofactor binding,” and “response to stimulus” (Supplementary Figure S4A). The DEGs were enriched in 39 pathways, found using a Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, 27 of which were different from those previously reported (He et al., 2012; Yang et al., 2020). The significant KEGG terms were primarily associated with microbial metabolism in diverse environments, biosynthesis of secondary metabolites and antibiotics, bacterial chemotaxis, and sulfur metabolism (Supplementary Figure S4B).

We screened 22 DEGs representing different GO clusters from 139 DEGs (Table 2) for qRT-PCR verification. Based on the qRT-PCR results, the expression trends of these candidate genes were consistent with the RNA-Seq data (Figure 3). A comparison of the two methods indicated a high level of concordance ($R^2=0.7833$), indicating that the RNA-Seq data were reliable.

**Furfural Tolerance Mechanism by Functional Analysis of DEGs**

**Increased Intracellular NAD(P)H Levels Resist Furfural Stress**

The sulfur assimilation operon CysIHIH (ZCP4_1208–1,210) and CysGDNC (ZCP4_1211–1,214) were significantly downregulated in F211 in response to furfural stress (Table 2). Deletion strains of these genes were engineered to investigate their furfural tolerance. The cell densities of the deletion strains ΔCysIHIH and ΔCysND were increased by 20 and 9% compared to those of CP4 (Figure 5A), respectively, showing that the deletion of these genes could improve the furfural tolerance. The enzymes encoded by CysND and CysIHIH genes involved in cysteine synthesis coupled with ATP and NAD(P)H consumption (Figure 4; Miller et al., 2009; Takagi, 2019). And the conversion of NAD(P)H to NAD(P)+ was coupled with reduction in furfural to furfuryl alcohol (Boopathy, 2009; Yi et al., 2015). The concentration of NADH of the ΔCysND and ΔCysIHIH was 35.7-fold and 44.6-fold higher than that of the control CP4 in RM without furfural (Supplementary Figure S5A), respectively, indicating that deletion of these genes can reduce NADH consumption. While the NADH/NAD+ ratio was decreased by 49.5 and 34.5% for ΔCysND and ΔCysIHIH under furfural stress, respectively, compared with non-stress condition (Supplementary Figure S5B), demonstrating that the deletion strains consumed more NADH under furfural stress. Thereby, the downregulation of these genes could reduce the consumption of NADH, and the saved NADH may be used for the conversion of furfural to furfuryl alcohol, thus enhancing the detoxification of furfural. The damaged proteins and DNA were correspondingly reduced.

Interestingly, overexpression of the cysteine synthase operon CysCNDG (ZMO0003–0006) contributed to the production of cysteine, replenishing proteins damaged by furfural, thus improving the furfural tolerance of 8b (Miller et al., 2009; AB CD...
Yang et al., 2020). The destruction of sulfur assimilation pathway can reduce the synthesis of cysteine, a deficiency of amino acids for growth (Miller et al., 2009) and response stress. Therefore, the furfural tolerance characteristics of ΔCysJI and ΔCysND strain may be counteracted partially by the Cys deficiency. In our study, the downregulation of the CysCNDG operon in F211 may be caused by a mutation in the ZCP4_0270 gene, which regulates the content of c-di-GMP, causing physiological and biochemical changes such as cell flocculation (Tan et al., 2014).

The ZCP4_1,170 gene, encoding the efflux pump protein TauE, involved in efflux sulfite to the periplasmic space, is downregulated 3.0-fold under furfural stress. The overexpression and deletion strains of this gene were constructed to demonstrate their function. Compared with the control strain CP4 (pE), the cell density of the overexpression strain CP4 (pE-1,170) and deletion strain Δ1170 (pE) was increased and decreased by 21 and 22% (Figure 5B), respectively, showing that its upregulated expression improved furfural tolerance. Conversely, the expression of the ZCP4_1,170 gene is downregulated in furfural-tolerant F211 (Table 2), which results from feedback regulation by sulfate assimilation genes. The downregulation of CysH and CysDNC genes reduces sulfite synthesis and this may have corresponded with less TauE being needed; therefore, the genes were feedback downregulated.

The expression of the ZCP4_1,414 gene encoding succinate dehydrogenase was upregulated by 1.9-fold in F211 (Table 2). The enzyme catalyzes the conversion of succinic acid semialdehyde to succinic acid and concomitantly reduces NAD(P)⁺ to NAD(P)H. The overexpression strains CP4 (pE-1,414) and Δ1414 (pE-1,414) of this gene showed a 24 and 11% increase (Figure 5C) in cell density compared with the control strains CP4 (pE) and Δ1414 (pE), indicating that its upregulation improved furfural tolerance. Furthermore, its upregulation can accelerate the regeneration of NAD(P)H and provide more reducing activity for furfural detoxification, thereby resulting in enhanced tolerance.

**Responses of Molecular Chaperones and Stress Proteins to Furfural Stress**

The ZCP4_0558 gene encoding a universal stress protein CsbD, molecular chaperone yfdX (ZCP4_0600), and clpB (ZCP4_1707), was significantly upregulated in F211 under furfural stress (Table 2). The overexpression and deletion strains of the ZCP4_0558 gene were constructed to study
the effect of its expression levels on furfural tolerance. The specific growth rate of the overexpression strain CP4 (pE-0558) and the deletion strain Δ0558 (pE) were increased and decreased by 27.6 and 9.5%, respectively, compared with the control strain CP4 (pE) (Figure 5D), indicating that its upregulation could improve furfural tolerance. The mutated ZCP4_0270 gene may increase the activity of diguanylate cyclase, enhancing the content of c-di-GMP and resist stress. In addition, the chemotaxis (ZCP4_1147 and ZCP4_1150) and flagella (ZCP4_0645) genes were downregulated in F211 (Supplementary Table S5), which may reduce cell motility and facilitate cell flocculation, thus improving furfural tolerance (Jeon et al., 2012; Xia et al., 2018). A stronger flocculation ability was observed in F211 compared to CP4 (Figure 2A), demonstrating the potential furfural tolerance mechanism.

Cell Flocculation
Two genes (ZCP4_0241 and ZCP4_0242) related to cellulose synthesis were upregulated in F211 (Table 2). The increased cellulose content on the cell surface contributes to cell flocculation (Jeon et al., 2012; Xia et al., 2018). CsbD from Methylocystis was upregulated under stress of starvation, high heat, and acid (Han et al., 2017). CsbD from Bacillus coagulans also showed upregulated expression in response to furfural stress (van der Pol et al., 2016). To our knowledge, this study provides the first evidence that its upregulation can improve the furfural tolerance of Z. mobilis. Chaperones play important roles in cell survival, and they can promote the folding of peptide chains and repair stress-damaged proteins, thus ensuring protein homeostasis in cells (Liu et al., 2016; Ghazaei, 2017). Furfural inhibits synthesis of various proteins and affects cell growth and survival (Fraiden et al., 2009; He et al., 2012). Therefore, the upregulated expression of molecular chaperones in F211 may promote protein synthesis, reduce damage to cells, and improve furfural tolerance.

Reduce Energy Consumption of Efflux Pump
The ATP-binding cassette (ABC) transporter family (ZCP4_1623, ZCP4_1624, and ZCP4_1,625) genes and the resistance-nodulation-division (RND) family (ZCP4_1702 and ZCP4_1703) genes were downregulated in F211 (Table 2). They consume

| Functional group and gene | Description | Log2 fold change in RNA-Seq | Log2 fold change in qRT-PCR |
|---------------------------|-------------|-----------------------------|-----------------------------|
| Transporter               |             |                             |                             |
| ZCP4_0708                 | Metl-like ABC transporter transmembrane protein | 1.858 | 1.225 |
| ZCP4_1,170                | Anion permease, Sulfite exporter TauE/SatE | −2.986 | −0.750 |
| ZCP4_1331                 | Alginate export | −2.033 | −0.554 |
| ZCP4_1481                 | Aspartate-alanine antiporter | 2.006 | 1.090 |
| ZCP4_1624                 | Multidrug efflux pump subunit AcrB | −1.359 | −1.147 |
| ZCP4_1,625                | Efflux transporter outer membrane factor lipoprotein, NodT family | −1.474 | −0.310 |
| ZCP4_1703                 | NodT family RND efflux system outer membrane lipoprotein | −1.665 | −0.475 |
| Stress related            |             |                             |                             |
| ZCP4_0558                 | General stress protein CsbD | 2.138 | 0.424 |
| ZCP4_0600                 | YidK protein, putative chaperone | 1.785 | 1.382 |
| ZCP4_0601                 | OsmC protein, putative chaperone | 1.701 | 1.062 |
| ZCP4_1707                 | ATP-dependent chaperone CipB | 1.467 | 0.359 |
| Metabolism                |             |                             |                             |
| ZCP4_1209                 | Sulfite reductase subunit beta, Cysl | −1.414 | −0.746 |
| ZCP4_1212                 | Sulfate adenylyltransferase subunit 1, CysD | −2.331 | −0.410 |
| ZCP4_1213                 | Sulfate adenylyltransferase subunit 2, CysN | −1.842 | −0.839 |
| ZCP4_1,414                | Succinate-semialdehyde dehydrogenase | 1.945 | 0.761 |
| ZCP4_1673                 | Acyltransferase of alpha/beta superfamily | 2.836 | 1.629 |
| Membrane related          |             |                             |                             |
| ZCP4_0241                 | Cellulose synthase operon C domain protein, BscC | 1.670 | 0.635 |
| ZCP4_0242                 | Cellulose synthase subunit B, BscB | 1.035 | 1.341 |
| ZCP4_0439                 | Squalene-hopene cyclase | −1.604 | −0.108 |
| ZCP4_0441                 | Squalene synthase HpnD | −1.367 | −0.006 |
| Regulator                 |             |                             |                             |
| ZCP4_0221                 | AsnF family transcriptional regulator | 1.109 | 0.943 |
| ZCP4_0806                 | Transcriptional regulator | 1.431 | 0.300 |

FIGURE 3 | Correlation between RNA-seq and qRT-PCR results for RNA-seq data verification. The gene expression ratios of RNA-seq and qRT-PCR data for the genes in Table 2 were log transformed (base 10), and the RNA-seq log2 ratio values were plotted against the qRT-PCR log2 values.
ATP to export lipids, amino acids, heavy metals, etc., to the outside of the cell (Saier and Paulsen, 2001). Deletion of an operon encoding the RND efflux system could improve the furfural tolerance of Z. mobilis ZM4 (Yang et al., 2020). The downregulation of these efflux pump genes may reduce the energy consumption of cells, providing more energy for cell
growth and increasing furfural tolerance. In future, corresponding functional verification experiment would be performed to consolidate the current discussion.

**CONCLUSION**

In conclusion, F211 resists furfural stress by accelerating the detoxification of furfural, improving cell flocculation, enhancing emergency response, and reducing energy consumption. This study lays the foundation for the rational design of high furfural tolerance strains, which would accelerate the industrialization process of lignocellulosic ethanol.

**DATA AVAILABILITY STATEMENT**

The RNA-seq datasets for this study can be found in the NCBI under accession number PRJNA743578.

**AUTHOR CONTRIBUTIONS**

YM conceived of the project, analyzed the data, supervised the study, and revised the manuscript. MH supervised the study and revised the manuscript. DH and ZW performed the experiments and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.736583/full?supplementary-material
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