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Article

Bioinformatics Analysis of the Fruit-Weight like Gene Family and Identification the Role of *OsFWL8* in Arsenic Tolerance in *Oryza sativa* L.

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Abstract: The Fruit-Weight like (FWL) family of plants is ubiquitous in different species, and some members of this family have been shown to play an indispensable role in regulating plant growth, development, and response to heavy metal stress. This study comprehensively identifies and characterizes the *FWL* gene family in *Oryza sativa*, underscoring its significant role in arsenic stress response and metal ion homeostasis. Through bioinformatics analysis, eight *FWL* genes were identified and grouped into two subfamilies based on phylogenetic tree. These genes were further analyzed for conserved motifs, gene structures, and cis-acting elements, revealing their potential involvement in abiotic stress responses, hormone signaling, and developmental processes. Particularly, the study focused on *OsFWL8*, analyzing its expression patterns and response to arsenic stress. Real-time PCR demonstrated that *OsFWL8* expression is highly induced in arsenic-exposed rice, suggesting a role in arsenic stress response. In addition, our study highlighted that overexpression of *OsFWL8* enhances arsenic sensitivity, whereas knockout mutants showed increased tolerance. These findings suggested that the *FWL* gene family, especially *OsFWL8*, plays a crucial role in arsenic stress response in rice, providing insights that could lead to the development of rice varieties with enhanced yield and metal stress tolerance.

Keywords: *FWL* gene family; arsenic stress; rice; *OsFWL8*

Introduction

Arsenic (As) is a metalloid, and excessive accumulation of arsenic in the human body can directly lead to health problems such as diabetes, cardiovascular disease, and even cancer. (Wang et al., 2021). The primary routes of arsenic exposure include consumption of contaminated water and foods with high arsenic levels, posing health risks to over 150 million people globally (Moreno-Jiménez et al., 2012). Inorganic arsenic is predominantly found as usually arsenate (AsV) and arsenite (AsIII) in environmental matrices such as soil and water. Industrial activities such as mining, chemical metallurgy, and the extensive application of pesticides and herbicides have exacerbated the accumulation of arsenic in these matrices, thereby elevating the potential for crop yield reductions and severe health issues (Lomax et al., 2012; Tijo Joseph et al., 2015).

Rice, a staple food for approximately 3.5 billion people worldwide, is particularly susceptible to arsenic accumulation due to the solubility and toxicity of its inorganic forms in paddy soils. Under aerobic soil conditions, arsenate (AsV) prevails, while under anaerobic conditions, such as those found in flooded paddy fields, highly bioavailable arsenite (AsIII) dominates (Abedin et al., 2002; Islam S et al., 2017). These anaerobic conditions, coupled with the unique physiological traits of rice, facilitate the efficient uptake and sequestration of arsenite, making rice a significant source of inorganic arsenic intake in humans, with levels approximately ten times higher than in other grains (Zhang et al., 2022). Arsenic enters rice predominantly through aquaporins. Research indicates that

OsLsi1 is crucial for the uptake of As(III), while *OsLsi2* assists in its transfer to the xylem, facilitating arsenic accumulation within the plant (Zhao et al., 2010; Ma et al., 2008). Within rice, arsenic is primarily sequestered in vacuoles, a process mediated by glutathione (GSH) and phytochelatins (PCs), with *OsABCC1* playing a key role in mitigating arsenic stress by transporting As-PC complexes into vacuoles. The disruption of *OsABCC1* function results in heightened arsenic sensitivity (Song et al., 2008; Song et al., 2014). Conversely, the suppression of *OsPCS1* and *OsPCS2* genes decreases cadmium (Cd) and arsenic content, whereas overexpression of *OsWNK9* and *OsPRX38* enhances arsenic tolerance in rice (Kidwai et al., 2018; Manuka et al., 2021). Nevertheless, the intricate mechanisms underlying rice's response to arsenic stress necessitate further exploration.

The plant *Fruit-Weight-like* (FWL) family, characterized by a cysteine-rich PLAC8 domain, includes several proteins known for their roles in regulating grain size and metal ion homeostasis. In maize, *ZmCNR1* and *ZmCNR2* regulate cob size, whereas in Arabidopsis, *AtPCR1* enhances cadmium tolerance (Song et al., 2004). *AtPCR2*, another member of this family, functions as a zinc efflux transporter, essential for maintaining zinc balance under varying environmental conditions (Song et al., 2010). In rice, some FWL genes have been implicated in grain morphology and metal transport. For example, mutations in *OsFWL4* reduced cadmium transport from roots to shoots, enhancing Cd tolerance, while the roles of *OsFWL3*, *OsFWL6*, and *OsFWL7* in yeast Cd tolerance and the function of *OsFWL8* remain less understood (Xiong et al., 2018; Gao et al., 2020). The gene *OsFWL5/OsPCR1* has been demonstrated to positively affect grain weight and zinc content, while concurrently reducing cadmium accumulation in rice (Song et al., 2015). Additionally, *TGW2* has been identified as a negative regulator of grain width in rice (Ruan et al., 2020). Despite these insights, the involvement of the FWL gene family in arsenic transport and response remains to be elucidated. In this study, through bioinformatics methods, we identified genes encoding FWL in rice (*Oryza sativa* L.) and analyzed their evolutionary relationships, gene structures, and promoter elements. Furthermore, we identified the expression level and physiological roles of *OsFWL8* in arsenic stress and tolerance through overexpression and CRISPR/Cas9-mediated mutation. Overall, this study broadens our understanding of the FWL gene family in rice and provides foundational insights for further investigations into their functions involved in abiotic stress response.

Materials and Methods

Plant Materials and Arsenic Treatment

Japonica rice cultivar Nipponbare (wild-type, WT) and transgenic lines harboring either overexpressed (OE-*OsFWL8*) or knockout (*Osfwl8*) vectors against the Nipponbare background were utilized. Rice seeds were germinated for two days and then exposed to 25 μ M As(III) for seven days. Additionally, uniformly grown 21-day-old WT and transgenic seedlings underwent a 3-day treatment with or without 40 μ M As(III) (Huang M et al., 2024).

Vector Creation and Genetic Transformation

Gene-specific primers were designed to amplify the coding sequence (CDS) of *OsFWL8* from the Nipponbare genome, which was subsequently cloned into the *pCAMBIA1300-GFP* vector to create the 35S::*OsFWL8*::*GFP* construct. The *Osfwl8* mutant line was developed using CRISPR/Cas9 technology. These vectors were then introduced into Nipponbare via agrobacterium-mediated transformation to produce the transgenic lines (Chunyan Li et al., 2024). Primer sequences used for are listed in Table S2.

Quantitative Real-Time PCR

Total RNA was extracted from rice samples using the Plant RNA Easy Fast Kit (TIANGEN, China). After DNase I treatment, complementary DNA (cDNA) was synthesized using Hifair III 1st Strand cDNA Synthesis SuperMix (YEASEN, China). Quantitative real-time PCR (RT-PCR) was

performed using gene-specific primers (Table S1) with Hieff qPCR SYBR Green Master Mix (No Rox) (YEASEN, China) on a BIO-RAD CFX96 Real-Time PCR system (BIO-RAD, USA). The thermal profile included an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. OsUBQ5 (*LOC_Os01g22490*) served as the internal control, and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Zhao et al., 2023). Primer sequences used for RT-PCR are listed in Table S2.

Data Analysis

Statistical analyses were performed using SPSS v22.0. ANOVA and Tukey's test assessed differences between treatments or samples, considering P-values < 0.05 as statistically significant. Graphs were generated using GraphPad Prism v10.0 (Zhao et al., 2023).

Identification of the FWL Gene Family

The FWL protein signature domain Hidden Markov Model was downloaded from the Pfam database (number: PF04749). Candidate genes were identified in the complete genome protein sequence of rice (*Oryza sativa*) using HMMER v3.1 software, and FWL gene family members were finalized by comparison with the Arabidopsis FWL family (Nie F et al., 2024). Basic information on members of the FWL gene family in rice in Table S1.

Phylogenetic Analysis of FWL Genes

FWL proteins were aligned using MAFFT, and unconserved sites and gaps were removed with trimAL. The maximum likelihood phylogenetic tree was constructed using iqtree, exporting the Newick Tree file. The tree was visualized and enhanced using the iTOL website.

Analysis of FWL Gene Structures

FWL gene structure was analyzed using the GFF3/GTF file of *Oryza sativa*, with annotation information extracted and visualized using the Gene Structure View (Advanced) tool in TBtools. Conserved motifs were predicted using the MEME suite, and conserved domains were identified using the NCBI- Conserved Domain Database (CDD) database, with results visualized in TBtools (Bailey et al., 2015).

Analysis of Gene Promoter Cis-Acting Elements

The first 1000 bp nucleotide sequence upstream of the FWL gene start codon was extracted using Samtools, analyzed for cis-acting elements on the PlantCare website, and the results were visualized using the R package ggplot2 after simplifying the data.

Results

Identification and Evolutionary Analysis of FWL Genes

The Hidden Markov Model signature domain for FWL proteins was downloaded from the Pfam database (Accession: PF04749). Using HMMER v3.1 software, we searched for candidate FWL genes in the complete genome protein sequence of *Oryza sativa* and compared these with the FWL family from *Arabidopsis thaliana* (Table S1). This analysis enabled the identification of FWL family members in rice. To elucidate the evolutionary relationships among FWL proteins across species, we performed sequence alignments and constructed a phylogenetic tree of FWL proteins from rice (Figure 1). Phylogenetic analysis divided the eight FWL members from rice into two subgroups, each containing four members: Subgroup I includes Os03g61440, Os03g61480, Os03g61500, Os03g61470; Subgroup II includes Os02g36950, Os02g52550, Os02g36940, Os10g02300.

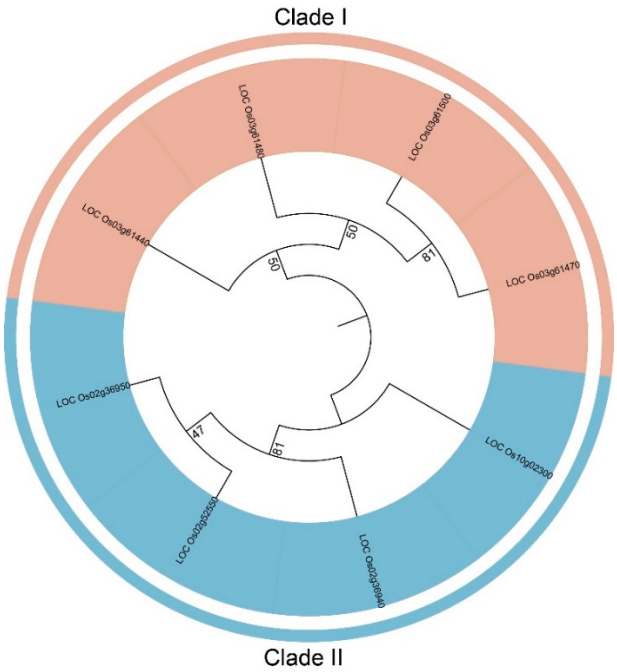


Figure 1. Phylogenetic Tree of FWLs Proteins in Rice. Using HMMER v3.1 software, candidate genes were searched for genome-wide protein sequences in rice (*Oryza sativa*) and the FWL gene family members were finalized by comparison with the FWL family of Arabidopsis. The tree shows two phylogenetic subfamilies (Clade I and Clade II) and color-coded.

Analysis of Conserved Motifs, Domains, and Gene Structure of FWL

The gene structure and annotation details of the FWL gene family in *Oryza sativa* were analyzed with the General Feature Format version 3 (GFF3) / Gene Transfer Format (GTF) files format. The conserved motifs of FWL proteins were predicted using the MEME suite, and their domains were identified using the NCBI's Conserved Domain Database (CDD) database (Figure 2). Analysis revealed that *OsFWL* genes contain between one and six motifs. Members of Subgroup II all possessed six motifs, whereas those in Subgroup I, such as *Os03g61440* and *Os03g61480*, had only five and two motifs, respectively. Each *OsFWL* gene contains 2-4 coding sequences (CDS); genes in Subgroup I have only 2 CDS while those in Subgroup II have 4 CDS. The genes in Subgroup II also have complete upstream and downstream regulatory regions (UTRs), unlike those in Subgroup I.

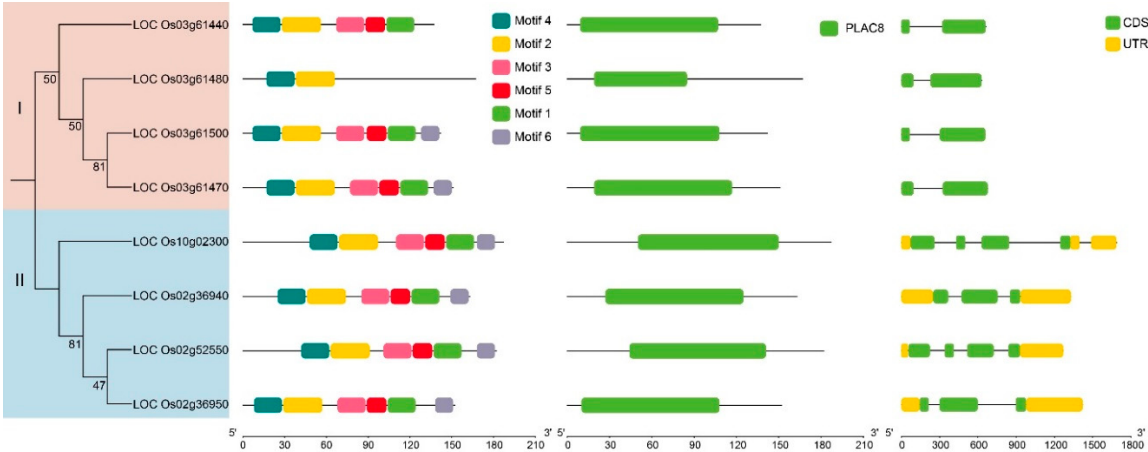


Figure 2. Prediction of conservative motif (Left) and gene structure (Right) of *OsFWLs* Protein in Rice. The different conserved motifs predicted on the left are color-coded and positioned. The panel on the right labels the CDS region and UTR region for each gene.

[illegible]

OsFWL8 as a Novel Gene in Response to Arsenic Stress in Rice

In order to investigate the function of *OsFWLs* gene family in response to arsenic stress, we selected *OsFWL8*, whose function has not been reported, as the object of further study. Real-time PCR analysis was employed to study the expression patterns of the *OsFWL8* gene in various rice tissues, using arsenic-tolerant japonica rice variety Nipperbare including roots, stems, leaves in the seedling stage, and roots, stems, flag leaves, leaf sheaths, nodes, young spikelets (1-10 cm), and spikes (>15 cm) at the booting stage. The expression of *OsFWL8* was predominantly observed in the roots and stems, particularly during the seedling and mature stages, where it was significantly higher compared to other tissues (Figure 4A). To determine the response of the *OsFWL8* gene to arsenic stress, 21-day-old seedlings were transferred to a hydroponic solution containing 0 and 40 μM As(III). The expression levels of *OsFWL8* were measured after 6 and 24 hours of treatment. Results indicated that arsenic stress significantly influenced *OsFWL8* expression, which was initially suppressed at 6 hours post-treatment but significantly up-regulated with prolonged exposure, suggesting that *OsFWL8* may be involved in the regulatory pathways responding to As(III) stress (Figure 4B).

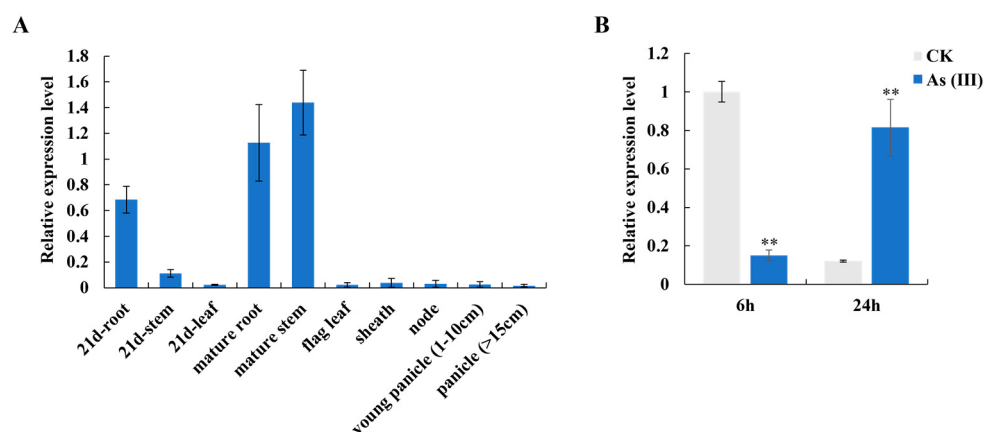


Figure 4. Analysis of the expression pattern of *OsFWL8* gene in rice. A. Expression of *OsFWL8* in different tissues of rice; B. After treatment with 0, 40 μ M As(III) for different times, changes in the expression of *OsFWL8* in the roots of rice seedlings at 21 days. ** Indicates a significant difference at the $P < 0.01$ level, each samples were subjected to three biological replicates.

Identification of *OsFWL8* Knockout and Overexpression Material

To elucidate the biological function of *OsFWL8*, we employed CRISPR/Cas9 gene editing technology. Primers targeting the exons of *OsFWL8* were designed, and gene editing was performed against the Nipponbare rice background. This resulted in the generation of two distinct homozygous *OsFWL8* knockout mutants, designated as *fwl8-1* and *fwl8-2*. In *fwl8-1*, a deletion of three bases and a single base substitution in the second exon resulted in a frameshift mutation, disrupting the normal protein translation sequence (Figure 5A and B). The *fwl8-2* mutant exhibited a deletion of three bases (CGG) at the same position, leading to the omission of an arginine at the 90th amino acid position (Figure 5A and B). Fluorescence quantification PCR revealed a significant reduction in the transcriptional levels of *OsFWL8* in both *fwl8-1* and *fwl8-2* seedlings compared to the wild type (Figure 5C). Additionally, the *OsFWL8* coding sequence was cloned into the *pCAMBIA1300S* vector, and the resulting overexpression transgenics, OE-*FWL8-1* and OE-*FWL8-2*, were obtained using Nipponbare as the recipient background. Real-time PCR verification showed that *OsFWL8* expression in OE-*FWL8-1* and OE-*FWL8-2* transgenic plants was upregulated by 887-fold and 1482-fold, respectively, compared to the wild type (Figure 5C).

Observation of Arsenic Tolerance in *OsFWL8* Knockout and Overexpression Materials

To assess the impact of *OsFWL8* on arsenic tolerance in rice, 21-day-old seedlings of Nipponbare, *fwl8-2*, OE-*OsFWL8-1*, and OE-*OsFWL8-2* were cultivated in nutrient solutions containing 0 and 40 μ M As(III) for 6 days, with each line having 12 biological replicates. Initially, new leaves in all lines exhibited inward rolling after one day, and by the second day, the leaves of Nipponbare and *fwl8-2* were partially unrolled while those of the transgenic lines remained tightly curled. After three days, the leaves of Nipponbare and *fwl8-2* returned to normal growth, whereas the old leaves of OE-*OsFWL8-1* remained curled and those of OE-*OsFWL8-2* were completely desiccated. By the sixth day, Nipponbare and *fwl8-2* showed healthy growth with only minor senescence at the leaf tips, whereas all plants in the OE-*OsFWL8-2* line were withered, and most of the OE-*OsFWL8-1* plants exhibited yellowing stems and only retained 1 to 2 new leaves (Figure 6). Post-treatment weighing revealed that the fresh weight of Nipponbare and *fwl8-2* was reduced by only about 8% compared to controls, whereas the fresh weight of OE-*OsFWL8-1* and OE-*OsFWL8-2* decreased by approximately 30% and 56%, respectively. These results underscore the significant influence of *OsFWL8* expression levels on arsenic tolerance in rice, with overexpression rendering the rice highly susceptible to As(III), particularly in the OE-*OsFWL8-2* line. The differential response of *fwl8-2* to As(III) stress compared to Nipponbare suggests inherent tolerance in Nipponbare.

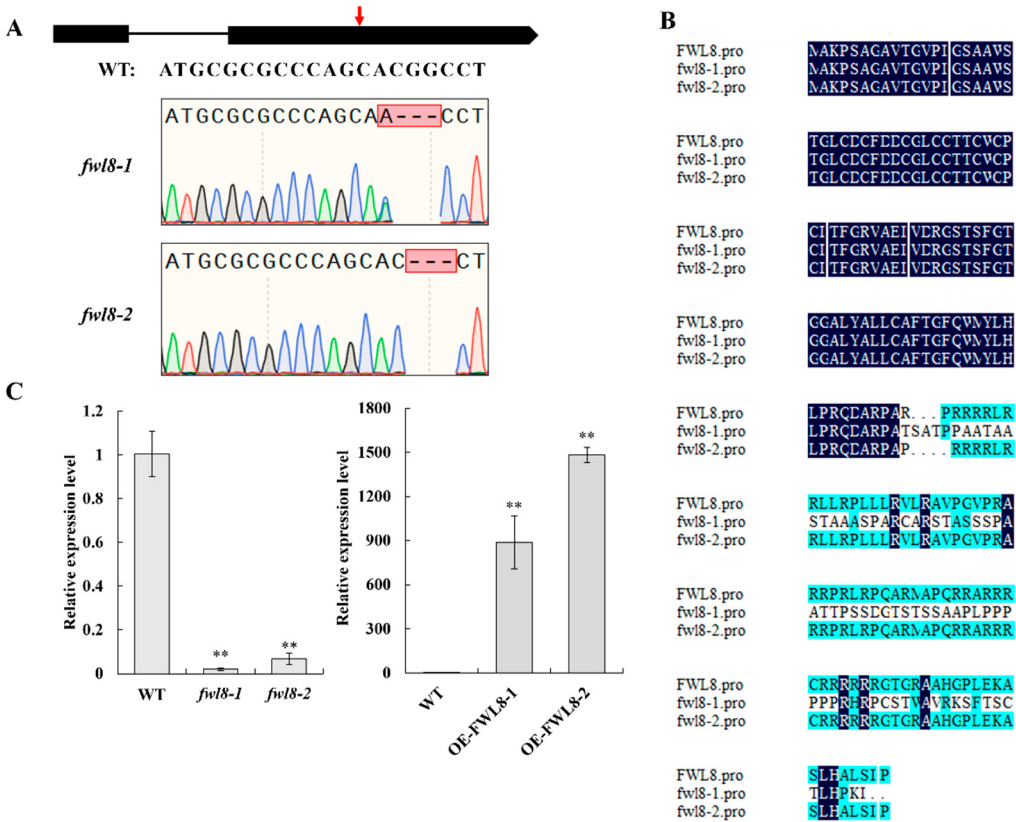


Figure 5. *OsFWL8* knockout and overexpression material identification. A. Editing sites of two *OsFWL8* knockout mutants, *fwl8-1* and *fwl8-2*, obtained using Crispr/Cas9 technology, with red arrows indicating mutation locations; B. Amino acid sequence alignment of *OsFWL8* in wide type, *fwl8-1* and *fwl8-2*; C. Real-time PCR was used to detect the expression levels of *OsFWL8* knockout and overexpression material.

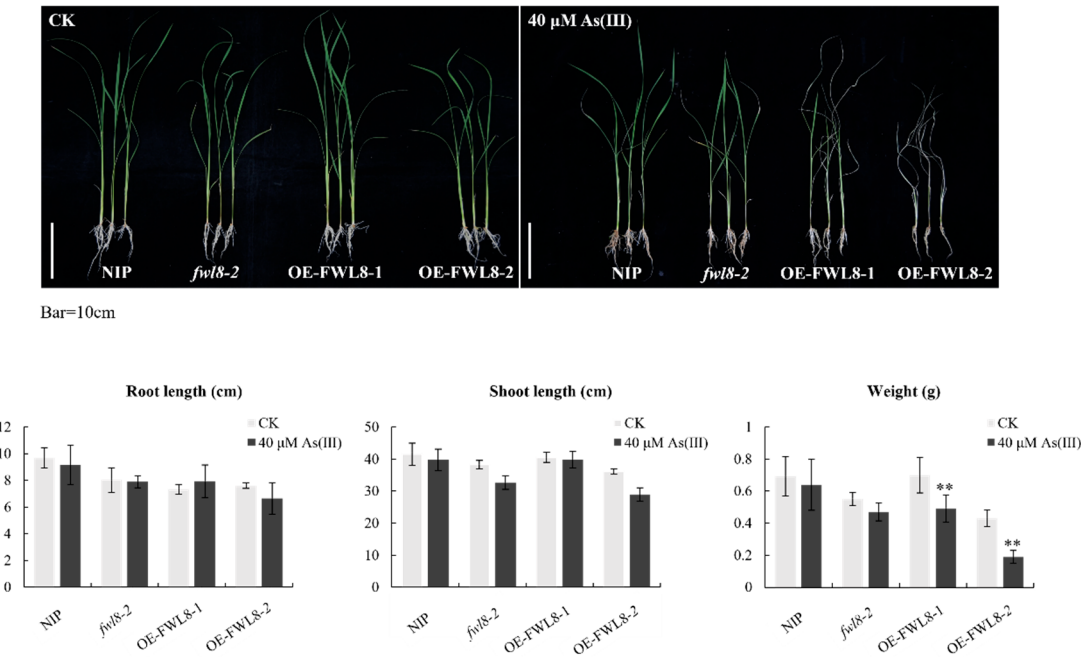


Figure 6. Analysis of arsenic tolerance phenotype in *OsFWL8* knockout and overexpression seedlings. The above picture is a phenotypic photograph of the treatment (Bar=10 cm), and the bottom picture is the statistical results of root length, shoot height and fresh weight of each plant. ** Indicates a significant difference at the $P<0.01$ level, each Samples were subjected to twelve biological replicates.

Rice will suffer from osmotic stress and excessive As transport when exposed to high As(III) concentrations. Therefore, we evaluated the relative expression levels of the key phytochelatin synthase-related gene (*OsPCS2*) and arsenic transporter-related genes (*OsABCC1*, *OsLsi1*, *OsLsi2*, *OsLsi6*) in WT, OE-*OsFWL8* and *fwl8-1* seedlings under normal and arsenic treatment conditions by qRT-PCR (Guo et al., 2024; Li et al., 2024). The results showed that the expression levels of *OsABCC1* and *OsPCS2* were significant increased in mutants compared with wide type and OE-*OsFWL8* under arsenic stress, which also indicates involvement of *OsFWL8* in the arsenic response process (Figure 7). Overall, these findings demonstrate that *OsFWL8* plays a crucial role in modulating arsenic tolerance in rice.

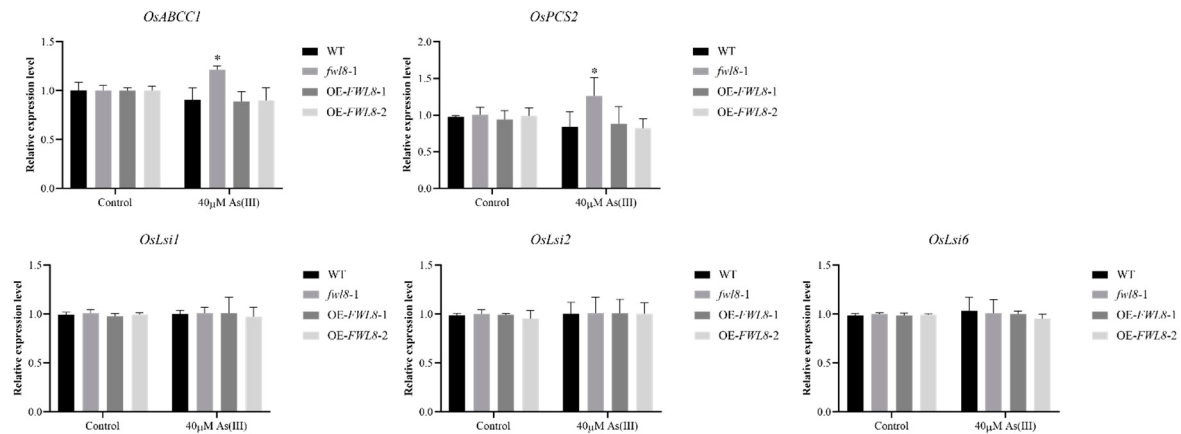


Figure 7. Expression analysis of genes related to arsenic stress tolerance and arsenic transport in wide type, *OsFWL8* knockout and overexpression seedlings with or without arsenic treatment. Each sample was subjected to three biological replicates. * Indicates a significant difference at the $P<0.05$ level, each Samples were subjected to three biological replicates.

Discussion

Previous studies have demonstrated that the *FWL* gene family plays a critical role in plant growth, development, and stress resistance. However, research on the *FWL* gene family has predominantly focused on species other than rice, revealing significant gaps in our understanding of its functions in this important crop. For example, recent studies have shown that overexpression of the *TaCNR2* gene in wheat, *Arabidopsis thaliana*, and rice enhances plant tolerance to cadmium (Cd), zinc (Zn), and manganese (Mn), while also reducing Cd accumulation in rice brown rice (Qiao et al., 2019). Additionally, *SaPCR2* and *BjPCR1* have been identified as efflux transporters involved in the tolerance to heavy metals like Cd and calcium (Ca^{2+}), playing essential roles in ion homeostasis within plants (Song et al., 2011; Lin et al., 2020).

In rice, the *FWL* gene family consists of eight members, named *OsFWL1* to *OsFWL8*. Most of these genes have been associated with grain weight and heavy metal transport. For instance, *OsFWL1* and *OsFWL3* negatively regulate grain width and weight (Xu et al., 2013; Ruan et al., 2020). *OsFWL4*, in particular, functions as a direct transporter, and mutations in this gene impair the transport of Cd from the roots to the shoots, thereby enhancing Cd tolerance. Other members of the *FWL* family, including *OsFWL3*, *OsFWL6*, and *OsFWL7*, have also been shown to increase yeast tolerance to Cd to varying extents, further emphasizing the potential of *FWL* genes in metal transport and stress tolerance (Xiong et al., 2018). *OsFWL4* has recently been implicated in the negative regulation of rice yield, highlighting its potential as a valuable target for genetic manipulation to develop non-transgenic rice varieties that are both Cd-tolerant and high-yielding (Gao et al., 2020). Similarly, *OsFWL5* has been identified as a key player in positively regulating grain weight, increasing Zn content in grains, and reducing Cd accumulation, thus contributing to improved grain quality (Song et al., 2015).

Despite these insights, the role of *OsFWL8* in rice stress responses, particularly in relation to As tolerance, has remained unclear. Our study fills this gap by generating mutants and overexpression lines of *OsFWL8* under various editing conditions. Through As(III) treatment, we found that overexpressed *OsFWL8* lines were more sensitive to arsenic stress, whereas the knockout mutants exhibited enhanced tolerance. These findings suggest that *OsFWL8* may play a crucial role in regulating arsenic transport and stress response in rice. Furthermore, our exploration of the molecular mechanisms underlying *OsFWL8*-mediated arsenic transport provides new insights into the complex regulatory network of arsenic stress tolerance in rice, which could enrich our understanding of the broader arsenic transport mechanisms in plants.

In addition to its potential role in arsenic transport, the *FWL* gene family likely regulates grain shape and the transport of toxic metals, which could open new avenues for improving rice varieties. By manipulating *OsFWL* genes, it may be possible to create rice cultivars that not only exhibit increased yield but also enhanced tolerance to toxic metals like arsenic. These insights hold promise for the development of non-transgenic rice varieties that are both high-yielding and arsenic-resistant, providing a valuable resource for global food security and environmental sustainability.

Conclusion

In this study, we systematically identified and characterized eight genes encoding FWL proteins in rice, which were classified into two subgroups based on phylogenetic analysis. Each member of the *FWL* gene family contained 1 to 6 conserved motifs, and the promoter regions of these genes were found to harbor numerous stress-responsive elements, plant hormone-responsive elements, and growth/development-related elements. We observed that the expression of *OsFWL8* was induced by As(III) treatment in 21-day-old rice seedlings, suggesting that this gene may play a role in regulating arsenic tolerance. The treatment results demonstrated that the overexpressed lines (OE-*FWL8*-1 and OE-*FWL8*-2) were more sensitive to arsenic stress compared to the wild type. In contrast, the mutants exhibited enhanced tolerance to arsenic stress. These findings suggest that the *FWL* gene family plays a significant role in stress adaptation in rice. Overall, this study not only broadens our understanding of the *FWL* gene family in rice but also provides foundational insights for further investigations into their biological functions and mechanisms.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1. Basic sequence information on members of the *FWL* gene family in rice and *Arabidopsis*; Table S2. Primer sequences used in this paper.

Author Contributions: QL and JZ conceived and designed research; MW, XFM, FN and XM performed the experiments and analyzed the data; XFM and LL contributed reagents and materials; JZ and MW completed the writing. All authors read and approved the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest

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