Heliyon 10 (2024) e27929

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

The role of the transcriptional repressor CssR in *Corynebacterium glutamicum* in response to phenolic compounds

Ju Zhang [a,b,1], Yuying Zhao [c,1], Zhaoxin Peng [c], MingFei Yang [c], Wenyu Zou [c], Xinyu Wu [c], Chenghui Wang [c], Meiru Si [c,**], Can Chen [a,*]

[a] *Key Laboratory of Plant Genetics and Molecular Breeding, Henan Key Laboratory of Crop Molecular Breeding & Bioreactor, College of Life Science and Agronomy, Zhoukou Normal University, Zhoukou, 466001, Henan, China*
[b] *College of Horticulture, Agricultural University of Hebei/Key Laboratory for Vegetable Germplasm Enhancement and Utilization of Hebei/Collaborative Innovation Center of Vegetable Industry in Hebei, Baoding, 071001, China*
[c] *College of Life Sciences, Qufu Normal University, Qufu, 273165, Shandong, China*

ARTICLE INFO

Keywords:
CssR
Transcription regulation
Phenolic compound
Corynebacterium glutamicum

ABSTRACT

The *cssR* gene (*ncgl1578*) of *Corynebacterium glutamicum* encodes a repressor of the TetR (tetracycline regulator) family. Its role in the stress response to antibiotics/heavy metals has been investigated, but how CssR functions in response to phenolic compounds in *C. glutamicum* has been rarely studied. In this study, we applied transcriptomic analysis, β-galactosidase analysis, qRT-PCR, and EMSAs to analyze the target genes and functions of CssR in response to phenolic compounds. Consistent with the upregulation of genes involved in the degradation of phenolic compounds, the Δ*cssR* mutant was more resistant to various phenolic compounds than was the wild-type strain. Furthermore, the addition of phenolic compounds induced the expression of corresponding genes (*ncgl0283*, *ncgl1032*, *ncgl1111*, *ncgl2920*, *ncgl2923*, and *ncgl2952*) *in vivo*. However, the DNA binding activity of CssR to the promoter of phenolic compound-degrading genes was undetected *in vitro*. Additionally, we also found that CssR indirectly negatively regulates the expression of cell wall/membrane/envelope biogenesis-related genes, which may enhance resistance to stress caused by phenolic compounds. Together, our findings demonstrate that CssR is a key regulator that copes with stress conditions induced by phenolic compounds, thus greatly expanding our understanding of the functions of TetR family transcription factors.

1. Introduction

Corynebacterium glutamicum, a nonpathogenic gram-positive soil bacterium widely used in industrial L-amino acid production and a model microorganism for systems biology, unavoidably generates or encounters a series of adverse circumstances during fermentation process [1]. These include oxidants, alkylating agents, antibiotics, high osmotic pressure, low pH, variations in temperature, and toxic aromatic compounds (including phenolic compounds) [2–4]. Thus, to survive within the diverse fermentation environments, *C. glutamicum* gradually develops a series of repair mechanisms, tolerance mechanisms, and internal regulatory mechanisms during the

* Corresponding author.
** Corresponding author.
E-mail addresses: smr1016@126.com (M. Si), chenc02@126.com (C. Chen).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2024.e27929
Received 14 August 2023; Received in revised form 5 March 2024; Accepted 8 March 2024
Available online 10 March 2024

natural evolution to protect its cellular constituents from reactive oxygen species (ROS) and effectively utilize aromatic compounds. Notably among these defense strategies are the low-molecular-weight (LMW) defense mechanism, the thickened cell wall, and regulatory proteins [5].

The regulatory proteins of *C. glutamicum* play essential roles in survival under various stressful conditions by detecting changes in environmental conditions through the action of specific regulatory systems and developing coordinated cellular responses to adapt to new conditions [6]; these proteins include the MarR (multiple antibiotics resistance regulators) family [7], the LysR (DNA-binding transcriptional dual-lysine regulator) family [8], the XRE (xenobiotic-response element) family [9], and the TetR (tetracycline repressor protein) family [10]. Among these families, the TetR family is a widespread bacterial transcriptional repressor protein family and is the largest family, with up to 16 members in *C. glutamicum* [11]. The protein was named after the tetracycline resistance repressor protein, the first member of the family [12]. The TetR family of regulators have a high degree of sequence similarity in DNA-binding domains. The three-dimensional structure of the TetR monomer is stabilized by hydrophobic helix-to-helix contacts [12,13]. In addition, these proteins are generally homodimers whose subunits consist of two domains, the N-terminal operator-binding domain and the C-terminal contiguous regulatory domain [14]. Most TetR transcriptional regulators act as repressors to regulate gene expression. The binding of inducers to regulatory domains results in structural changes in the protein that prevent the binding of repressors to their operators; thus, repressors are molecular switches that function in either operator-binding or inducer-bound forms [15]. It has been reported that TetR family regulators mainly regulate genes related to morphological changes in bacteria, biofilm formation, biosynthesis, the tricarboxylic acid cycle, and antibiotic resistance [12].

In *C. glutamicum*, TetR family regulators act as sensors to monitor the cell environment and regulate gene expression in many cases. For example, PaaR regulates phenylacetic acid (PAA) catabolism [16], RolR regulates resorcinol catabolism [17], BioQ regulates biotin metabolism [18], OsrR mediates H_2O_2 resistance [10], the multidrug resistance-related transcription factor CgmR [15], the aconitase repressor AcnR [19], the central regulator of the nitrogen starvation response AmtR [20], and the l-methionine biosynthesis repressor McbR [21]. However, the regulatory mechanism of this TetR-type regulator on antibiotic resistance, oxidative stress, heavy metals, and aromatic catabolism has not been not been fully elucidated. Thus, an in-depth analysis of the regulatory effects of TetR family regulators on multiple environmental stimuli is vital.

The genome of *C. glutamicum* contains several gene clusters encoding enzymes related to aromatic compounds catabolism. Therefore, *C. glutamicum* can utilize a large variety of aromatic compounds as the sole source of carbon for growth [1,22]. Therefore, we propose that *C. glutamicum* may also harbor a TetR homolog that play a role in response to aromatic compounds (including phenolic compounds). Our previous study demonstrated the crucial role of *C. glutamicum* CssR, a member of the TetR family, in a variety of stress responses [23], which prompted us to investigate whether CssR is also related to the response to phenolic compounds. In this study, we found that compared with those in the WT, the β-galactosidase activity and the relative mRNA levels of the genes related to phenolic compound degradation were increased in the Δ*cssR* strain, and a high sensitivity to phenolic compound stress was shown in the Δ*cssR* strain. In addition, the CssR was found to indirectly negatively control the genes involved in phenolic compound degradation. To our knowledge, this is the first report demonstrating the ability of CssR to regulate the response to phenolic compounds.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The plasmids and bacterial strains used in this study are shown in Supplementary Table S1. *C. glutamicum* and *Escherichia coli* were grown at 30 and 37 °C, respectively, in Luria-Bertani (LB) media as previously reported [24]. Sorbitol-containing brain-heart broth (BHIS) (0.5 M) medium was used for producing *C. glutamicum* mutants [24]. Mineral salts medium (MM) containing glucose (Glu) or phenolic compound was used for morphological or expression analysis [24]. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was used to induce the expression of the pXMJ19 derivatives in *C. glutamicum*. The *lacZY* fusion reporter plasmids were subsequently transformed into relevant *C. glutamicum* strains by electroporation to produce chromosomal fusion reporter strains. When needed, antibiotics were added to the medium as previously reported [24].

2.2. Plasmid construction

The primers used in this study are listed in Supplementary Table S2.

The fusion reporter vector pK18*mobsacB*-$P_{ncgl0283}$*::lacZY* was produced by cloning an overlap PCR product into pK18*mobsacB* to maintain the expression of the β-galactosidase *lacZY* reporter gene under the *ncgl0283* promoter DNA (corresponding to nucleotides +15 to −545 relative to the translational start codon (GTG) of the *ncgl0283* gene) [7]. First, the *lacZY* DNA fragment and the 560-bp promoter DNA fragment of *ncgl0283* were amplified with the primers lacZY-F1/lacZY-R and PNCgl0283-F1/PNCgl0283-R1, respectively. Second, the $P_{ncgl0283}$*::lacZY* overlap PCR fragments were generated by overlap PCR using two template products from the first round of PCR and the primer pair PNCgl0283-F1/lacZY-R; the fragments were subsequently digested with *Sma*I/*Pst*I and inserted into the *Sma*I/*Pst*I-restricted pK18*mobsacB* [7]. The other *lacZY* fusion reporter vectors used in this study were constructed via a similar method [7]. The fidelity of all the constructs was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

2.3. Protein expression and purification

The protein expression and purification methods for CssR were performed as described in previous studies [23]. *E. coli* BL21 (DE3)

(pET28a-*cssR*) strain was grown in KAN (50 μg/ml)-containing LB medium at 37 °C to an $OD_{600\ nm}$ of 0.5 and induced with 0.5 mM IPTG for an additional 12 h at 22 °C. After the cell pellet was harvested via centrifugation and disintegrated by sonification, fractured mixtures were centrifuged at 15,000×*g* for 60 min and then His_6-tag CssR in the supernatant was purified using Ni-nitrilotriacetic acid (NTA)-agarose chromatography (Novagen, Madison, WI). The purified CssR was detected as a single 27-kDa band by Coomassie blue staining and SDS-PAGE. The resultant proteins were dialyzed with PBS and stored at −80 °C until use.

2.4. Survival assays

To measure the response to various phenolic compounds, the experiment was performed according to our previous studies [23,25]. The percentage survival was calculated as follows: [(CFU ml^{-1} after challenge under different stresses)/(CFU ml^{-1} before stress challenge)] × 100.

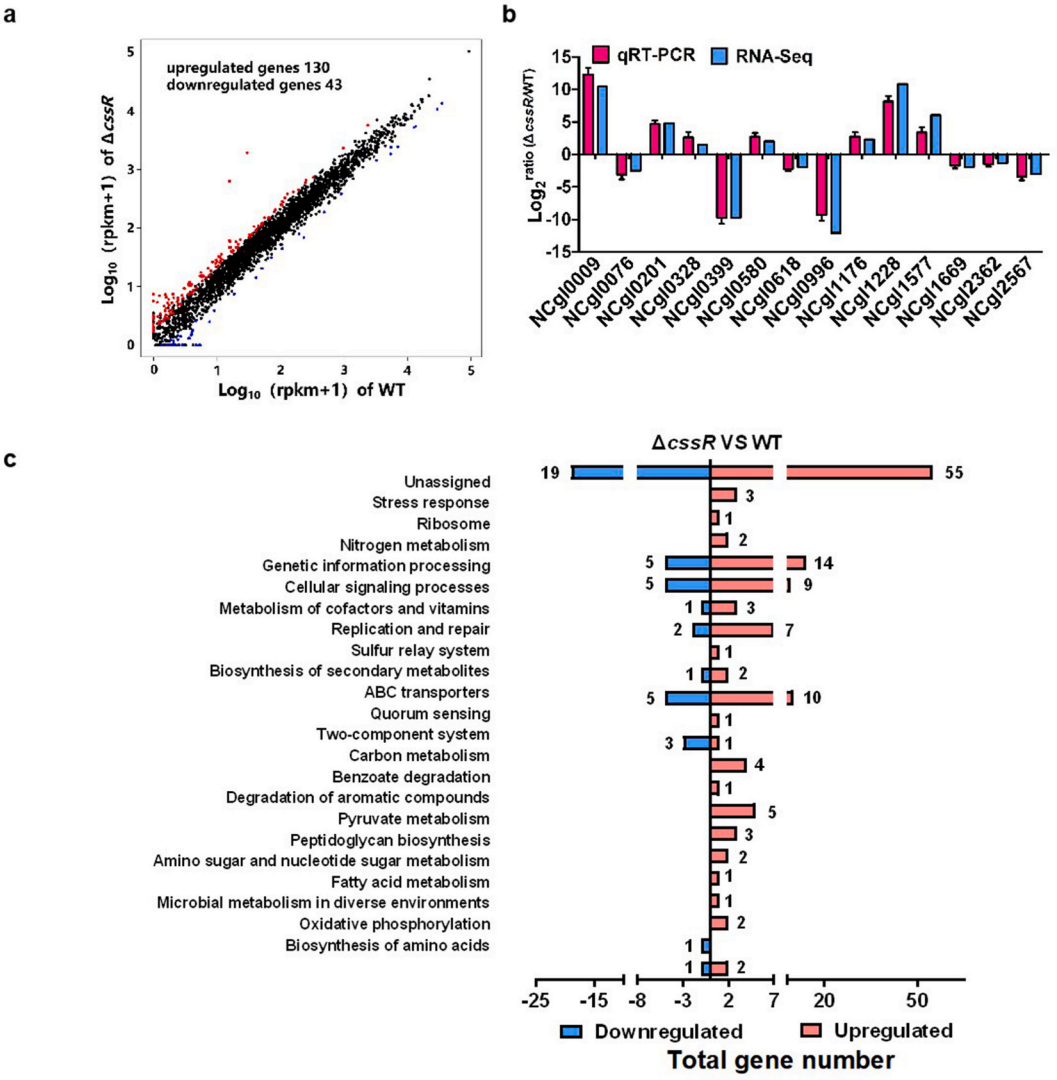

Fig. 1. RNA-seq analysis of CssR regulated genes in *Corynebacterium glutamicum*. (**a**) Scatter plot of differentially expressed genes. The genes with significant differences were indicated by red (upregulation) and blue dots (downregulation). (**b**) Relative transcript levels of selected potential CssR-dependent genes in *C. glutamicum* Δ*cssR*/*C. glutamicum* RES167 parental strain (WT) measured by qRT-PCR and transcriptomic analyses. 14 representative genes were chosen to validate the RNA-Seq data by qRT-PCR. The red bars represented the log_2 conversion multiple of the qRT-PCR values obtained for three biological replicates. The blue bars represented RNA-Seq data. The results were the average of three independent experiments; the error bars indicated the standard deviation (SD). (**c**) KEGG pathway analysis of differentially expressed genes (*cssR* mutant vs wild-type). The blue and red bars represent down- and up-regulated genes, respectively, and the numeric labels represent the number of genes related to that pathway.

Table 1
Genome-wide comparison of mRNA levels in *C. glutamicum cssR mutant (ΔcssR)* and *C. glutamicum* RES167 parental strain (WT) using RNA-seq analysis.

Accession no.	Gene name	Predicted function	Fold change[a]	*p*-value[b]
Genes with an enhanced mRNA level in Δ*cssR* mutant				
NCgl0009		Transcriptional regulator	10.40	8.0E-04
NCgl0010		Hypothetical protein	2.67	0.01
NCgl0014		Hypothetical protein	1.26	2.79E-04
NCgl0015		LysR family transcriptional regulator	1.58	5.35E-06
NCgl0018		Protein-disulfide isomerase	1.16	0.02
NCgl0052		Hypothetical protein	1.19	1.3E-03
NCgl0082		MarR family transcriptional regulator	1.84	4.0E-04
NCgl0084		Urease subunit beta	1.43	3.74E-03
NCgl0097		Hypothetical protein	9.89	0.01
NCgl0108		Mannitol 2-dehydrogenase	1.26	0.04
NCgl0116		Hypothetical protein	1.36	2.58E-03
NCgl0122		Hypothetical protein	2.04	0.02
NCgl0154		GntR family transcriptional regulator	1.16	4.69E-04
NCgl0173		ArsR family transcriptional regulator	1.48	0.01
NCgl0201		Hypothetical protein	4.83	4.27E-06
NCgl0204		Hypothetical protein	2.54	5.0E-04
NCgl0227		Hypothetical membrane protein	1.24	3.29E-04
NCgl0231	*malE*	Malic enzyme	1.52	0.03
NCgl0242*		Glutamine amidotransferase	1.07	1.26E-03
NCgl0243*		UDP-N-acetylmuramyl tripeptide synthase	1.10	5.37E-04
NCgl0268		Two-component system, response regulator	1.42	0.03
NCgl0270		Hypothetical protein	1.71	0.02
NCgl0279		Acyl-CoA synthetase	1.44	0.02
NCgl0280		MarR family transcriptional regulator	1.64	1.38E-03
NCgl0283		Glutaryl-COA dehydrogenase	2.29	1.55E-05
NCgl0295		Hypothetical protein	1.24	2.62E-03
NCgl0308		Uncharacterized phage-associated protein	1.59	3.82E-07
NCgl0328		Nitroreductase	1.45	7.61E-06
NCgl0348		Transposase	3.26	0.03
NCgl0354		Acetyltransferase	1.50	7.60E-06
NCgl0358		XRE family transcriptional regulator	1.4	5.28E-06
NCgl0393		Hypothetical protein	2.80	0.02
NCgl0400	*pspH*	Phosphoserine phosphatase	1.24	3.06E-03
NCgl0405		Transcriptional regulator	1.75	3.04E-06
NCgl0411		Iron (III) transport system ATP-binding protein	1.74	3.27E-05
NCgl0485		Acetyl-coa hydrolase	3.58	0.02
NCgl0498		Hypothetical protein	1.32	3.42E-05
NCgl0545		Hypothetical protein	2.01	2.0E-03
NCgl0580		Multidrug DMT transporter permease	2.01	0.02
NCgl0597		Phytoene dehydrogenase	1.31	1.93E-03
NCgl0609		D-methionine transport system ATP-binding protein	1.7	6.35E-04
NCgl0629		Methylisocitrate lyase	1.48	1.20E-06
NCgl0630		Citrate synthase	1.28	9.92E-05
NCgl0653		Hypothetical protein	4.56	2.06E-04
NCgl0664		2-methylcitrate dehydratase	1.25	6.60E-05
NCgl0704		Helicase	1.18	1.71E-03
NCgl0760		Hypothetical protein	1.16	7.42E-03
NCgl0822		Hypothetical ABC transport system ATP-binding protein	9.78	0.03
NCgl0862		Hypothetical transposase	1.38	1.28E-05
NCgl0863		Hypothetical transposase	1.34	2.59E-04
NCgl0867		Hypothetical transposase	1.38	1.28E-05
NCgl0868		Hypothetical transposase	1.34	2.59E-04
NCgl0871		Mg-dependent DNase	1.16	4.75E-04
NCgl0942	*pspC*	Stress-responsive transcriptional regulator	1.65	0.01
NCgl0992		Hypothetical protein	11.00	2.77E-04
NCgl1002		Hypothetical protein	1.52	2.784–04
NCgl1005		Nucleoside-diphosphate-sugar epimerase	2.00	1.21E-04
NCgl1024	*nadA*	Quinolinate synthase	1.71	4.37E-05
NCgl1026		DMT family transporter	1.62	4.83E-06
NCgl1032	*podA*	4-hydroxybenzoate 3-monooxygenase	1.7	4.0E-03
NCgl1038		Hypothetical protein	1.23	7.15E-04
NCgl1068		Hypothetical protein	1.24	3.30E-04
NCgl1069		Hypothetical protein	1.25	1.86E-04
NCgl1111		Protocatechuate 3,4-dioxygenase beta subunit	1.23	0.04
NCgl1171		Hypothetical protein	1.21	6.28E-03
NCgl1176		ABC Transport system substrate-binding protein	2.29	0.01

(*continued on next page*)

Table 1 (*continued*)

Accession no.	Gene name	Predicted function	Fold change[a]	*p*-value[b]
NCgl1180		Hypothetical protein	1.96	5.69E-09
NCgl1190		Hypothetical protein	1.95	0.01
NCgl1204		ABC transporter duplicated ATPase	1.27	1.75E-03
NCgl1212		8-hydroxy-5-deazaflavin: NADPH oxidoreductase	1.26	1.83E-03
NCgl1228		Nitrate/nitrite transport system substrate-binding protein	10.83	2.42E-04
NCgl1256		Hypothetical protein	12.65	9.73E-03
NCgl1259		Hypothetical protein	1.17	1.07E-03
NCgl1284		Hypothetical protein	1.20	7.37E-04
NCgl1286		Hypothetical protein	1.21	1.08E-03
NCgl1289		Hypothetical protein	1.19	0.02
NCgl1295		Hypothetical protein	12.18	8.07E-04
NCgl1296		Hypothetical protein	1.92	0.05
NCgl1300		Major facilitator superfamily permease	1.83	6.98E-04
NCgl1318		NAD(P)H dehydrogenase	1.30	2.78E-05
NCgl1379	*zupT*	Zinc transporter	1.28	6.97E-05
NCgl1427		Hypothetical protein	1.74	6.83E-03
NCgl1473		Hypothetical protein	2.67	0.03
NCgl1485		Hypothetical protein	2.35	0.02
NCgl1563		ArsR family transcriptional regulator	1.28	0.01
NCgl1564		Iron complex transport system permease protein	1.72	5.97E-07
NCgl1576		ABC transporter permease	5.42	5.97E-60
NCgl1577		ABC transporter ATP-binding protein	6.05	4.24E-70
NCgl1579		CBS domain-containing protein	2.04	3.32E-05
NCgl1580		Coenzyme F420-dependent N5, N10-methylene tetrahydromethanopterin reductase	2.00	1.81E-05
NCgl1589		Hypothetical protein	1.59	2.31E-05
NCgl1652		Hypothetical protein	1.74	0.03
NCgl1671		Hypothetical protein	1.85	4.14E-04
NCgl1751		Hypothetical protein	1.17	1.84E-03
NCgl1816		Hypothetical protein	2.95	3.32E-04
NCgl1881		Hypothetical protein	2.06	1.17E-06
NCgl1936		Hemin transport system permease protein	1.89	0.04
NCgl1965	*thiF*	Thiamine biosynthesis protein	9.50	0.02
NCgl1988		Hypothetical protein	1.48	4.24E-06
NCgl2034		ArsR family transcriptional regulator	1.23	2.20E-04
NCgl2182		Hypothetical protein	1.68	7.76E-04
NCgl2334		Transposase	2.66	0.02
NCgl2379		Integrase	10.09	0.02
NCgl2412		Hypothetical protein	1.26	5.93E-05
NCgl2488		Hypothetical protein	1.21	2.04E-04
NCgl2566		Threonine efflux protein	3.09	4.22E-03
NCgl2584		Antibiotic biosynthesis monooxygenase	1.16	9.49E-03
NCgl2593		Hypothetical protein	3.31	3.1E-07
NCgl2632		Hypothetical protein	1.38	9.97E-06
NCgl2637		Multicomponent Na+:H+ antiporter subunit F	3.56	1.02E-03
NCgl2638		Multicomponent Na+:H+ antiporter	1.51	2.51E-03
NCgl2704		Adenosylhomocysteine nucleosidase	1.62	2.45E-05
NCgl2713		Permease	1.39	0.04
NCgl2736		Inosine-uridine nucleoside N-ribohydrolase	1.28	6.74E-04
NCgl2785	*uppP*	Undecaprenyl-diphosphatase	1.92	0.03
NCgl2786		Putative transposase	2.17	7.72E-04
NCgl2807		Glycerophosphoryl diester phosphodiesterase	1.32	2.79E-05
NCgl2817		L-lactate dehydrogenase	1.23	1.39E-04
NCgl2844		23S RNA-specific pseudouridylate synthase	1.50	3.00E-05
NCgl2858		Hypothetical protein	1.31	1.18E-03
NCgl2861		Hypothetical protein	1.67	2.21E-03
NCgl2868		Crp/Fnr family transcriptional regulator	11.12	8.07E-04
NCgl2869		Copper chaperone	1.22	1.75E-04
NCgl2882		Hypothetical protein	1.28	6.59E-03
NCgl2899	*rtcB*	tRNA-splicing ligase	1.37	1.70E-04
NCgl2920	*genR*	Gentisate 1,2-dioxygenase	1.16	3.51E-03
NCgl2921*	*nagR*	IclR-type regulator	0.91	0.01
		regulator		
NCgl2923		3-hydroxybenzoate 6-monooxygenase	2.47	3.43E-04
NCgl2925		Hypothetical protein	1.24	5.84E-05
NCgl2935		ABC-2 type transport system ATP-binding protein	1.17	2.21E-04
NCgl2947		Short chain dehydrogenase	1.29	2.48E-03
NCgl2952		Maleylacetate reductase	2.78	3.12E-06
NCgl2960		Hypothetical protein	9.63	5.17E-03
Genes with a decreased mRNA level in Δ*cssR* mutant				

(*continued on next page*)

Table 1 (*continued*)

Accession no.	Gene name	Predicted function	Fold change[a]	*p*-value[b]
NCgl0076		Hypothetical protein	−2.51	0.03
NCgl0146		Methylated DNA-protein cysteine methyltransferase	−1.44	4.21E-04
NCgl0167		LacI family transcriptional regulator	−2.44	6.46E-05
NCgl0171		Cold shock protein	−1.53	6.03E-10
NCgl0281		Short-chain dehydrogenase	−2.00	7.72E-04
NCgl0303		Cold shock protein	−1.26	1.56E-07
NCgl0314		Zn-dependent hydrolase or glyoxylase	−1.39	1.67E-08
NCgl0350		Acyltransferase	−1.28	3.04E-07
NCgl0399		Hypothetical protein	−11.85	9.73E-03
NCgl0424	*resA*	Thiol-disulfide oxidoreductase	−1.37	1.18E-06
NCgl0428		Hypothetical protein	−2.46	0.05
NCgl0509		Energy-coupling factor transport system substrate-specific component	−1.61	3.37E-06
NCgl0560		Hypothetical protein	−2.51	4.40E-04
NCgl0618		Iron complex transport system substrate-binding protein	−1.98	3.06E-04
NCgl0645		Iron complex transport system ATP-binding protein	−1.47	1.43E-04
NCgl0687		Nitrilotriacetate monooxygenase	−1.88	0.04
NCgl0759		Hypothetical protein	−10.19	9.73E-03
NCgl0823		ParR family transcriptional regulator	−9.67	0.04
NCgl0860		Hypothetical protein	−9.26	5.17E-03
NCgl0996		Hypothetical protein	−12.07	4.4E-04
NCgl1110*	*rolR*	TetR-type repressor, RolR	−0.92	8.7E-03
NCgl1455		Protein-tyrosine-phosphatase	−2.16	2.66E-03
NCgl1491		Hypothetical protein	−10.93	5.17E-03
NCgl1644		Hypothetical protein	−10.89	9.73E-03
NCgl1669		Putative DNA primase/helicase	−1.99	7.72E-04
NCgl1684		Hypothetical protein	−8.50	0.04
NCgl1819		Hypothetical protein	−10.59	0.04
NCgl1847		Hypothetical protein	−9.30	2.77E-03
NCgl1875		Glutamate transport system ATP-binding protein	−1.18	5.29E-07
NCgl1991		Hypothetical protein	−9.85	0.04
NCgl2149		Hypothetical protein	−2.16	3.57E-07
NCgl2308*	*pcaR*	IclR-type regulator	−0.89	0.04
NCgl2333		Hypothetical protein	−1.19	4.21E-07
NCgl2362		Hemoglobin-like protein	−1.31	4.85E-08
NCgl2400		Hypothetical protein	−3.05	1.58E-03
NCgl2406		Major facilitator superfamily permease	−1.19	1.44E-06
NCgl2464		Putative ABC transport system permease protein	−1.77	2.55E-12
NCgl2469		Hypothetical membrane protein	−1.38	1.38E-08
NCgl2514		Proton-dependent oligopeptide transporter	−1.63	0.02
NCgl2517	*ompR*	Two-component system, OmpR family, sensor kinase	−1.23	1.90E-10
NCgl2522		Major facilitator superfamily permease	−1.32	4.12E-08
NCgl2567		ArsR family transcriptional regulator	−3.03	6.89E-03
NCgl2744		Hypothetical protein	−12.11	1.33E-04
NCgl2752		Hypothetical protein	−1.17	0.01
NCgl2845		Hypothetical protein	−1.46	2.38E-09

The mRNA ratios represent mean values from three independent experiments starting from independent cultures. The strains were cultivated in the LB medium, and mRNA was isolated in the exponential growth phase. [a] Fold change was defined by $\log_2$(the gene expression ratio of the *Corynebacterium glutamicum* Δ*cssR* mutant to *Corynebacterium glutamicum* RES167 parental strain (WT)). Fold change values of higher than +1.16 or lower than −1.16 (corresponding to mRNA ratio Δ*cssR*/WT of 2.23 and 0.45, respectively) were considered to be significant. [b]Significance determined by *p*-value ($p < 0.05$). The mRNA ratios for the genes *ncgl0242*, *ncgl0243*, *ncgl1110*, *ncgl2308* and, *ncgl2921* were marked with an asterisk, as the mRNA ratios (*ncgl0242*, *ncgl0243*, *ncgl1110*, *ncgl2308* and, *ncgl2921*) were not within the defined range. However, the genes were included, as they were part of operons of which the other genes fulfilled the selected criteria. The genes are ordered according to their position on the genome.

2.5. RNA sequencing (RNA-seq) experiment

RNA-seq was performed according to the methods described in previous reports [23,25]. Total RNA was extracted from the exponentially growing *C. glutamicum* RES167 parental strain and the Δ*cssR* mutant (3 biological replicates) via the RNeasy Mini Kit (Qiagen, Hilden, Germany) along with the DNase I Kit (Sigma-Aldrich, Taufkirchen, Germany). RNA degradation and contamination were monitored on 1% agarose gels, RNA purity was checked using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA), and RNA integrity was assessed using a Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 5 μg of RNA per sample was used as input material in RNA sample preparations for subsequent cDNA library construction. All 6 samples had RIN values above 7.0. Sequencing libraries were generated using an Illumina HiSeq™ 2000 RNA Sample Preparation Kit (Illumina, San Diego, USA) following the manufacturer's recommendations and four index codes were added to attribute the sequences to each sample. Differential expression analysis was performed using the NOIseq method (Sonia Tarazona 2100). *P* values were adjusted using the Benjamini & Hochberg method. A corrected *P*-value of 0.05 and a $\log_2$ (fold change) of 1.16 were set as the thresholds for significantly differential expression. Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was performed with the GOseq R

package, in which the gene length bias was corrected. GO terms with corrected *P* values less than 0.05 were considered to indicate significant enrichment of DEGs.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using a previously described method [7,23].

2.7. β-galactosidase assay

The *lacZY* fusion reporter strains were subsequently grown in LB broth medium to $OD_{600\ nm}$ of 0.6–0.7 for determination of β-galactosidase activity. For phenolic compound research, the WT(pXMJ19), Δ*cssR*(pXMJ19), and Δ*cssR*(pXMJ19-cssR) strains were first grown in triplicate in 100 mM Glu-containing MM until the stationary phase and subsequently harvested and transferred into MM at a 1.0% inoculum concentration containing 2 mM Glu or different phenolic compounds. Overnight cultures of MM grown with Glu or phenolic compound were used for analysis of β-galactosidase activity. β-galactosidase activities were assayed with o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate.

2.8. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

qRT-PCR was performed as previously described in a CFX Connect RealTime PCR Detection System (Bio-Rad) in 20-μl reaction volumes using iQ SYBR green Supermix (Bio-Rad) with the primers listed in Table S2 in the supplemental material at a final concentration of 200 nM each [23]. Relative expression levels were estimated using the $2^{-\Delta\Delta CT}$ (where CT was the threshold cycle) method, and the 16S rRNA gene served as a reference for normalization [23].

3. Results

3.1. The impact of the cssR mutation determined by transcriptome analysis

RNA-seq-based transcriptomic experiments were conducted to elucidate the transcriptional changes caused by the deletion of *cssR*. An overview of the changes in gene expression is shown in Fig. 1a. A total of 173 genes exhibited at least 2.23-fold alterations in the mRNA ratio in cells in the exponential growth phase cultivated in LB medium; these are listed in Table 1. Table 1 also included a few genes that did not meet the criteria but were part of operons containing genes that met the criteria.

43 genes exhibited a ≥2.23-fold decrease in the mRNA concentration in the Δ*cssR* mutant. This group included, for example, the *ncgl0146* gene coding for a methylated DNA-protein cysteine methyltransferase; the *ncgl0687* gene coding for a nitrilotriacetate monooxygenase; the *ncgl0171* and *ncgl0303* genes coding for cold shock proteins; the *ncgl0167* gene coding for a LacI family transcriptional regulator; the *ncgl2567* gene coding for an ArsR family transcriptional regulator; and the *ncgl0860*, *ncgl1644* and other genes coding for hypothetical proteins.

A total of 130 genes were found to have a ≥2.23-fold increase in mRNA concentration in Δ*cssR* mutant. The group showing strong increases included, for example, the *ncgl0283* and *ncgl2952* genes related to the degradation of phenolic compounds; the *ncgl0009* gene related to global regulatory pathways; and the *ncgl1176*, *ncgl1576*, and *ncgl1577* genes coding for ABC-type transporter systems.

To verify the results obtained by the RNA-seq-based transcriptomic analysis, qRT-PCR was performed for 14 representative genes with altered mRNA levels in the Δ*cssR* mutant, namely, *ncgl0076*, *ncgl0399*, *ncgl0618*, *ncgl0996*, *ncgl1669*, *ncgl2362* and *ncgl2567*, as examples of down-regulated genes; *ncgl0009*, *ncgl0201*, *ncgl0328*, *ncgl0580*, *ncgl1176*, *ncgl1228* and *ncgl1577* as examples of up-regulated genes. As shown in Fig. 1b, the $\log_2$-transformed mean values of qRT-PCR from three biological replicates for all genes were very consistent with the $\log_2$-transformed fold changes in RNA-seq-based transcriptomic data from three biological replicates, thus confirming the results of the RNA-seq-based transcriptomic experiments. KEGG pathway analysis performed to determine the functions of the differentially expressed genes. Twenty-three pathways were identified among the DEGs, including those related to the stress response, degradation of aromatic compounds, cell wall/membrane/envelope biogenesis, genetic information processing, cellular signaling processes, and ABC transporters (Fig. 1c). The percentage of the differentially expressed genes among the predicted genes in each KEGG pathway was greater for "cellular signaling processes", "replication and repair", "genetic information processing" and "stress response" (Fig. S1). Taken together, these results provide an overview of the transcriptome analysis of the Δ*cssR* mutation.

3.2. Differentially expressed genes related to the degradation of aromatic compounds, stress response, cell wall/membrane/envelope biogenesis, and global regulatory pathways

Through the transcriptome analysis and KEGG pathway analysis of CssR-regulated genes, we investigated the genes involved in the degradation of aromatic compounds, the stress response, cell wall/membrane/envelope biogenesis, and global regulatory pathways. Therefore, the relevant genes were further analyzed separately, and the results showed that the expression ratios of all relevant genes were greater for the Δ*cssR* mutant than for the *C. glutamicum* RES167 parental strain (WT) (Fig. S2). The expression levels of the genes involved in the degradation of aromatic compounds, the stress response, and cell wall/membrane/envelope biogenesis were between 1 and 3, and the expression levels of the genes involved in global regulatory pathways were between 1 and 11. Therefore, the deletion of *cssR* could have an impact on these processes.

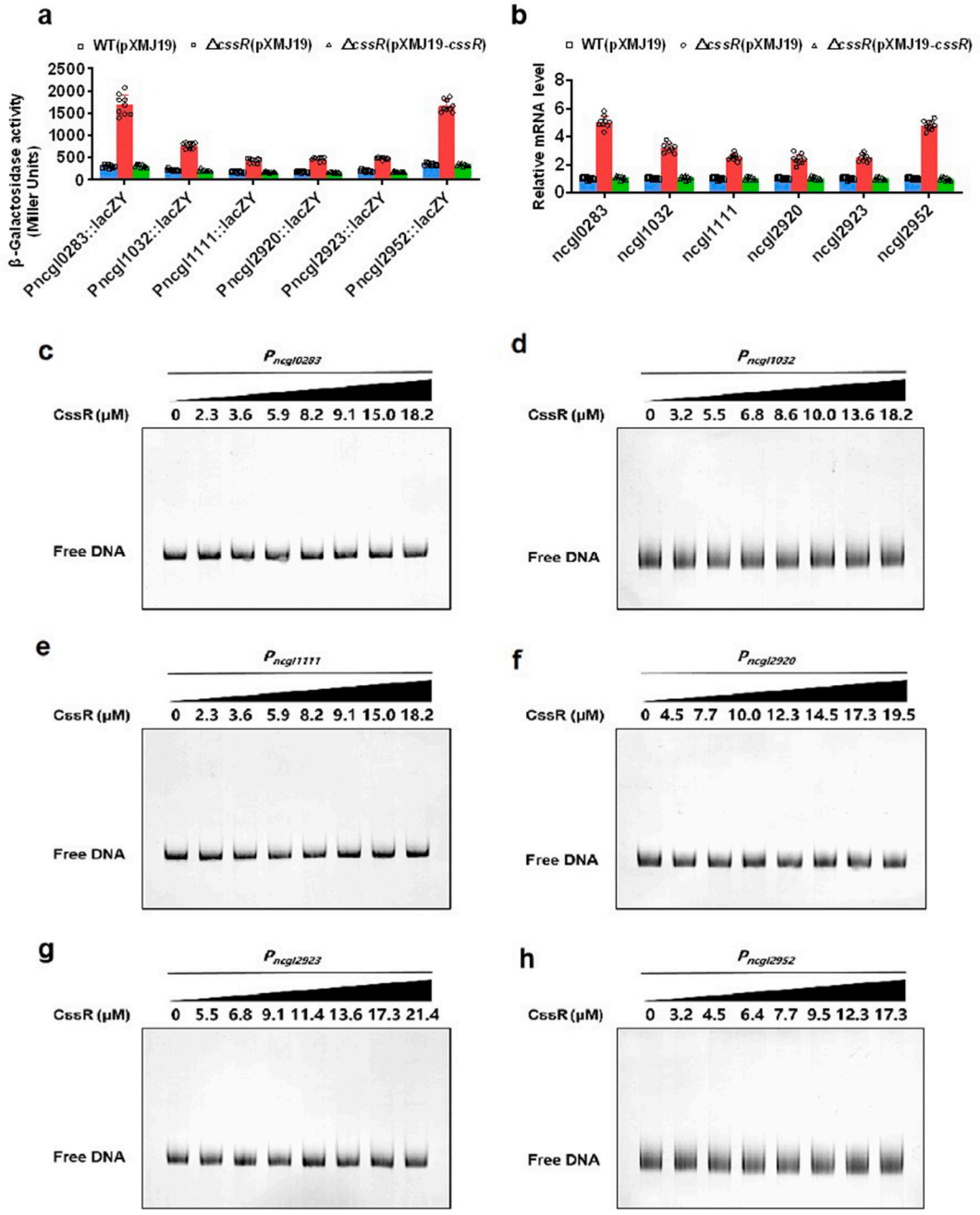

Fig. 2. Negative regulation of the phenolic compounds degrading genes by CssR. (**a**) β-galactosidase activities of the promoters of the phenolic compounds degrading-genes in WT(pXMJ19), Δ*cssR*(pXMJ19) mutant, and complementary Δ*cssR*(pXMJ19-cssR). (**b**) Quantitative RT-PCR analyses of the expression of the phenolic compounds degrading-genes in WT(pXMJ19), Δ*cssR*(pXMJ19) mutant, and complementary Δ*cssR*(pXMJ19-cssR) strains. The levels of gene expression in each sample were calculated as the fold expression ratio after normalization to 16S rRNA gene transcript levels. The mRNA levels were presented relative to the value obtained from WT(pXMJ19) cells. The relative transcript level of WT(pXMJ19) strains was set at a value of 1.0. For a and b, the data were shown as the averages of three independent biological experiments (three technical replicates were taken for each biological experiment), and error bars indicated the SDs from three independent experiments, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (**c-h**) CssR bound indirectly to the promoter regions of the phenolic compound-degrading genes. EMSA was performed to analyze the interactions between CssR and promoters of *ncgl0283* ($P_{ncgl0283}$), *ncgl1032* ($P_{ncgl1032}$), *ncgl1111* ($P_{ncgl1111}$), *ncgl2920* ($P_{ncgl2920}$), *ncgl2923* ($P_{ncgl2923}$), and *ncgl2952* ($P_{ncgl2952}$). These genes are involved in the degradation of phenolic compounds. The raw figures for EMSA were provided in the Supplementary Figs. S5–10.

3.3. CssR negatively regulates the expression of genes related to the degradation of phenolic compounds

To verify the role of CssR in the expression of genes related to the degradation of phenolic compounds, the fusion of the promoter to the *lacZY* reporter gene was introduced into the chromosomes of WT (pXMJ19), Δ*cssR* (pXMJ19), and Δ*cssR* (pXMJ19-cssR). The β-galactosidase activities of the promoters of the phenolic compound-degrading genes were quantitatively measured (Fig. 2a). Compared to that in the WT (pXMJ19) strain, the activity of Δ*cssR* (pXMJ19) increased significantly, and this increase could be reversed in the complementary strain Δ*cssR* (pXMJ19-cssR), confirming that CssR negatively regulates the expression of genes related to the degradation of phenolic compounds. The negative regulation of genes related to the degradation of phenolic compounds by CssR was further confirmed via qRT-PCR (Fig. 2b), which revealed that the expression of the phenolic compound-degrading genes, for example, *ncgl0283*, *ncgl1032*, *ncgl1111*, *ncgl2920*, *ncgl2923*, and *ncgl2952*, was increased in Δ*cssR*, and this increase could be reversed in the complementary strain Δ*cssR* (pXMJ19-cssR). To test whether the CssR regulatory effect was direct, EMSA was performed using purified His_6-CssR and the phenolic compound-degrading gene promoter regions [*ncgl0283* ($P_{ncgl0283}$), *ncgl1032* ($P_{ncgl1032}$), *ncgl1111* ($P_{ncgl1111}$), *ncgl2920* ($P_{ncgl2920}$), *ncgl2923* ($P_{ncgl2923}$), and *ncgl2952* ($P_{ncgl2952}$)]. His_6-CssR and its promoter regions showed no detectable binding (Fig. 2c–h); therefore, CssR was bound indirectly to the promoters of phenolic compound-degrading genes. Taken together, these results suggest that CssR indirectly negatively regulates the expression of genes related to the degradation of phenolic compounds.

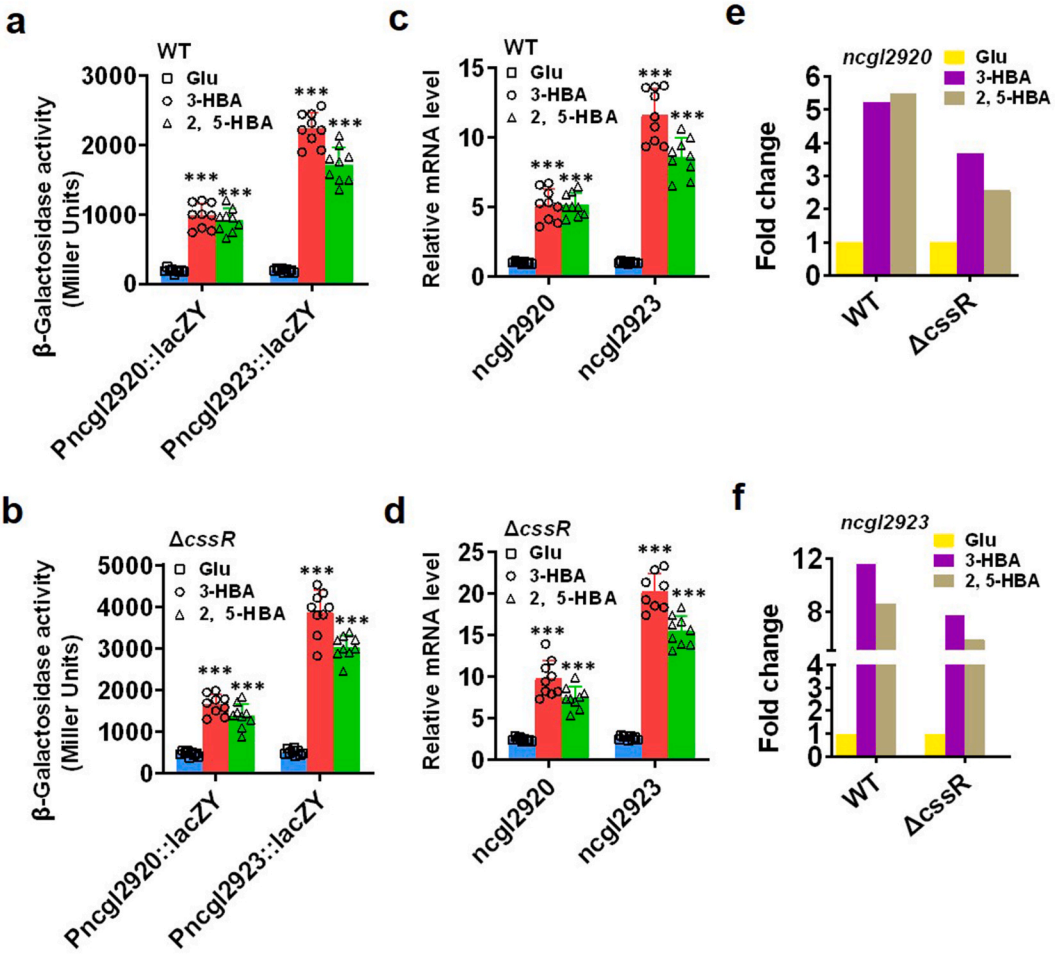

Fig. 3. Expression of *ncgl2920* and *ncgl2923* was induced by aromatic compounds in a CssR-dependent manner. (**a and b**) β-galactosidase analysis of the promoter activities of *ncgl2920* and *ncgl2923* was performed using the transcriptional chromosomal fusion reporter expressed in WT and Δ*cssR* mutant exposed to different phenolic compounds. (**c and d**) qRT-PCR assay was performed to analyze the expression of *ncgl2920* and *ncgl2923* in indicated strains exposed to different phenolic compounds. For a-d, the data were shown as the averages of three independent biological experiments (three technical replicates were taken for each biological experiment), and error bars indicated the SDs from three independent experiments. ***P < 0.001. The mRNA levels were presented relative to the value obtained from WT cells without treatment. Relative transcript levels of WT strains without stress treatment were set at a value of 1.0 (**e and f**). Fold change of the transcription level was calculated according to the data from c and d by the equation: the value obtained from the strains exposed to stress/the value obtained from the corresponding strains cultivated glucose (Glu). The data were shown as the average of three independent experiments.

3.4. Expression of genes was induced by phenolic compounds in a CssR-dependent manner

ncgl2920 and *ncgl2923* are important key enzymes involved in the degradation of several phenolic compounds, such as 3-hydroxybenzoic acid (3-HBA) and 2,5-dihydroxybenzoic acid (2, 5-HBA). Thus, we used these phenolic compounds as effector molecules to test the promoter activities of *ncgl2920* and *ncgl*2923 by β-galactosidase analysis in the WT strain and Δ*cssR* mutant. As shown in Fig. 3a and b, β-galactosidase levels in *ncgl2920* and *ncgl2923* promoters were very low for 2 mM glucose (Glu) in the WT, but the levels were greater in the Δ*cssR* mutant. After the addition of 3-HBA and 2,5-HBA as inducers, the promoter activities of *ncgl2920* and *ncgl2923* were increased. However, the increase in the *ncgl2920* and *ncgl2923* promoter activities in the WT strain was more significant than that in the mutants. Similar results were also observed at the mRNA transcriptional level by qRT-PCR analysis (Fig. 3c and d). The relative transcript levels of the WT strains cultured only with Glu were set at a value of 1.0. The other mRNA levels are presented relative to the values obtained from the WT without stress. The mRNA levels of *ncgl2920* and *ncgl2923* were greater in the Δ*cssR* strain than in the WT strain with Glu, but the levels increased significantly in the WT strain after the inducers were added. For example, the fold increase in mRNA levels of *ncgl2920* exposed to 3-HBA was greater than 5 in the WT but less than 4 in the Δ*cssR*. Therefore, the relative mRNA level of *ncgl2920* induced by CssR was equal to the level in the WT strain minus the level in the Δ*cssR* strain (Fig. 3e and f). The same result was observed for other genes related to the degradation of phenolic compounds (*ncgl0283, ncgl1032*, *ncgl1111*, and *ncgl2952*) by β-galactosidase analysis and qRT-PCR assay (Fig. S3). These results indicated that CssR responded to the induction of phenolic compounds and was able to regulate these genes.

3.5. The ΔcssR mutant has a high survival rate in response to phenolic compound stress

Phenolic compounds have become important environmental pollutants due to their difficultly degrading chemicals and wide use, which has caused increasing pressure on eco-environmental systems. Biodegradation, especially the degradation of phenolic compounds by microorganisms, is one of the most cost-effective methods for managing such pollution. *C. glutamicum* can grow using a variety of phenolic compounds as sole carbon and energy sources. There are multiple different metabolic pathways associated with phenolic compounds within cells. *cssR* was found to be associated with phenolic compound degradation genes. Thus, to assess the role of CssR in response to stress by phenolic compounds, we tested the sensitivity of the of Δ*cssR* strain phenotype to various phenolic compounds by survival assays (Fig. 4). As shown in Fig. 4, the Δ*cssR* strain exhibited increased resistance to phenolic compounds compared to the WT strain. Moreover, survival rate of the complemented strain was similar to that of the WT strain.

3.6. CssR negatively regulates the expression of genes related to cell wall/membrane/envelope biogenesis

To verify the role of CssR in the expression of genes related to cell wall/membrane/envelope biogenesis, the β-galactosidase activities were quantified (Fig. S4a). Compared to that in the WT (pXMJ19) strain, the activity of Δ*cssR* (pXMJ19) increased significantly, and this increase could be reversed in the complementary strain Δ*cssR* (pXMJ19-cssR), confirming that CssR negatively regulates the expression of genes related to cell wall/membrane/envelope biogenesis. Quantitative RT-PCR analysis further confirmed the negative regulation of these genes by CssR (Fig. S4b), indicating that the expression of the cell wall/membrane/envelope biogenesis-related gene *ncgl0242*, *ncgl2785*, and *ncgl2807* increased in Δ*cssR*, and this increase could be reversed in the complementary strain Δ*cssR* (pXMJ19-cssR). To test whether the CssR regulatory effect was direct, EMSA was performed using His_6-CssR and the cell wall/membrane/envelope biogenesis-related gene promoter regions [*ncgl0242* ($P_{ncgl0242}$), *ncgl2785* ($P_{ncgl2785}$), and *ncgl2807* ($P_{ncgl2807}$)]. His_6-CssR and its promoter regions showed no detectable binding (Figs. S4c–e); therefore, CssR binds indirectly to cell wall/membrane/envelope biogenesis-related gene promoters. These results suggest that CssR indirectly negatively regulates the expression of

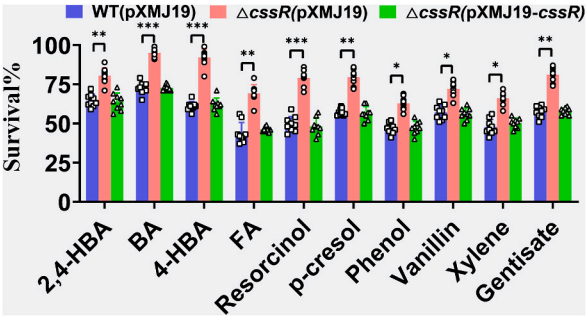

Fig. 4. Δ*cssR* mutant was highly sensitive to phenolic compound stress compared to WT. Survival of the WT (pXMJ19) strain (the *C. glutamicum* RES167 parental strain with the empty plasmid pXMJ19), Δ*cssR* (pXMJ19-cssR) mutant (the *cssR* deletion mutant expressing pXMJ19), and Δ*cssR* (pXMJ19-cssR) (the Δ*cssR* mutant expressing the WT *cssR* gene in the shuttle vector pXMJ19) was assessed after exposure to various phenolic compounds [4.26 mM 2, 4-dihydroxybenzoate (2,4-HBA), 0.39 mM benzoic acid (BA), 1.39 mM 4-hydroxybenzoate (4-HBA), 10 mM fumaric acid (FA), 6 mM resorcinol, and 7.5 mM *p*-cresol] for 30 min. The results were shown as the averages of three independent biological experiments (three technical replicates were taken for each biological experiment), and error bars indicated the SDs from three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

genes related to cell wall/membrane/envelope biogenesis.

4. Discussion

In this study, we investigated the regulatory function of the TetR-type sensor CssR (NCgl1578) in *C. glutamicum*. Transcriptomic analysis revealed that 173 genes exhibited at least 2.23-fold altered transcription in the *cssR*-deleted (Δ*cssR*) mutant which was primarily associated with the degradation of phenolic compounds, oxidative stress, cell wall/membrane/envelope biogenesis, and global regulatory pathways. The Δ*cssR* mutant was found to be more resistant to phenolic compounds, consistent with the upregulated expression of many phenolic compound degradation-related genes (*ncgl0283*, *ncgl1032*, *ncgl1111*, *ncgl2920*, *ncgl2923*, and *ncgl2952*). It has been shown in *C. glutamicum* that *ncgl0283*, *ncgl1032*, *ncgl1111*, *ncgl2920*, *ncgl2923*, and *ncgl2952* encode glutaryl-CoA dehydrogenase, 4-hydroxybenzoate 3-monooxygenase, resorcinol 4-hydroxylase (RolH), gentisate 1,2-dioxygenase, 3-hydroxybenzoate 6-monooxygenase, and maleylacetate reductase, respectively. A previous study revealed that the *rolRHMD* gene cluster (from *ncgl1110* to *ncgl1113*) was involved in resorcinol catabolism [17]. Despite no reports about these genes thus far, NCgl1032 and NCgl2952 showed high amino acid identities with RolH and RolM (NCgl1112), respectively [1]. Moreover, *ncgl2920* and *ncgl2923* play pivotal roles in the gentisate pathway. Deletion of *ncgl2920* and *ncgl2923* resulted in a significant decrease in the degradation activities of phenolic compounds [6,11,24]. These results indicated that the impact of the overproduction of NCgl0283, NCgl1032, NCgl1111, NCgl2920, NCgl2923, and NCgl2952 on the restoration of phenolic compounds damage should be very great in the Δ*cssR* mutant strain. Transcriptomic analysis revealed that many genes involved in the oxidative stress response, such as *ncgl0018*, *ncgl1212*, *ncgl2736* were upregulated in the Δ*cssR* mutant. It has been reported that NCgl0018 was vital for the survival of *C. glutamicum* under oxidative stress [26], indicating the impact of the overproduction of NCgl0018, NCgl1212, and NCgl2736 on the restoration of oxidative damage should be great in the Δ*cssR* mutant strain. These findings further confirmed our previous finding that the oxidant resistance of the of Δ*cssR* strain might be attributable to increased reducing power levels [23]. In addition, many genes involved in cell wall/-membrane/envelope biogenesis, including *ncgl0242*, *ncgl0243*, *ncgl2785*, and *ncgl2807*, were also found to be negatively controlled by CssR. Like *M. tuberculosis*, the cell envelope of *Corynebacterium glutamicum* can be divided into multiple layers: the cell membrane, the thicker peptidoglycan-arabinogalactan layer, the mycoacid layer, and the top layer [27]. This multilayer structure could enhance tolerance to a variety of adverse environmental factors. Compared to those in the WT, the cell walls and intact membranes in the Δ*cssR* mutant were thicker, indicating that CssR might play a protective role by influencing the structure of the cell envelope. Taken together, these results indicate that CssR plays an important role in stress resistance and adaptation for survival, thereby significantly expanding our knowledge of the functionality of TetR family transcription factors and providing new insights into the response of *Corynebacterium glutamicum* to phenolic compounds.

Data availability statement

All the data generated or analyzed during this study are included in the manuscript and its additional file. The RNA-seq data in this study have been deposited in the NCBI SRA under BioProject accession number PRJNA939092.

Ethics declarations

Review and/or approval by an ethics committee was not needed for this study.
Informed consent was not required for this study.

CRediT authorship contribution statement

Ju Zhang: Writing – original draft, Investigation, Data curation, Conceptualization. **Yuying Zhao:** Writing – original draft, Investigation, Formal analysis, Data curation. **Zhaoxin Peng:** Investigation. **MingFei Yang:** Investigation. **Wenyu Zou:** Investigation. **Xinyu Wu:** Investigation. **Chenghui Wang:** Investigation. **Meiru Si:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Can Chen:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31500087), Training Plan of Young Backbone Teachers in Colleges and Universities of Henan Province, China (2021GGJS139).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27929.

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