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# *LmGSTF3* Overexpression Enhances Cadmium Tolerance in *Lemna minor*

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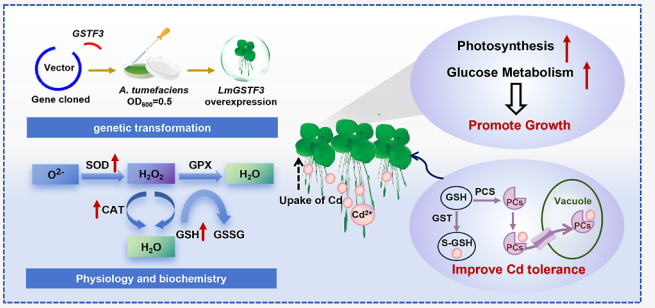
**ABSTRACT:** Glutathione S-transferase (GST) has been established to play an important role in regulating the responses of plants to stress, although its function and mechanisms of action in the cadmium (Cd)-tolerant *Lemna minor* remain unclear. In this study, we sought to identify a Cd-responsive *GST* gene from *Lemna minor* for functional analysis and mechanistic characterization. We accordingly identified a member of the *GST* gene family, *LmGSTF3*, which plays a positive role in adaptation of *Lemna minor* to Cd. Having successfully obtained overexpressing (OE) strains via genetic transformation, we established that these strains were characterized by elevated Cd tolerance compared with the wild-type strain, as evidenced by significant increases in growth rate, chlorophyll content, antioxidant enzyme activities, and Cd removal rate. At the transcriptome level, the OE strains were found to have a stronger regulatory ability in response to Cd, particularly with respect to photoprotection, antioxidant defense, and glycolytic metabolism, which may be key factors contributing to the Cd tolerance of *Lemna minor*. Our findings provide a basis for further elucidating the biochemical and molecular mechanisms underlying the Cd tolerance conferred by *GST* genes in *Lemna minor* and will potentially contribute to the utilization of *Lemna minor* in remediating aquatic pollution.




**KEYWORDS:** *LmGSTF3 gene, overexpression, cadmium (Cd), tolerance, Lemna minor*



## INTRODUCTION

As a consequence of accelerated industrialization, large quantities of inadequately treated industrial wastewater are being discharged into waterbodies, resulting in increasingly serious pollution of the aquatic environment with heavy metals, such as cadmium (Cd).[1−3] In nature, having entered water, Cd will not be readily removed, thereby posing a long-term threat to the environment.[4] By affecting normal physiological and biochemical functions, Cd can have toxic effects on aquatic plants,[5−7] and in terms of the risk to human health, Cd in water directly or indirectly ingested can lead to deformities and diseases, such as osteoporosis and cancer.[8−10] However, although a number of approaches have been adopted in an effort to address the problem of Cd pollution in water, these tend to be expensive and inefficient.[11,12] Consequently, there is an urgent need to develop more economic and efficient methods for remediating aquatic Cd pollution.

In recent years, phytoremediation has emerged as an important approach for restoring heavy metal-contaminated waterbodies.[13] Phytoremediation is an eco-friendly method that utilizes plants to sequester and degrade environmental pollutants[14−16] and, accordingly, identifying suitable heavy metal hyperaccumulators is a key factor contributing to effective remediation.[17] Compared with other macrophytes, *Lemna minor* is often considered a plant of choice for phytoremediation, given its wide distribution and high metal-accumulating potential.[18,19] In particular, with respect to Cd uptake from water, the *Lemna minor* is known for its Cd accumulation, which is approximately 30 times of the hyperaccumulators.[20] Generally, Cd hyperaccumulators are those with Cd concentrations up to 100 mg·kg$^{-1}$ (dry weight).[21] In addition, its rapid growth in aquatic environments with a wide range of water qualities makes this plant a suitable option for aquatic restoration.[22,23] Moreover, given its combined properties of sewage remediation and bioenergy production, *Lemna minor* is increasingly being proposed as a novel high-quality biomass feedstock, and these favorable attributes have accordingly stimulated research on the phytoremediation properties of *Lemna minor*.[24,25]

The enzyme glutathione *S*-transferase (GST) is a member of a superfamily of multifunctional proteins that play essential

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roles in primary and secondary metabolism in plants,[26] and GSTs from an increasing number of species have been reported to be associated with the regulation of plant responses to Cd stress.[27] On the basis of genomic organization, sequence similarity, and functions, the GST superfamily is further categorized into the eight classes Tau (U), Phi (F), Theta (T), Zeta (Z), Lambda (L), docosahexaenoic acid (DHA), tetrachlorohydroquinone dehalogenase (TCHQD), and microsomal GSTs;[28,29] among which, the Phi and Tau classes are considered to be plant specific.[30] In *Arabidopsis thaliana*, it has been found that the *GSTF8* gene is involved in the vesicular binding and transport of Cd, thereby reducing the cellular accumulation of Cd and enhancing Cd tolerance.[31] Furthermore, GST has been demonstrated to function as a glutathione peroxidase (GPX) that attenuates cytotoxicity by binding to GSH.[32] GSH conjugates are more water soluble and can be further chelated into a plant cell vacuole via ATP-binding cassette transporters on vacuoles.[33] It has also been reported that the GST activity of *Lemna minor* increases in response to Cd treatment and that the *GST* gene plays a role in this response.[34] At present, however, the molecular mechanisms underlying the tolerance of *Lemna minor* to Cd remain poorly understood, thereby highlighting the need for a transcriptome analysis of *Lemna minor*.

In this study, we identified a Cd-responsive *GST* gene in *Lemna minor*, designated *LmGSTF3*, which was overexpressed to determine the mechanisms associated with Cd tolerance. Physiological and biochemical analyses showed that overexpression elevates Cd tolerance with prolonged Cd exposure time, as evidenced by significant increases in growth rate, chlorophyll content, antioxidant enzyme activities, and the Cd removal rate. Furthermore, transcriptomic analysis provided evidence to indicate the mechanisms whereby *LmGSTF3* enhances the Cd tolerance of overexpressing (OE) strain, thereby providing new germplasm for the remediation of Cd-contaminated waterbodies.

## MATERIALS AND METHODS

**Plant Materials and Growth Conditions.** For the purposes of this study, we used duckweed *Lemna minor* 0009 obtained from the Guizhou University Duckweed Germplasm Bank. The *Lemna minor* was inoculated into a complete Hoagland culture medium containing 1.5% sucrose for 7 days and then transferred to an open 1/5 Hoagland medium for expanded culture at 5000 lx light intensity, 25 °C, a 16 h/8 h photoperiod (L/D), and 75% humidity.[35]

**Cloning of the *LmGST* Gene and Expression Analysis.** To characterize the *LmGST* gene, we extracted total RNA from *Lemna minor* according to the instructions of an Eastep Super Total RNA Extraction Kit (Promega, USA) and reverse-transcribed the extracted RNA to cDNA using a TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China). Specific primers (Table S1) were used to amplify the coding sequence of *LmGST*, and the PCR products were inserted into a pMD20-T vector and sequenced. The sequences thus obtained were subsequently aligned by using DNAMAN software. The nucleotide and encoded amino acid sequences of *LmGST* are shown in Text S1. The neighbor-joining method was used for phylogenetic tree construction by using MEGA-X software.

To determine the relative expression levels of *LmGST*, we PCR amplified these gens using a Fast SYBR Mixture (Jiangsu Cowin Biotech, China), and calculated expression relative to that of the *18S* internal reference using the $2^{-\Delta\Delta C_t}$ method. cDNA was also prepared from the WT and OE strains of *Lemna minor*, with or without Cd treatment using the primer sequences, as shown in Table S1.

**Prokaryotic Expression Analysis.** In order to further assess the function of the cloned *LmGSTF3* gene, the coding sequence was inserted into a pET-30a vector at the *Bam*HI and *Sac*I restriction sites, which was used to transfect *Escherichia coli* BL21 for the prokaryotic expression. Generally, the *E. coli* expression system was a common prokaryotic expression mode.[36] The sequences of the primers used for amplification are shown in Table S2. The control and BL21 pET30a-*LmGSTF3* bacteria were then used to inoculate Luria−Bertani liquid medium containing 1 mM IPTG and 50 $mg{\cdot}L^{-1}$ kanamycin for shaking culture (37 °C and 220 rpm). The growth status, colony formation, GST enzyme activity, and Cd removal rate of BL21 pET30a-*LmGSTF3* compared with those of the control bacteria were examined at 0, 200, 400, and 600 $mg{\cdot}L^{-1}$ Cd concentrations.[37] The GST enzyme activity was determined by using a GST assay kit (Solarbio, China).

**Vector Construction and Genetic Transformation.** To functionally characterize the *LmGSTF3* gene with respect to Cd tolerance, the coding sequence of *LmGSTF3* was isolated using the primers shown in Table S3 and subsequently inserted into a pCAMBIA-Ubi-1300 vector using *Hin*dIII and *Kpn*I sites. The plasmid construct was used to transform an *Agrobacterium* strain using the freeze−thaw method and this strain was subsequently used to transform calli of *Lemna minor* 0009,[38] as a consequence of which, we obtained between 8 and 18 positive transgenic strains. Genomic DNA was extracted from these strains using the CTAB method and was thereafter PCR amplified (using the primers shown in Table S1) and sequenced to determine whether *LmGSTF3* was genetically modified. The overexpressing strains (OE-1, OE-2, and OE-3) verified by PCR were used in subsequent experiments. The calli induction process and the steps of calli genetic transformation are detailed in Texts S2 and S3.

**Quantification of *Lemna minor* Physiological and Biochemical Indices.** To investigate the physiological and biochemical differences between WT and OE strains in response to Cd treatment, we obtained samples from *Lemna minor* treated with a 1/5 Hogland medium containing 0 and 10 $mg{\cdot}L^{-1}$ $CdCl_2$[39] and inoculated in 14.5 × 9.5 × 5.4 cm culture boxes at 1.5 g fresh weight (achieve 100% surface coverage to inhibit the propagation of algae and harmful microorganisms) for 7 days. The basis of Cd concentration selection is shown in Text S4. The activities of peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), and GST enzymes and the contents of hydrogen peroxide ($H_2O_2$) and glutathione (GSH) in *Lemna minor* were measured after 24 h using the respective assay kits (Solarbio, China). In addition, samples were collected at 12 h, 24 h, 3 days, and 7 days for measurement of growth rate, root length, and chlorophyll content.[20]

**Determination of Cd Concentration.** In order to further compare the Cd accumulation and removal of WT and OE strains, samples of roots and fronds were dried in an oven at 60 °C to constant weight and ground with a mortar, and the coarse *Lemna minor* powder was sieved and weighed for dry weight. Then, the powder and 4 mL concentrated nitric acid were successively added to the digester tube, and the infrared digester (LWY84B; Siping, Jilin) was used for heating and digester to obtain clear and transparent digester solution, and the volume was fixed to 50 mL with distilled water. The Cd


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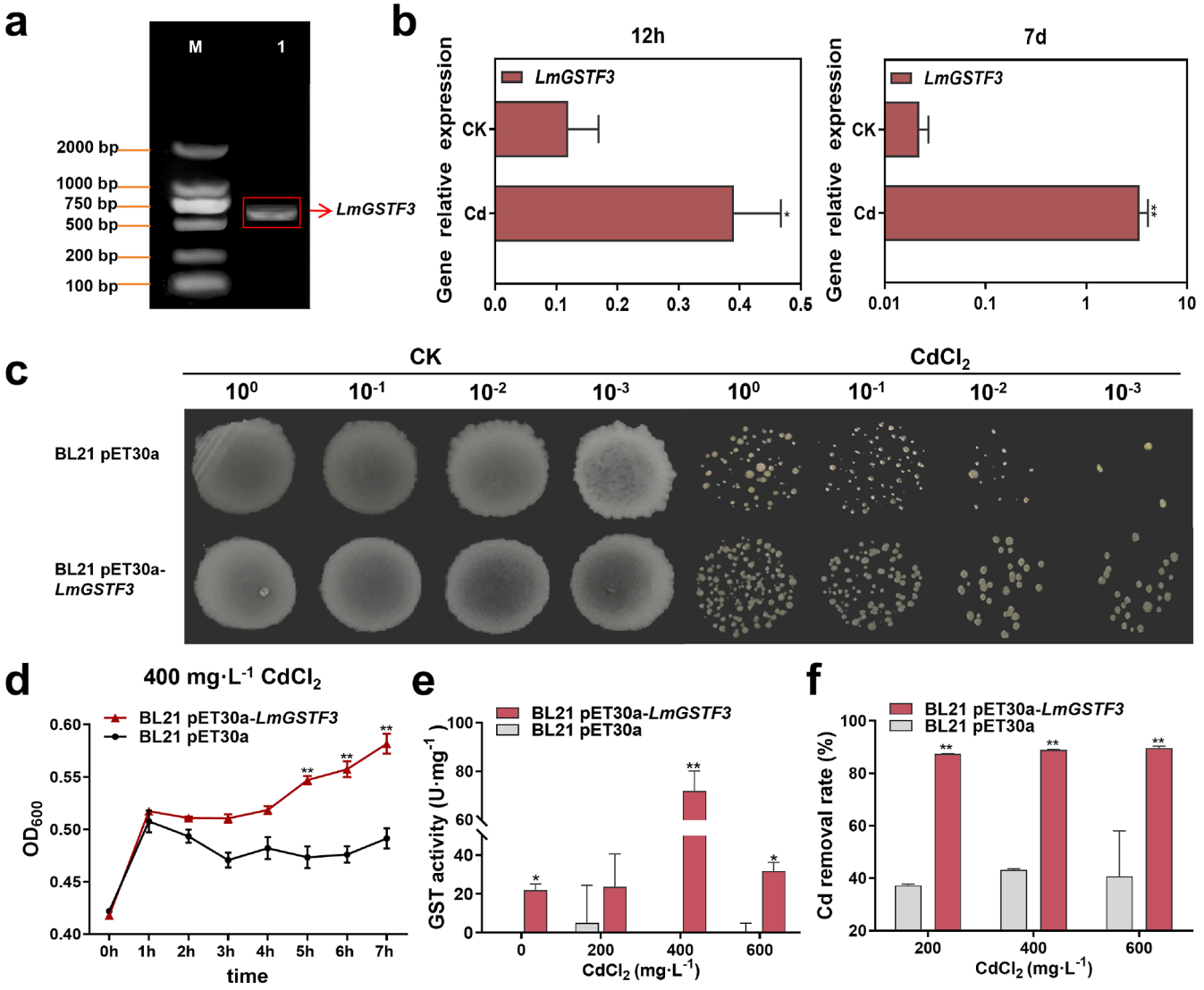


**Figure 1.** Effects of cloning *LmGSTF3* gene and prokaryotic expression. (a) Amplification electrophoresis of *LmGSTF3*. M: DNA Marker; 1: PCR amplification products. (b) Expression level of the *LmGSTF3* gene in *Lemna minor* through quantitative RT-PCR (qRT-PCR) analyses. CK: none Cd-treated group; Cd: 10 mg·L$^{-1}$ Cd treated group. (c) Colony formation of *E. coli* on Cd-treated plates. The left and right plates were normal and $CdCl_2$ plate, and the dilution from left to right in each figure was $10^0$, $10^{-1}$, $10^{-2}$, and $10^{-3}$ times, from top to bottom were control bacteria and BL21 pET30a-*LmGSTF3*, respectively. (d) Growth curves, (e) GST enzyme activity, and (f) Cd removal rate at different Cd concentrations of *E. coli*. Data were shown as the mean ± standard deviation, compared with CK (*$p$ < 0.05; **$p$ < 0.01; $n$ = 3).

content was determined by a flame atomic absorption spectrometer (Analytik Jena NovaAA 400P, Germany). Typically, the ability of heavy metals to migrate in the roots and fronds of plants was evaluated by the translocation factor (TF).[40] The specific formulas used for calculations of Cd content, bioconcentration factor (BCF), Cd removal rate, and TF are shown in Text S4.

**RNA Sequencing and qRT-PCR Validation.** To elucidate the biochemical and molecular mechanisms of the *GST* gene-mediated tolerance to Cd, the OE and WT strains of *Lemna minor* were treated with 10 mg·L$^{-1}$ of Cd for 12 h, with untreated *Lemna minor* being used as a control. For each group, three replicates were used for RNA-seq, performed by Majorbio Biopharm Biotechnology Co., Ltd. (Shanghai, China). Specific details of the methods used for RNA extraction, library construction, and sequencing were presented in Texts S5 and S6.

To further explore the changes of gene expression levels of related pathways in *Lemna minor* overexpressing *LmGSTF3* under Cd stress. According to the most critical GSH metabolic pathway, with *18S* as the internal reference gene, the relative expression levels of *GR*, *GPX*, *GGT*, *pepA* and *pepN* were detected by quantitative PCR. The primer sequences are shown in Table S1.

**Data Analysis.** GraphPad Prism 6.02 and Origin 2021 software were used for plotting, and multiple *t*-tests were used to compare the differences between the experimental and control groups by using SPSS 22.0 software. All data were presented as the means ± standard deviations of three replicate treatments for each sample, with a $p$-value < 0.05 being considered statistically significant.

## RESULTS

***LmGSTF3* Gene Is Highly Responsive to Cd.** Transcriptome analysis revealed that the expression of *GST* in



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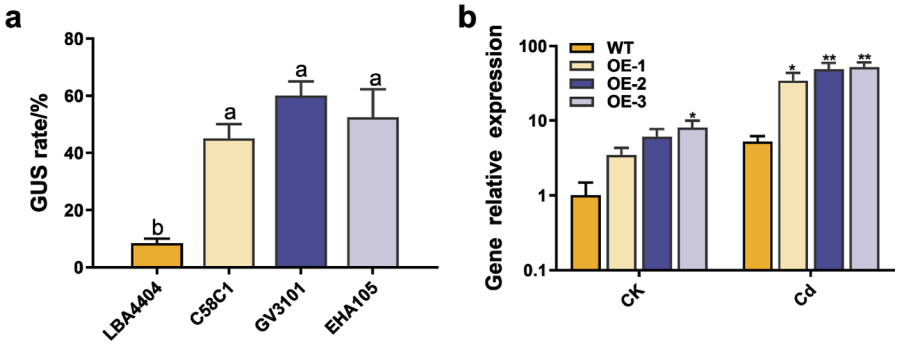


**Figure 2.** *Lemna minor* genetic transformation system. (a) Infection efficiency of different types of *Agrobacterium*. Different lowercase letters indicate significant difference ($p < 0.05$). (b) Effect of Cd on *GST* gene expression of WT and OE strains under CK- or Cd-treated conditions. CK: none Cd-treated group; Cd: 10 mg·L$^{-1}$ Cd-treated group; each group was repeated three times and expressed as mean ± SD. Analysis was performed by *t*-test and compared with WT, *$p < 0.05$, **$p < 0.01$.

*Lemna minor 0009* was upregulated in response to Cd treatment (Figure S1). We further cloned the *LmGST* gene using RACE technology (Figure 1a) and accordingly established that the protein-coding sequence of the gene was 561 bp in length and encoded a protein containing 186 amino acids (Figure S2). A phylogenetic tree constructed based on GST sequences revealed that this *Lemna minor* GST, designated *LmGSTF3*, was a member of the F-class of the GST family, which was genetically closest to GSTs in *Zea mays* and *Oryza brachyantha* (Figure S3). In addition, qRT-PCR analysis revealed that *LmGSTF3* was significantly upregulated, particularly on day 7 of treatment, at which time, the expression was approximately 152-fold higher than that of the control (Figure 1b, $p < 0.05$).

To functionally characterize the *LmGSTF3* gene, we used the gene to transform *E. coli* and assessed the Cd tolerance. Compared with the control bacteria, we found that BL21 pET30a-*LmGSTF3* produced more colonies on Cd-treated plates (Figure 1c) and that growth of these transformants was invariably superior, particularly under the 400 mg·L$^{-1}$ Cd treatment (Figure 1d, $p < 0.05$). In addition, we detected an initial increase in GST enzyme activity in response to exposure to increasing Cd concentrations, which peaked at 400 mg·L$^{-1}$ Cd (Figure 1e, $p < 0.05$). Moreover, BL21 pET30a-*LmGSTF3* bacteria were found to have a Cd removal rate of up to 89%, which was 2.2-fold higher than that of the control (Figure 1f, $p < 0.05$).

***LmGSTF3*-Overexpressing Strains Were Obtained via Genetic Transformation.** *Lemna minor* 0009 was used to establish and optimize the genetic transformation system based on *Lemna minor* calli, with the best effects being achieved using 5 $\mu$M TDZ and 45 $\mu$M 2,4-D (Figure S4). We also found that the addition of 0.1 mg·L$^{-1}$ IAA can effectively induce frond regeneration (Figure S5), and the calli induction and frond regeneration of *Lemna minor* are shown in Figure S6. Having constructed the overexpression recombinant vector Ubi-1300-*LmGSTF3*, we used this to transform *Lemna minor* calli (Figure S7). PCR analysis confirmed that the OE strains expressed *LmGSTF3*, and sequence comparisons revealed a greater than 90% similarity (Figure S8).

Furthermore, through the comparison of various *Agrobacterium* strains, it was found that EHA105, GV3101, and C58C1 had a higher infection efficiency than LBA4404 (Figure 2a, $p < 0.05$) and transgenic *Lemna minor* were effectively obtained. RT-qPCR analysis also revealed that expression of the *GSTF3* gene in Cd-treated OE strains was higher than that in the WT strain (Figure 2b, $p < 0.05$), thereby indicating that these OE strains would be suitable for characterizing the mechanisms associated with Cd tolerance in *Lemna minor*.

**Overexpression of *LmGSTF3* Enhances *Lemna minor* Growth.** In response to prolonging the exposure to Cd, we detected significant increases in chlorophyll content of OE-3 strains by 70% on the seventh day compared to WT, consistent with morphology changes (Figure 5a,b, $p < 0.05$). Additionally, compared with the WT strain under Cd stress, the root length, and growth rate of OE strains increased significantly on the seventh day and that of OE-3 strains increased by 225 and 177%, respectively (Figure S9a,b, $p < 0.05$). Moreover, compared with the control group, we observed increases in the activities of antioxidant enzyme in response to Cd stress (Figure 3a–c, $p < 0.05$), with increases of 179, 233, and 110% being recorded for the activities of POD, CAT, and SOD, respectively, in the OE-3 strain. The results were further confirmed by NBT staining (Figure 3f). Exposure to Cd was also found to promote increases (94.15 U·mg$^{-1}$ and 1438.68 Ug·g$^{-1}$, respectively) in the GST activity and GSH content in OE-2 strains, which were 84 and 50% higher than the corresponding values in the WT strain (Figure 3d,e, $p < 0.05$).

**Overexpression of *LmGSTF3* Enhances Cd Accumulation in *Lemna minor*.** Compared with the WT strain, we recorded higher values for Cd concentration, BCF, and removal rate in OE strains with a prolongation of the Cd treatment time. Specifically, on the third and seventh days, the highest Cd concentrations in OE strains reached 2330.94 and 3735.01 mg·kg$^{-1}$, respectively, which were 1.36-fold and 1.83-fold higher than the corresponding WT strain values (Figure 3g, $p < 0.05$). Furthermore, the BCF of the OE-3 strain peaked at 1856 on day 7, which was 3.9-fold higher than that of the WT strain (Figure 3h, $p < 0.05$), and the Cd removal rate recorded on the same day was 81%, which was 2.2-fold higher than that of the WT strain (Figure 3i, $p < 0.05$). Moreover, the OE strains accumulated 1.33-fold and 1.37-fold higher concentrations of Cd in the roots than WT, respectively, at the 24th hour and on the third day. By contrast, on the third and seventh days, the Cd concentrations in the fronds of the OE strains were 1.92–2.44 times higher than WT (Figure S9c, $p < 0.05$). Notably, on day 3 and day 7 of Cd exposure, the TF of OE-3 strain increased significantly from 1.38 to 1.93 compared with a WT strain (Figure S9d, $p < 0.05$).

**Overexpression of *LmGSTF3*-Altered Gene Expression Pattern.** In order to gain further insight into the regulatory network after transferring the *LmGSTF3* gene into


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Environ. Sci. Technol. 2025, 59, 2711–2721

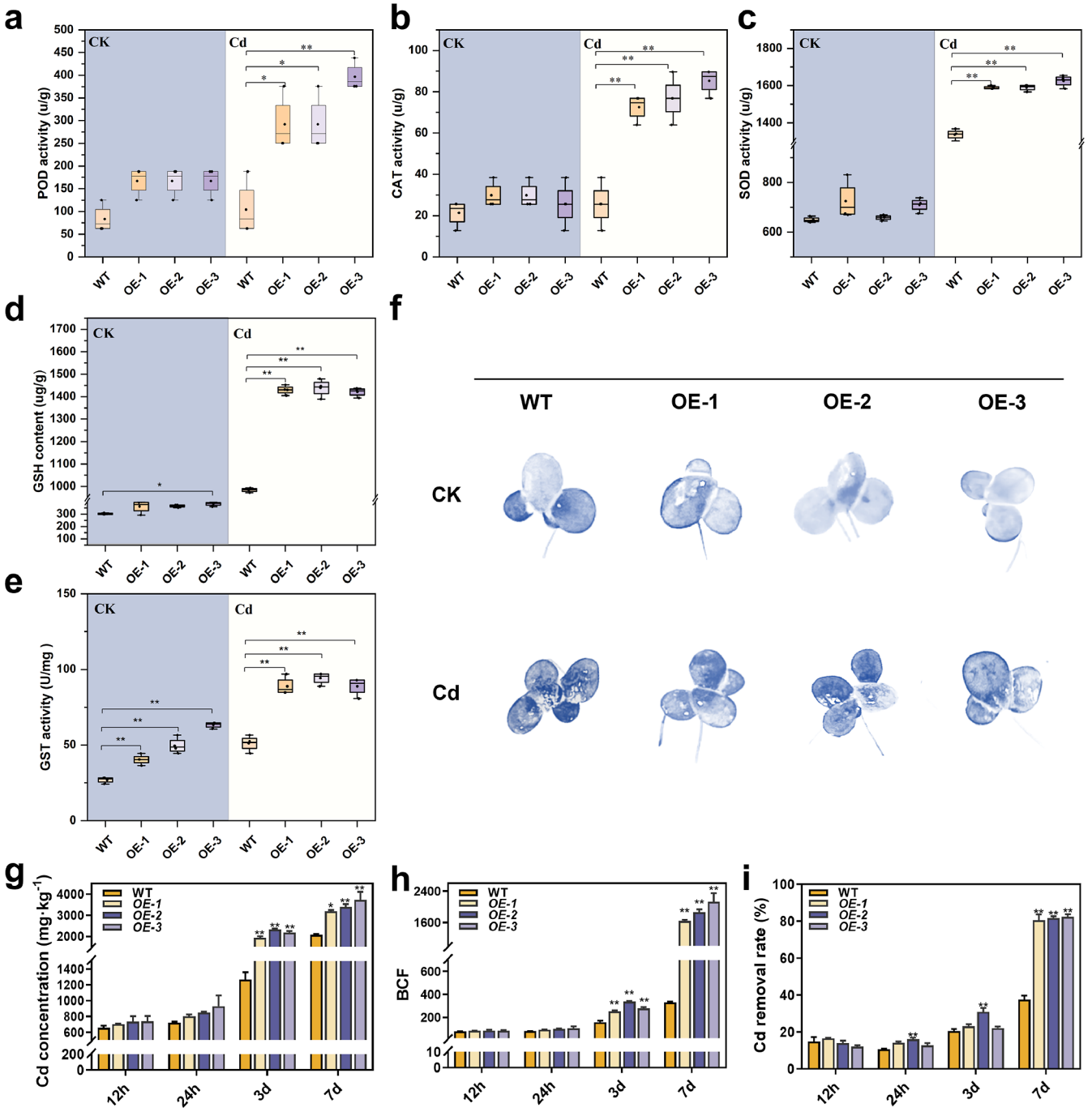


**Figure 3.** Effect of Cd treatment on biochemical indices related to *Lemna minor*. (a) POD activity, (b) CAT activity, (c) SOD activity, (d) GSH content, and (e) GST activity of WT and OE strains under CK- or Cd-treated conditions. (f) NBT staining of WT and OE strains under CK- or Cd-treated conditions. (g) Cd concentration, (h) BCF, and (i) Cd removal rate of WT and OE strains under CK- or Cd-treated conditions. CK: none Cd treated group; Cd: 10 mg·L$^{-1}$ Cd treated group. Data were shown as the means ± SD of three independent groups. The differences between OE and WT strains were analyzed using the *t*-test, *$p$ < 0.05, **$p$ < 0.01.

*Lemna minor*, we performed transcriptome analysis. Table S4 shows the quality and read-access statistics of the WT and OE strain transcriptome databases. Correlation and PCA analysis revealed the reliability of the subsequent experiments as well as the rationality of our sample selection (Figures 4a and S10a). Unigene expression was found to be clustered between 0 and 2 (Figure S10b). Moreover, under Cd treatment, we detected 453 differentially expressed genes (DEGs) compared with the WT strain, among which 208 and 245 were upregulated and downregulated, respectively (Figure 4b). Under control treatment, a total of 1732 DEGs were identified versus the WT strain, of which 523 and 1209 were upregulated and downregulated, respectively (Figure S11b). Most obviously, OE and WT produced 338 DEGs after Cd treatment (Figure S11a).

**Overexpression of *LmGSTF3* Altered Functional Gene Expression.** With respect to gene function, we initially focused on transcription factors involved in plant stress resistance, among which MYB family members were found to have the highest proportion (16%) of upregulated DEGs,



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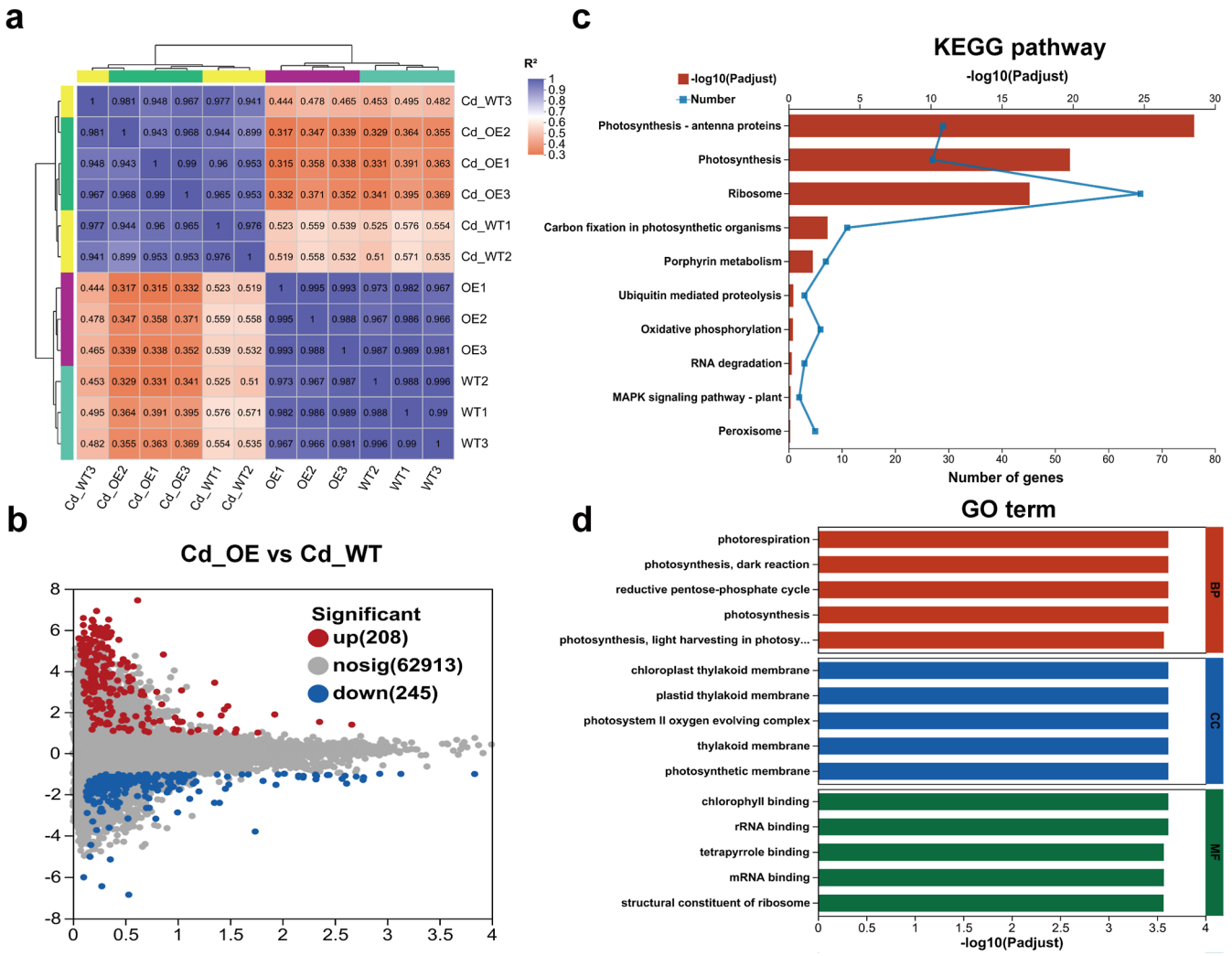


**Figure 4.** DEGs in WT and OE strain under Cd treatment. (a) Correlation heatmap of the CK- and Cd-treated groups. (b) Scatter plots of DEGs between WT and OE strains. Red: upregulated genes, gray: unchanged genes; blue: downregulated genes. (c) Top 10 DEGs in KEGG enrichment for Cd_OE vs Cd_WT ($P < 0.05$). (d) GO enrichment of DEGs (The top 5 GO terms were taken according to the $p$-value of the selected classification, respectively).

indicating that these factors may play a positive regulatory role under Cd stress (Figure S12). GO enrichment analysis indicated that DEGs could be categorized into three main GO functional categories: biological processes, cellular components, and molecular functions. In the CK_OE groups, upregulated DEGs were found to be mainly involved in photosynthesis, structural constituents of cytoskeleton, and anchoring junction (Figure S11 and Table S5). While in the Cd_OE groups, they were mainly involved in photorespiration, chloroplast thylakoid membrane, and chlorophyll binding (Figure 4d and Table S6). Furthermore, KEGG pathway analysis revealed significant annotation to metabolism-related pathways, with ribosome, photosynthesis, and porphyrin metabolism pathways significantly annotated under both treatments, which may indicate that overexpression itself also requires a higher energy support (Figures 4c and S11d, Tables S7 and S8). Notably, we found that the expression levels of amino acid metabolism and oxidative phosphorylation pathway DEGs in OE strains were further induced under Cd stress. It is accordingly hypothesized that the aforementioned significantly enriched pathways may play pivotal roles in conferring the enhanced Cd tolerance associated with the *LmGSTF3* overexpression.

## DISCUSSION

**Overexpression of *LmGSTF3* Enhances Cd Tolerance.** The *LmGSTF3* gene has been established to contribute to the promotion of Cd tolerance.[41,42] Moreover, heterologously expressed *LmGSTF3* in *E. coli* enhanced the Cd tolerance of these bacteria (Figure 1c), which is consistent with the findings of Lee et al.[43] Notably, with increasing Cd concentration, although GST enzyme activity peaked at 400 mg·L$^{-1}$ Cd, there was no corresponding concentration-dependent change in the Cd removal rate (Figure 1e,f, $p < 0.05$). This anomaly suggested that GST may not be the dominant factor in the overall Cd tolerance of BL21 but still played a certain auxiliary role. Further, the Cd removal rate demonstrated the possible existence of other Cd detoxification strategies, such as metal-ion efflux systems, cell surface adsorption, and transformation of heavy metals.[44−47] What is more, the next step is to detect the sensitivity of *E. coil* to Cd utilizing glutathione biosynthesis inhibitors to further reveal the role of GST in enhancing Cd tolerance.[48]


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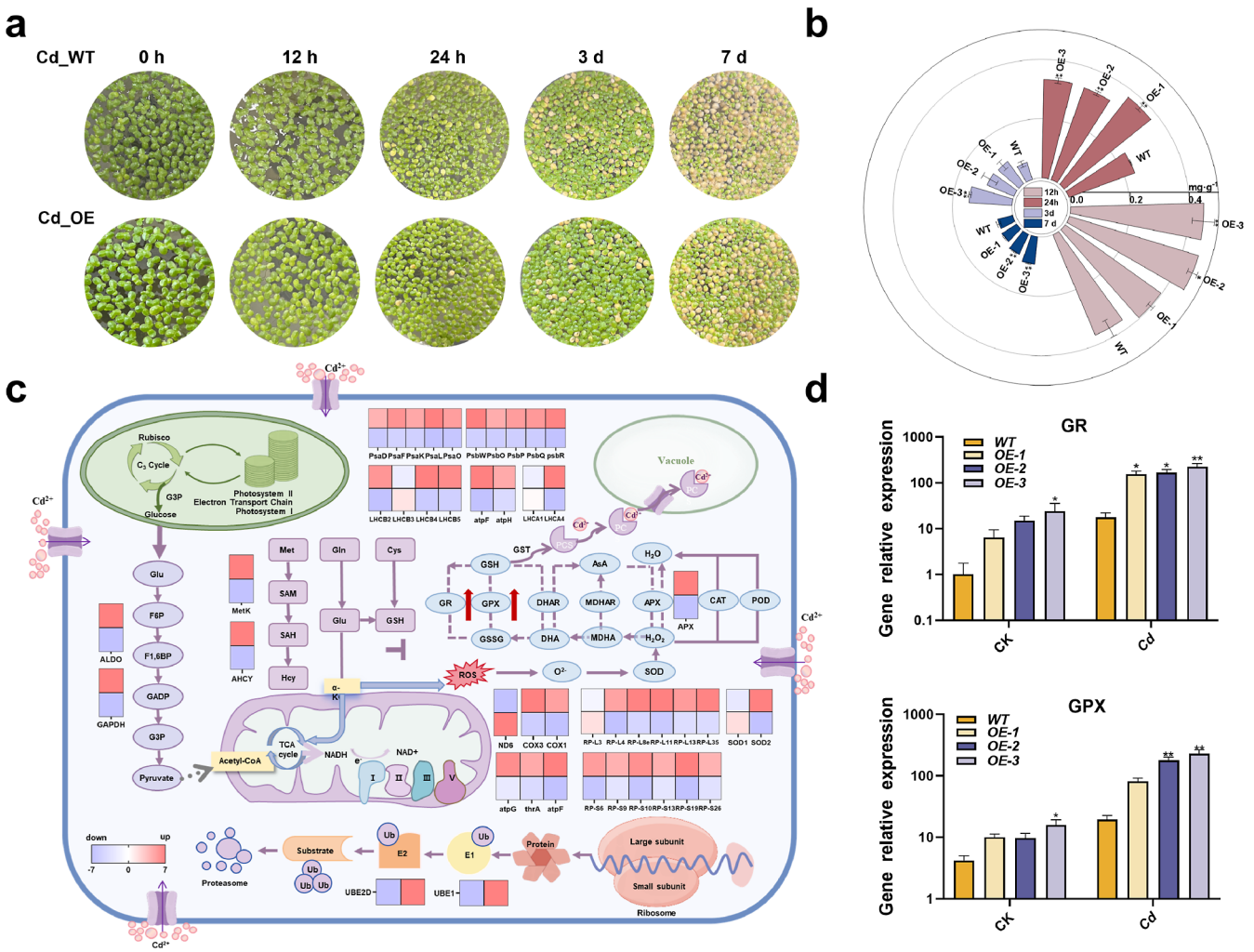


**Figure 5.** Mechanism of overexpression of *LmGSTF3* improved Cd tolerance. Effect of Cd on the (a) apparent morphology, (b) chlorophyll content, and (d) expression levels of genes related to GST metabolic pathway of WT and OE strains under CK- or Cd-treated conditions. (c) Regulatory metabolic networks of *Lemna minor* response to Cd. Red boxes indicate genes that increased in abundance under Cd treatment and blue boxes vice versa. CK: none Cd treated group; Cd: 10 mg·L$^{-1}$ Cd treated group; each group was replicated three times and expressed as mean ± SD. Analyzed by *t*-test and compared with WT, $*p < 0.05$, $**p < 0.01$.

To further comprehend the role of *LmGSTF3*, OE strains of *Lemna minor* were created via *Agrobacterium*-mediated genetic transformation (Figure 2),[49] which continuously enhanced the Cd tolerance (Figure 5a,b). In plants, GST promotes the binding of GSH to $Cd^{2+}$, which plays a prominent role in the cellular resistance to Cd toxicity.[50,51] Furthermore, through the production of antioxidant enzymes and nonenzymatic antioxidants, the antioxidant defense system can tightly regulate the amount of ROS and protect plants from oxidative damage.[52,53] Further evidence of this effect was provided by the upregulation of GST, POD, SOD, CAT activity, and GSH content of OE strains (Figure 3a–e, $p < 0.05$). Similar to our findings, overexpression of *OsGSTL2* in *Arabidopsis* may enhance antioxidant properties which participate in providing tolerance toward heavy metal stresses.[54] Typically, plants resist Cd toxicity through vacuolar sequestration and cell wall immobilization,[55,56] and effectively remove heavy metals from water through biosorption and intracellular accumulation.[57] Our study observed with prolonged Cd stress time, OE strains promoted the transport of Cd from roots to fronds compared with WT (Figure S9c,d, $p < 0.05$). This phenomenon may be attributed to the fact that heavy metals initially entered the plant through root system. Previous studies have found that approximately 60% of Cd accumulated in the root system, and then efficiently translocated Cd into the fronds.[58,59] Notably, similar detoxification mechanisms have been found in *Arabidopsis*.[60] Collectively, OE strains sequestered transported Cd into the vacuole to reduce Cd cytotoxicity, similar to previous findings.[61]

**Molecular Mechanisms of *LmGSTF3* Overexpression in Cd Tolerance.** Global DEGs profiling in the OE strains showed that *LmGSTF3* regulates multiple biological pathways. KEGG analyses revealed that the overexpression itself had an effect on the gene expression network of *Lemna minor* under control treatment.[62] However, the OE strains had markedly heightened pathways associated with mitochondrial oxidative phosphorylation, amino acid metabolism, and photoprotective and antioxidant mechanisms under Cd treatment. A well-established of plants maintain cellular redox balance by activating a series of antioxidant enzymes.[63] Among the components of antioxidant systems, SOD2 has been demonstrated to enhance Cd tolerance by scavenging free radicals. In plants, the APX of AsA-GSH cycle reduces ROS toxicity by converting $H_2O_2$ to $H_2O$.[64,65] Our study detected


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Environ. Sci. Technol. 2025, 59, 2711−2721

upregulation expression of SOD and APX in OE strains upon exposure to Cd (Figure 5c and Table S9). Moreover, the upregulated expression of *GR*, *GPX*, *pepA*, *pepN*, and *GCT* genes in RT-qPCR validated the enhanced Cd tolerance in the transcriptome (Figures 5d and S13, $p < 0.05$). Interestingly, inefficient oxidative phosphorylation, in turn, produces ROS, which can lead to mitochondrial dysfunction.[66] In response, plants maintain intracellular metabolic homeostasis and energy supply by increasing carbohydrates.[67] Moreover, basic biological processes related to amino acid metabolism and ubiquitin-mediated proteolysis play pivotal roles in plant responses to abiotic stress.[68,69] For instance, overexpression of methionine and AHCY promoted plant growth under Cd treatment.[70,71] Our study found that the upregulated expression of methionine and AHCY in OE strains compared to WT, indicating a positive effect on Cd resistance. Similarly, overexpression of *NtUBC1* in tobacco enhanced its tolerance to Cd.[72] The increased expression of UBE1 and UBE2D was also observed in our study. Collectively, these results indicated that overexpression of *LmGSTF3* resulted in the transcriptional reprogramming of Cd-stress response genes, thereby enhancing *Lemna minor* tolerance to Cd.

Next, our results showed that *Lemna minor* tends to regulate an energy metabolic flux in response to Cd cytotoxicity. Specifically, the promotion of photosynthesis for glucose production is the initiating step, through the glycolysis pathway to convert glucose into energy and synthesis of other organic materials, and finally through oxidative phosphorylation to produce ATP, which provides energy for plants.[73] These pathways are linked together to help plants cope with Cd stress. Notably, because plant photosynthesis is sensitive to heavy metals,[74] many species have evolved specific Cd tolerance mechanisms impairing photosynthesis little.[75] For instance, *Picris divaricate* responded to Cd toxicity by protecting the PSII reaction center.[76] In this study, the DEGs of the photosynthesis pathway were significantly upregulated and dominated by the DEGs of photosystem II and photosystem I (Figure 5c and Table S9). Moreover, studies have shown that the downregulated expression of cytochrome *c* oxidase (COX) in pea seeds under Cd stress, induced oxidative cell stress.[77,78] Our study observed the upregulated expression of COX1, COX3, atpG, thrA, and atpF (Figure 5c and Table S9), implying that the OE strain may rely on the energy released to activate the antioxidant system against Cd toxicity. However, such a mechanism identified in this study refers specifically to the transcriptome level. Hence, it is urgent to carry out more research on multiomics analysis and key gene function verification to further reveal their molecular mechanism.

**Environmental Implications.** *Lemna minor* is extensively utilized in the remediation of pollution across diverse aquatic ecosystems due to its accelerated growth rate and high Cd accumulation (Figures S9 and 3g, $p < 0.05$). Moreover, *Lemna minor* used for sewage treatment has a nitrogen and phosphorus absorption capacity comparable to *Eichhornia crassipes* and converted it into its own biomass accumulation.[79] Further application to paddy fields revealed that *Lemna minor*-paddy cocropping could not only reduce Cd content in rice but also fix nitrogen, which was essential for environmental protection.[80] In addition, recent studies have shown that *Lemna minor* has the potential to combine heavy metal pollution control with sustainable energy development.[81,82] For instance, Ge et al. utilized agricultural wastewater to cultivate high-starch *Lemna minor* to produce bioethanol.[83] Consequently, our findings in this study have identified plausible regulatory mechanisms, whereby *LmGSTF3* may contribute to mediating Cd tolerance, thus providing a new perspective for utilizing *Lemna minor* in the remediation of heavy metal-polluted waterbodies.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.4c08749.

Primer sequences for amplification and quantification; primer sequences for prokaryotic expression; primer sequences for overexpression; quality and mapping statistics for transcriptome data from *Lemna minor*; analysis of GO function of CK_OE vs CK_WT; analysis of GO function of Cd_OE vs Cd_WT; KEGG enrichment analysis of CK_OE vs CK_WT; KEGG enrichment analysis of Cd_OE vs Cd_WT; Cd detoxification-related genes in *Lemna minor*; induction of calli by different concentrations of TDZ and 2,4-D; type of antibiotic added to electrocompetent cells of *Agrobacterium*; nucleotide and amino acid sequences for phylogenetic tree construction; Calli induction process; steps of genetic transformation of calli; determination of Cd content in *Lemna minor*; RNA extraction, library preparation, and sequence analysis; RNA-seq data processing; GST metabolic pathway in the transcriptome of *Lemna minor* 0009; nucleotide and amino acid sequence of *LmGSTF3* region; phylogenetic tree of the *LmGSTF3*; effects of TDZ and 2,4-D on calli induction of *Lemna minor*; effects of 6-BA and IAA on fronds regeneration of *Lemna minor*; *Lemna minor* calli induction and regeneration of fronds; expression vector profiles of Ubi-*LmGSTF3*; detection of overexpression effect of *LmGSTF3* gene; quantification of *Lemna minor* physiological and biochemical indices; effect of *LmGSTF3* overexpression on gene expression pattern; DEGs in WT and OE strain under control treatment; TF family of Unigenes; and validation of the expression pattern of three differential genes (PDF)

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Environ. Sci. Technol. 2025, 59, 2711−2721

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### Notes

The authors declare no competing financial interest.

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