Rice

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Exploring FKBP12's Role in Enhancing Drought Tolerance in Rice

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Abstract

Rice, as the largest consumer of global freshwater resources, faces significant challenges due to increasing drought conditions exacerbated by climate change. In this study, we explore the critical role of FKBP12, a molecular chaperone protein, in modulating drought tolerance in rice. Utilizing a T-DNA insertional mutant (*fkbp12*) and *FKBP12*-overexpressing lines, we investigated the gene's influence on rice under various drought conditions. Our results revealed that the *fkbp12* mutant exhibited significantly enhanced drought tolerance compared to the wild type, evidenced by improved water retention, reduced cellular damage, and an upregulated expression of key drought-responsive genes such as *OsNCED3*, *OsSNAC1*, and *OsDREB2A*. This suggests a compensatory upregulation of abscisic acid (ABA)-mediated pathways, enhancing the plant's ability to cope with water deficit. Conversely, overexpression of FKBP12 resulted in increased sensitivity to drought, likely due to disruption in stress signaling and reactive oxygen species (ROS) scavenging mechanisms. Additionally, we observed an impact on seed development, where the *fkbp12* mutant presented smaller seed sizes, indicating a potential trade-off between growth and stress tolerance. This comprehensive analysis not only highlights the diverse roles of FKBP12 in drought stress response but also its implications for rice yield and seed development, providing valuable insights for breeding more resilient rice varieties in the face of escalating climate challenges.

Keywords Drought tolerance, FKBP12, ABA, ROS, Rice

Introduction

Approximately 72% of global freshwater is utilized in agricultural crop production, positioning crops as the largest consumers of freshwater resources (Bouman et al., 2001). This is increasingly significant as environmental pollution and global warming intensify, leading to more frequent droughts and floods. In China, agriculture faces a severe water crisis, with an upward trend in affected regions. Annually, about 6.67 million hectares of irrigated farmland receive inadequate irrigation, resulting in a loss of up to 80 million tons of grain due to water shortages (Belder et al. 2005). Thus, drought poses a formidable threat to the stability of agricultural yields, and implementing efficient water-saving agricultural

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practices is essential for the sustainable development of rice production.

Ensuring food security is a primary goal to maintain national economic stability, with enhancing the drought tolerance of rice crops being a top priority in agricultural innovation. Traditionally, progress in breeding drought-tolerant rice has been slow. However, recent advancements in molecular biology have empowered researchers to identify new genes related to drought resistance, providing a valuable genetic resource for breeding drought-resistant crops (Gao et al., 2023, Wang et al. 2024). Yet, the response of rice to drought stress is governed by complex traits regulated by multiple genes, proteins, and metabolic pathways, which presents a substantial challenge in the field of drought tolerance research (Hu et al. 2009; Oladosu et al. 2019; Geng et al. 2024; Ruan et al. 2024a).

Drought stress significantly impairs plant growth and development by decreasing biomass accumulation. The primary impacts of drought on plant growth include reduced cell division and growth, diminished leaf size and stem height, disrupted water/nutrient utilization, altered stomatal behavior, decreased plant water content, and lowered water use efficiency (Farooq et al. 2009). Research indicates that water scarcity induces the biosynthesis of abscisic acid (ABA), leading to stomatal closure, reducing stomatal conductance, and thus minimizing water loss due to transpiration. This reduction in stomatal conductance also decreases leaf CO_2 uptake, ultimately reducing photosynthetic efficiency (Yinpeng et al., 2009, Tomimatsu et al., 2016). Moreover, as CO_2 uptake decreases, photorespiratory efficiency increases, leading to a decline in carbon reaction efficiency during drought stress. In C_3 plants like rice, Rubisco is the key enzyme for CO_2 assimilation, and its functionality depends on the intracellular CO_2/O_2 concentration. Under typical conditions, with open stomata and higher CO_2 concentrations than O_2, Rubisco acts as a carboxylase. However, under water stress, when stomata are closed and O_2 concentrations exceed those of CO_2, Rubisco functions as an oxygenase, reducing carbon fixation and ultimately lowering rice yields (Li et al. 2020).

The FKBP protein family comprises highly homologous molecular chaperone proteins, distinguished by an FK506-binding domain (FKBd). This domain acts as the binding site for FK506 and rapamycin and is also the active site for peptidyl-prolyl cis-trans isomerase. The FKBd domain consists of six antiparallel β-sheets, a short α-helix, and flexible loops at the 30s, 40s, 50s, and 80s positions (Gollan et al. 2012). In rice, there are 28 FKBP proteins, with OsFKBP64, OsFKBP65, and OsFKBP75 being particularly responsive to heat stress and playing critical roles in seed maturation (Breiman, 2006, Nigam et al. 2008; Nie et al. 2024). The study shows that heterologous expression of *OsFKBP20* in yeast conferred high-temperature tolerance. Through yeast two-hybrid screening, OsFKBP20 was found to interact with the SUMO conjugase OsSce1, orchestrating rice's response to high-temperature stress. Additionally, it has been reported that heterologous expression of *OsFKBP12* in Arabidopsis increases sensitivity to the pathogen Pseudomonas syringae, with interactions with OsYchF1 confirmed through yeast two-hybrid analysis, thereby negatively regulating both biotic and abiotic stresses (Cheung et al. 2020). In addition, the complex formed by OsDhn-Rab16D and OsFKBP plays a crucial role in the ABA-responsive drought stress signaling pathway in rice (Tiwari et al., 2019).

In our research, we identified a rice *OsFKBP12* gene T-DNA insertion mutant that exhibits significantly higher drought tolerance compared to the wild type, while plants with overexpressed transgenic rice show reduced drought tolerance. This study aims to delve deeper into the molecular mechanisms of *OsFKBP12* under drought stress, providing theoretical and technical support for developing new drought-resistant rice varieties. This ongoing research underscores the potential of genetic and molecular tools to enhance crop resilience, addressing critical agricultural challenges posed by climate change.

Materials and Methods

Plant Materials and Stress Treatments

The rice T-DNA insertional mutant *fkbp12* (PFG_2C-20288.L) and its wild-type counterpart, Dongjin (DJ), were sourced from the RiceGE database (http://signal.salk.edu/cgi-bin/RiceGE). Molecular characterization was performed using FKBP12-specific and T-DNA right border primers, the details of which can be found in Table S1. To generate FKBP12-overexpressing transgenic plants in the DJ genetic background, the entire 1200 bp cDNA of the target gene, spanning from the start to the stop codon, was amplified using DJ cDNA as a template. This segment included *Xba* I and *Pst* I restriction sites. The amplified and purified target fragment was cloned into the linearized 2 × 35 S-pCAMBIA1300 vector using the NovoRec® plus One-step PCR Cloning Kit. The correctly assembled fusion vector was transformed into Agrobacterium tumefaciens EHA105 and subsequently used to transform DJ callus tissue to produce overexpressing transgenic plants.

For germination, seeds were soaked in water at 28 °C for 2 days, followed by planting in either a bottomless 96-well plate or directly in soil. Two weeks post-planting, the seedlings were segregated into a control group (untreated) and a treatment group (subjected to drought stress using 18% PEG6000 and 180 mM mannitol). Subsequent to the treatment period, samples were collected

or physiological parameters were assessed. Leaves from both control and treated seedlings were immediately harvested and flash-frozen in liquid nitrogen, then stored at -80 °C for subsequent physiological or proteomic analysis.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from root, stem, leaf, stamen, leaf sheath, and opening inflorescence tissues using TRIzol reagent (Cowin Biotech, Taizhou, China). The qRT-PCR was conducted using the SuperStar Universal SYBR Master Mix (Cowin Biotech, Taizhou, China) and CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), following the methodology outlined by Zhao et al. (Zhao et al. 2022).

Subcellular Localization of FKBP12

The coding sequence of OsFKBP12 was amplified via PCR and cloned into the pCambia1300-GFP vector for transient expression in protoplasts derived from 2-week-old wild-type rice stems or 3-week-old tobacco leaves. The subcellular localization was determined by co-localization analysis of GFP fluorescence using a confocal laser scanning microscope (LSM710, Zeiss), according to the method described by Ruan et al. (Ruan et al. 2019).

Proline Content

Proline content in rice leaves was determined following the method by Zhao et al. (Zhao et al. 2023). Approximately 0.5 g of leaf tissue was ground to a powder and mixed with 5 mL of 3% sulfosalicylic acid. After centrifugation at 12,000×g for 10 min, 1 mL of the supernatant was mixed with 1 mL of ninhydrin reagent and 1 mL of glacial acetic acid, and then incubated at 100 °C for 1 h. Post-reaction, samples were cooled on ice, followed by the addition of 2 mL toluene to extract the colored product, which was quantified at 520 nm using a spectrophotometer.

Soluble Sugar Content

To generate a standard curve, glucose solutions of various concentrations (1 μM, 10 μM, 50 μM, 100 μM, 150 μM, 200 μM, 300 μM) were prepared, each with a volume of 1 mL. For the assay, 50 μL of each diluted glucose solution was mixed with 4.95 mL of anthrone reagent and boiled for 15 min. The optical density of glucose standards was measured at 620 nm using a spectrophotometer. Dried samples (0.1 g) were ground in liquid nitrogen and extracted in 50 mL tubes with 2 mL of 80% ethanol on an orbital shaker at 200 rpm for 1 h. After centrifugation at 6,000 × g for 10 min, the supernatant was transferred to a new 5 mL tube and mixed with an equal volume of chloroform, followed by centrifugation at 12,000 × g for 10 min. The aqueous phase was transferred to a new tube for optical density measurement, and the content of soluble sugars was calculated based on the standard curve.

Malondialdehyde (MDA) Content

MDA content was determined following the method described (Xiong et al. 2021). Approximately 0.5 g of rice leaf tissue was homogenized in 10 mL of 10% trichloroacetic acid (w/v) and centrifuged at 5,000 × g for 10 min. Two mL of supernatant was mixed with 2 mL of 0.6% thiobarbituric acid (w/v) in 10% trichloroacetic acid and reacted at 100 °C for 15 min. The mixture was then rapidly cooled on ice and centrifuged at 5,000 × g for 10 min. The absorbance of the supernatant was measured at 450 nm, 532 nm, and 600 nm, and the MDA content was calculated using the formula: $6.45 \times (OD_{532} - OD_{600}) - 0.559 \times OD_{450}$.

CAT and SOD Enzyme Activity and H_2O_2 Content Measurement

Catalase (CAT) and superoxide dismutase (SOD) activities were determined using commercial assay kits (BC0200 and BC5160, Solarbio, Beijing, China). Fresh leaves from four-leaf stage plants (0.1 g) were homogenized in 1 mL of phosphate buffer (50 μmol L^{-1}, pH 7.0) and centrifuged at 8,000 × g for 10 min at 4 °C. The supernatant was collected for crude enzyme extract and enzyme activity was measured spectrophotometrically. Hydrogen peroxide (H_2O_2) content was quantitatively analyzed using a hydrogen peroxide assay kit (BC3590, Solarbio), following previously described methods (Chen et al. 2021).

DAB and NBT Staining

For DAB staining, the second leaf from the base of rice seedlings was cut into approximately 1 cm sections and immersed in DAB solution (1 mg/ml). The sections were vacuum infiltrated for 10 min and then incubated at room temperature for 12 h. After incubation, the samples were decolorized in anhydrous ethanol in a 100 °C water bath until the chlorophyll was completely removed. The samples were then mounted on slides and images were captured using a stereo microscope.

For NBT staining, leaf sections were immersed in a 6 mM NBT solution and subjected to vacuum infiltration for 10 min followed by a 12-hour incubation at room temperature. Similar to DAB staining, after incubation, the samples were decolorized in anhydrous ethanol in a 100 °C water bath until complete removal of chlorophyll.

ABA Treatments

To determine the sensitivity of rice seedlings to abscisic acid (ABA), seeds of both wild-type and transgenic lines were sterilized and germinated on half-strength Murashige and Skoog (1/2 MS) medium for 36 h.

Uniformly germinated seeds were then transferred to 1/2 MS media containing 0, 1, and 5 mM ABA and grown in a growth chamber set at 28 °C with a 14-hour light/10-hour dark cycle for 10 days. Plant height was measured using ImageJ software.

Measurement of Chlorophyll Contents

The method for determining chlorophyll content was based on the previous description (Ruan et al., 2017). Equal amounts of leaf tissue (ranging from 0.05 to 0.1 g) were immersed in 10 mL of chlorophyll extraction solvent and extracted in the dark for 16 h. The supernatant was then used to measure chlorophyll content spectrophotometrically at wavelengths of 663 nm and 645 nm.

Scanning Electron Microscopy of Rice Glumes

Scanning electron microscopy (SEM) was performed following the method reported (Ruan et al. 2020). Fully developed seeds were coated with a thin layer of gold to enhance conductivity. SEM was used to image the samples, and ImageJ software was utilized to analyze the images to obtain detailed morphologies and cell dimensions of the rice glumes.

Western Blot Analysis

Total protein extraction was adapted from the methods described by Ruan et al. (Ruan et al. 2024b). The proteins were then resolved using 12% SDS-PAGE and subjected to Western blot analysis, strictly following the protocol outlined by Kurien and Scofield (Kurien et al., 2006).

Results

Induction of *FKBP12* Gene Expression by PEG6000 and ABA

Utilizing the NCBI BLAST tool, we identified 28 members of the OsFKBP protein family within the rice genome and conducted a phylogenetic analysis. The results revealed that OsFKBP12 shares a close evolutionary relationship with OsFKBP20 and OsFKBP20-1b (Fig. 1A). In Arabidopsis, *FKBP12* has been reported to participate in the ABA signaling pathway and stress responses (e.g., pathogen resistance), suggesting its potential role in plant stress responses (Cheung et al. 2020). Previous studies have shown that members of the rice FKBP family (e.g., *OsFKBP20*) play important roles in high-temperature stress responses (Nie et al. 2024), while the function of *FKBP12* has not been systematically investigated. Therefore, we selected *FKBP12* as the research subject to fill this gap in knowledge. To investigate the role of *FKBP12* in abiotic stress responses,

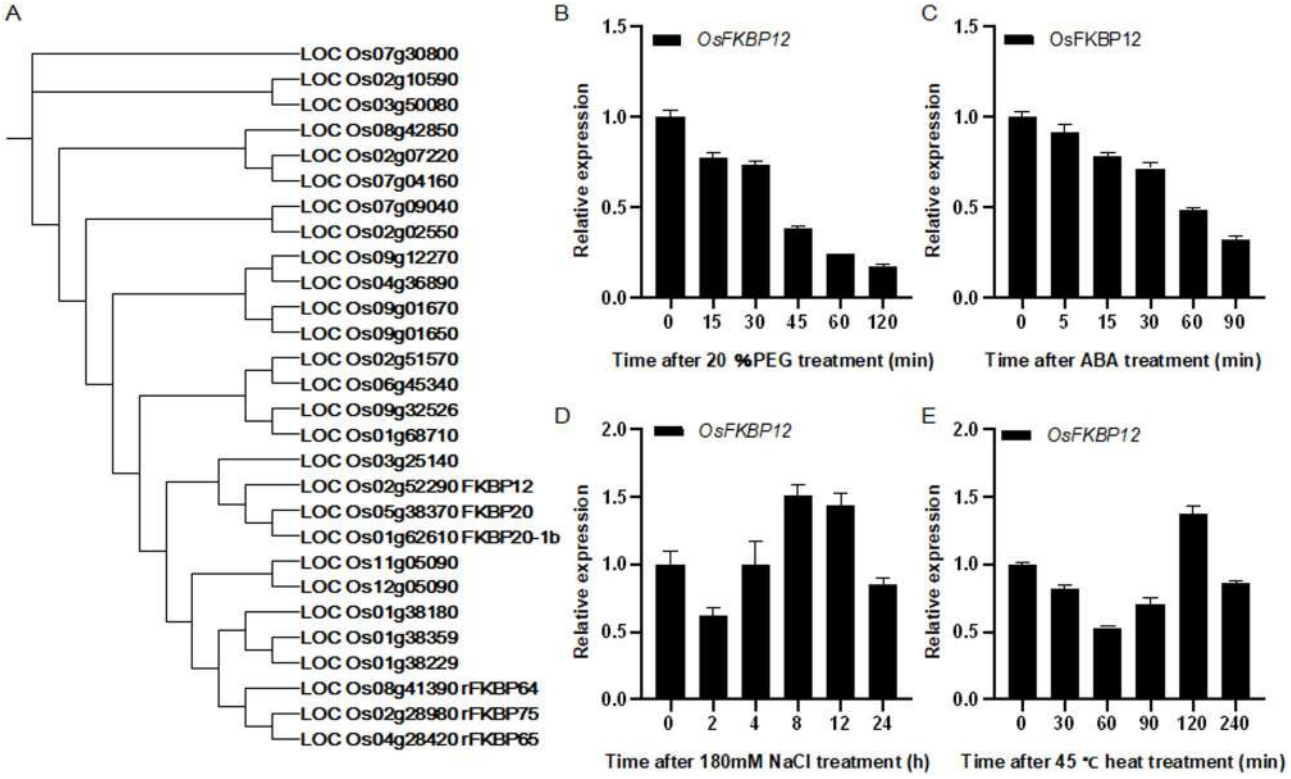

Fig. 1 Construction of the phylogenetic tree and gene expression analysis of OsFKBP12. (**A**) The phylogenetic tree analysis of OsFKBP12 homologous genes in rice. (**B**) RT-qPCR analysis of *OsFKBP12* in rice seedlings treated with 20%PEG. (**C**) RT-qPCR analysis of *OsFKBP12* in rice seedlings treated with 50 μm ABA. (**D**) RT-qPCR analysis of *OsFKBP12* in rice seedlings treated with 180 mM NaCl. (**E**) RT-qPCR analysis of *OsFKBP12* in rice seedlings treated with 45 ℃

wild-type DJ rice was subjected to treatments with 18% PEG6000, ABA, NaCl, and a high temperature of 45℃. Samples were collected at various time points for RNA extraction and cDNA transcription, followed by an analysis of *FKBP12* expression dynamics under each condition using quantitative real-time PCR. The findings indicate that the expression levels of *FKBP12* progressively decreased with time under 18% PEG6000 and ABA treatments (Fig. 1B and C). Conversely, under NaCl and high-temperature conditions, there were no notable trends in expression changes (Fig. 1D and F). These observations suggest that FKBP12 may play a significant role in the drought response mechanisms of rice.

Tissue Expression Patterns and Subcellular Localization of OsFKBP12

To delve deeper into the functional roles of FKBP12 in rice, we harvested various tissues (including roots, stems, leaves, leaf sheaths, and panicles) from wild-type DJ rice at the heading stage for RNA and protein analyses. Quantitative and semi-quantitative assays demonstrated that *FKBP12* is expressed across all tested tissues, notably exhibiting higher expression levels in leaves and leaf sheaths, and lower levels in roots (Fig. 2A and B). Furthermore, we employed specific FKBP12 polyclonal antibodies to examine protein abundance across these tissues. Immunoblotting results confirmed that protein expression patterns correspond with RNA levels, with the highest expression in leaves and the lowest in roots (Fig. 2C).

Moreover, to determine the subcellular localization of FKBP12, we employed transient expression techniques to introduce FKBP12-eGFP fusion proteins into rice protoplasts and tobacco leaf epidermal cells. Confocal laser scanning microscopy revealed that the FKBP12-GFP fusion, driven by the 35 S promoter, emitted green fluorescence in both the nucleus and cytoplasm (Fig. 2D and E). This observation provides crucial insights for

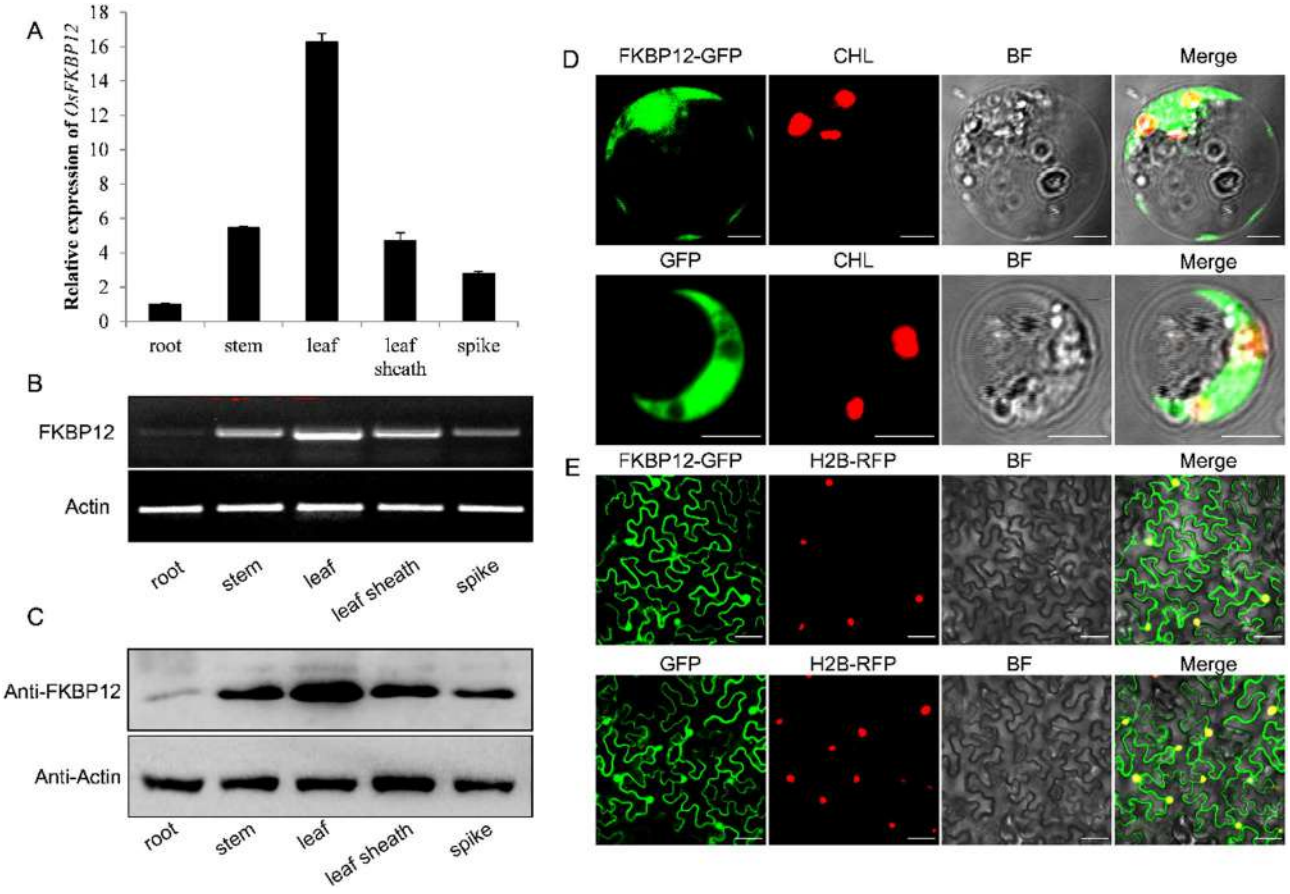

Fig. 2 The tissue expression patterns and subcellular localization of OsFKBP12. (**A**) Expression of *OsFKBP12* in various organs analyzed by qRT-PCR analysis. (**B**) Expression of *OsFKBP12* in various organs analyzed by RT-PCR analysis. (**C**) Immunoblot analysis of OsFKBP12 in various organs in the wild type. (**D**) Subcellular localization of the OsFKBP12 protein in rice protoplasts. Images showing rice protoplasts expressing the p35S::OsFKBP12::GFP (Top panel) or p35S::GFP (Bottom panel)fusion protein; H2B-RFP, nucleus marker; CHL, chloroplast. Fluorescence was observed using a confocal microscope. Bar = 5 μm. (**E**) Subcellular localization of the OsFKBP12 protein in tobacco epidermal cells. Nuclear localization signal-mCherry were used as nuclear markers (second column). Image showing tobacco epidermal cells expressing the p35S::OsFKBP12::GFP (Top panel) or p35S::GFP (Bottom panel)fusion protein; H2B-RFP, nucleus marker. Fluorescence was observed using a confocal microscope. Bar = 50 μm

further exploration of its cellular functions and regulatory mechanisms.

Enhanced Drought Tolerance in *fkbp12* Mutant

To further investigate the role of FKBP12 in rice, we utilized a T-DNA insertional mutant sourced from http://signal.salk.edu/cgi-bin/RiceGE, where the insertion occurred at 572 bp within the gDNA (Fig. 3A-B). Quantitative and semi-quantitative analyses indicated that this T-DNA insertion profoundly affected the transcription of the *FKBP12* gene in the *fkbp12* mutant (Fig. 3C-D). Additionally, proteins extracted from 14-day-old wild-type and *fkbp12* mutant plants were analyzed via Western

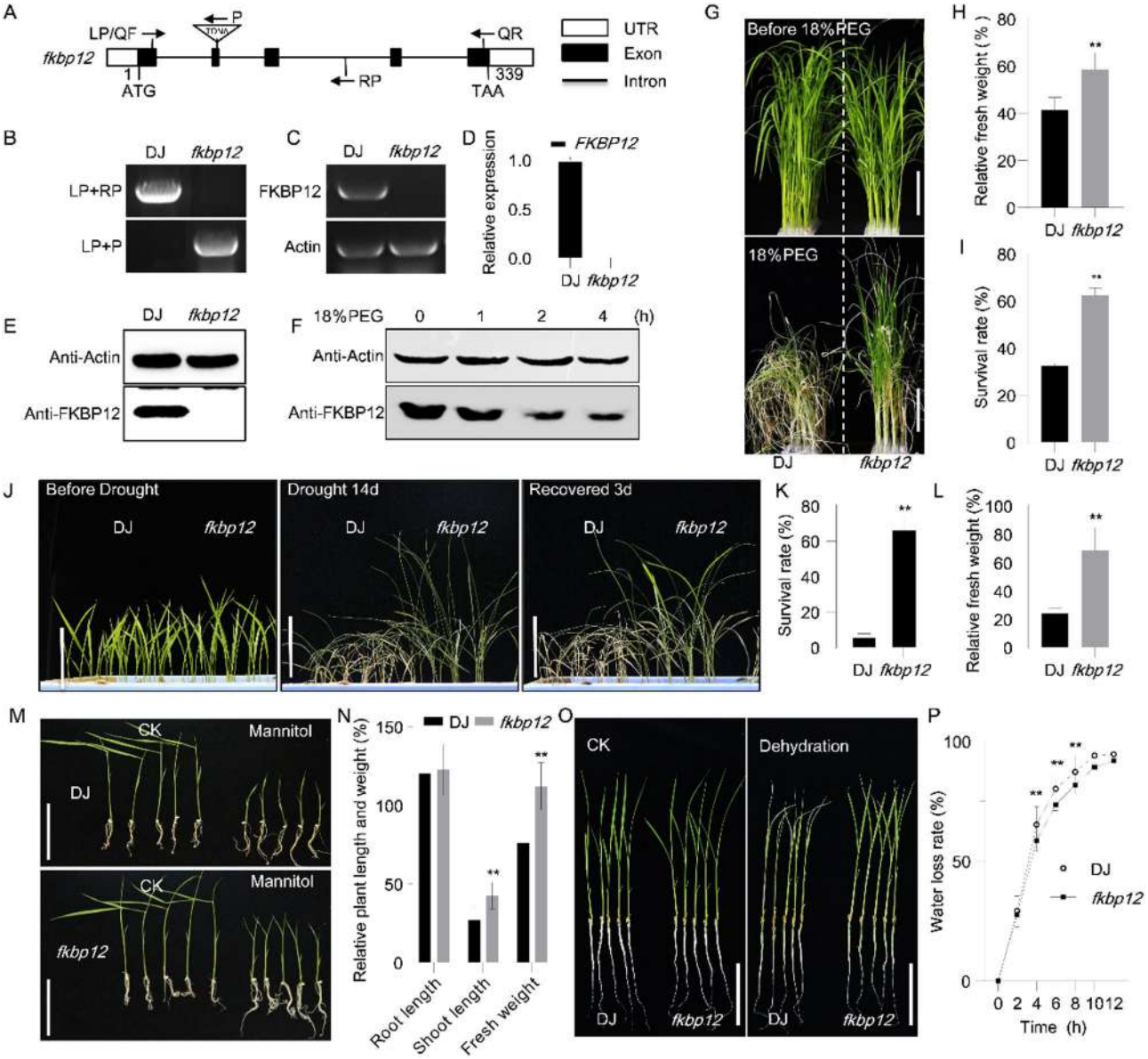

Fig. 3 Molecular identification and phenotypic analysis of *fkbp12*. (**A**) The *FKBP12* gene structure. (**B**) Genotyping of *fkbp12* mutants. (**C**) RT-PCR analysis of *FKBP12* in *fkbp12*. (**D**) qRT-PCR analysis of *FKBP12* in *fkbp12*. (**E**) Immunoblot analysis of OsFKBP12 in *fkbp12*. (**F**) Immunoblot analysis using anti-FKBP12 antibody to show FKBP12 protein levels after 18%PEG treatment for different times; actin was used as the control. (**G**) Phenotypic comparison of DJ and *fkbp12* seedlings under normal conditions or after 18%PEG stress for 14 d. and then allowed to recover for 3 d. Scale bar = 5 cm. H-I. Relative fresh weight (**H**), and survival rate (**I**) of DJ and *fkbp12* seedlings after 18%PEG stress for 14 d and then allowed to recover for 3 d. (**J**). Phenotypic comparison of DJ and *fkbp12* seedlings treated with drought stress. Scale bar = 5 cm. K-L. Survival rate (**K**), and fresh weight (**L**) of DJ and *fkbp12* seedlings treated with drought stress. M. Phenotypic comparison of DJ and *fkbp12* seedlings treated with dehydration stress for 2 h. Scale bar = 5 cm. N. Water loss rate of DJ and *fkbp12* seedlings treated with dehydration stress. O. Growth of DJ and *fkbp12* seedlings treated with 180 mM Mannitol after seed germination on 1/2 MS medium for 2 days. Scale bar = 5 cm. P. Relative shoot length and Relative root length of DJ and *fkbp12* seedlings treated with 180 mM Mannitol. Data are presented as mean ± SD ($n = 3$, $^{*}P \le 0.05$; $^{**}P \le 0.01$, Student's t test)

blot, revealing almost complete inhibition of FKBP12 protein translation in the mutant (Fig. 3E).

Previous studies utilizing qRT-PCR showed that *FKBP12* expression was downregulated under PEG6000 induction (Fig. 1B). Subsequent treatments of wild-type with PEG6000, and periodic sampling, demonstrated a consistent downregulation of FKBP12 protein levels under drought stress (Fig. 3F), aligning with our quantitative data and suggesting a role for FKBP12 in drought stress signaling. Further investigations were conducted on 21-day-old DJ and *fkbp12* hydroponic seedlings treated with 18% PEG6000, followed by a recovery period of 48 h in normal nutrient solution. The results showed significant lodging and irreversible leaf curling in wild-type rice, whereas the mutants maintained an upright posture (Fig. 3G). Comparison of fresh weight and survival rates between the wild-type and mutant revealed significantly higher values in the *fkbp12* mutant (Fig. 3H-I), indicating superior drought tolerance in the *fkbp12* mutant. To definitively confirm that *FKBP12* corresponds to *LOC_Os02g52290*, we meticulously inserted a 5.25 kb genomic fragment of *LOC_Os02g52290* into the binary vector pCAMBIA2300, followed by its introduction into the *fkbp12* mutant via transformation. This process yielded 11 independent T_0 transformants. Detailed phenotypic analysis clearly demonstrated that each transformant (FKBP12-RE) effectively mitigated the *fkbp12* mutant phenotype, as evidenced by decreased drought tolerance (Fig. S1, S5).

Additionally, both wild-type and *fkbp12* mutant seedlings were subjected to soil-based drought conditions during their seedling stage, and the mutants exhibited significantly higher survival rates and fresh weights than the wild-type (Fig. 3J-L), consistent with the results from PEG6000 treatments. To further confirm these findings, wild-type and *fkbp12* mutant seeds were germinated on 1/2 MS medium, then transplanted to 1/2 MS medium with or without 200 mM mannitol. After 7 days, the mannitol treatment notably reduced the shoot height of the wild type more than that of the mutants. Both types showed a significant increase in root length with no notable difference between them. However, the fresh weight of the wild type significantly decreased post-treatment, whereas that of the mutants remained nearly unchanged (Fig. 3M-N). These results further emphasize the enhanced drought tolerance of the *fkbp12* mutant.

A significant effect of drought stress on plants involves the regulation of stomatal aperture to reduce transpiration and water loss. We assessed the water loss rate in detached leaves from wild-type and *fkbp12* mutant, observing a significantly higher rate in the wild type compared to the *fkbp12* mutant (Fig. 3O-P). This indicates that the loss of *FKBP12* may impact the transpiration dynamics in rice leaves, reinforcing our insights into the role of FKBP12 in enhancing rice drought tolerance.

Increased Oxidative Stress Tolerance in the *fkbp12* Mutant

To deepen our understanding of how plants manage oxidative stress under adverse conditions, we examined the response of rice to drought stress. Typically, reactive oxygen species (ROS) produced during photosynthesis and respiration are effectively neutralized by various antioxidant mechanisms to maintain a physiological equilibrium. However, stress conditions such as drought disrupt this balance, significantly elevating ROS levels (Jing et al. 2022; Li et al. 2021a, b; Zhou et al. 2022). Superoxide dismutase (SOD) first catalyzes the breakdown of superoxide radicals, while catalase (CAT) and peroxidase (POD) decompose hydrogen peroxide (H_2O_2) into water and oxygen, crucial for ROS detoxification (Liu et al. 2023).

In this study, we compared superoxide anion levels, hydrogen peroxide content, CAT activity, and malondialdehyde (MDA) levels in both wild-type rice and *fkbp12* mutant before and after drought stress. DAB staining revealed no significant differences between the wild type and mutants before stress; however, post-stress, mutants exhibited noticeably fewer yellow-brown spots (Fig. 4A), NBT staining indicated that superoxide anion accumulation was significantly greater in the wild type under drought conditions (Fig. 4B), suggesting significantly lower H_2O_2 accumulation—a result further validated by direct H_2O_2 measurements (Fig. 4C).

Antioxidant enzyme activity analyses showed no significant differences in CAT and SOD activities or MDA levels between the wild type and mutants before drought exposure. Post-drought, however, CAT and SOD activities in the mutants were significantly enhanced, and there was a lesser increase in MDA levels (Fig. 4D, E, F). This indicates that *fkbp12* mutant possess improved antioxidative capabilities under drought conditions. Further, DAB staining following treatments with varying hydrogen peroxide concentrations showed that, although H_2O_2 accumulation increased with concentration, it was markedly less in mutants compared to the wild type (Fig. 4G, H), reinforcing the mutants' heightened antioxidative capacity.

Drought stress also leads to cellular dehydration in rice. To combat this, rice boosts the levels of soluble sugars and proline within cells, which lowers water potential and minimizes water loss. Our data indicate no significant differences in the levels of these osmolytes between the wild type and mutants under control conditions; however, after 24 h of drought treatment, both proline and soluble sugar levels were significantly higher in the mutants (Fig. 4I, J). This suggests that *fkbp12* mutant effectively mitigate water loss by increasing cellular osmotic pressure, thus enhancing their drought tolerance.

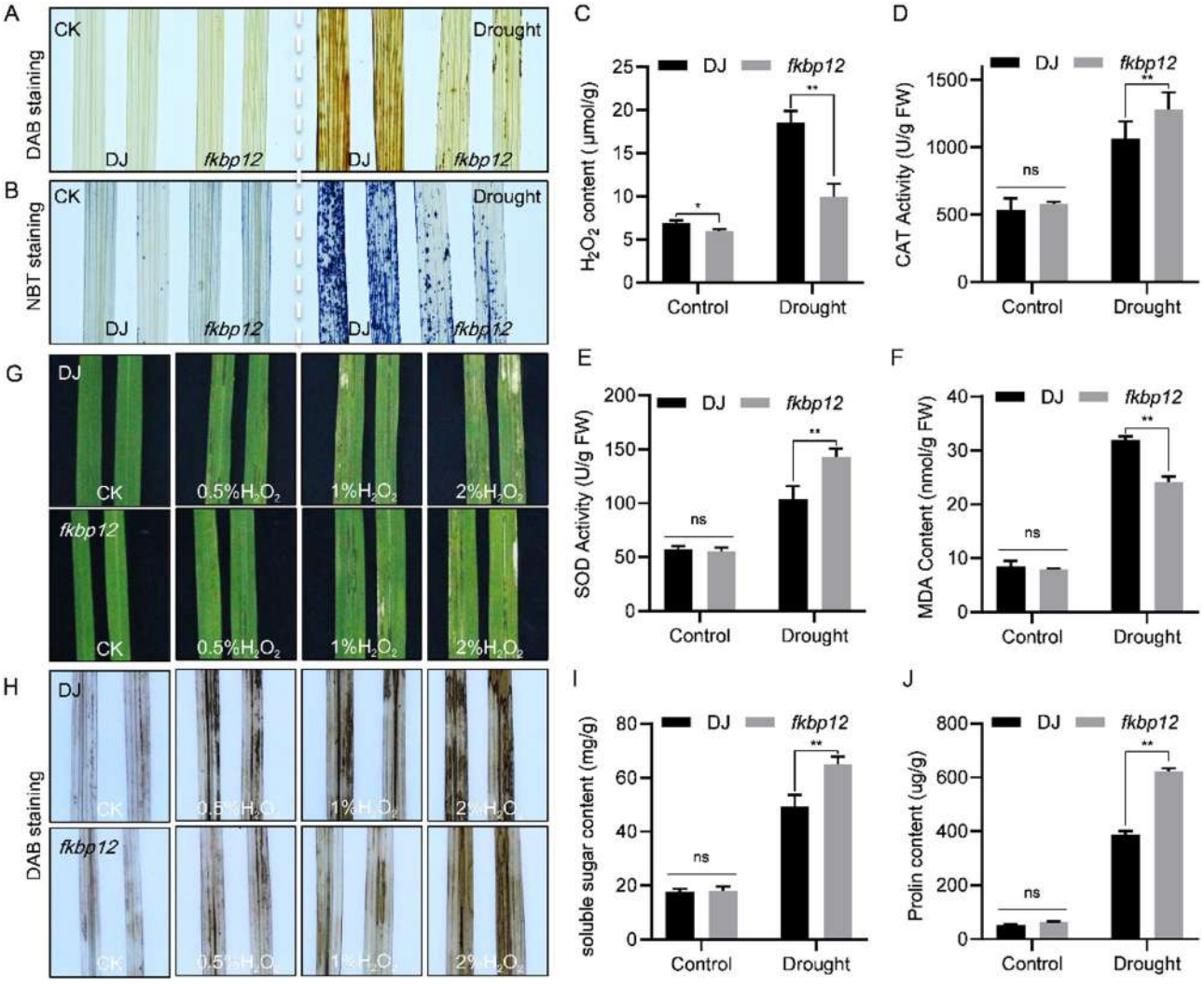

Fig. 4 Oxidative stress tolerance was improved in *fkbp12* plants. (**A**) DAB staining of the leaves of DJ and *fkbp12* plants at the 3-leaf stage under normal conditions or after 18%PEG stress for 4 d. (**B**) NBT staining of the leaves of DJ and *fkbp12* plants at the 3-leaf stage under normal conditions or after 18%PEG stress for 4 d. (**C**) Leaf phenotype of DJ and *fkbp12* plants at the 3-leaf stage under normal conditions or after H_2O_2 stress for 4 d. (**D**) DAB staining of the leaves of DJ and *fkbp12* plants at the 3-leaf stage under normal conditions or after H_2O_2 stress for 4 d. E-F. Activities of CAT (**E**) and SOD (**F**) of DJ and *fkbp12* plants at the 3-leaf stage under normal conditions or after 18%PEG stress for 2 d. G-J. Content of H_2O_2(**G**), proline (**H**), soluble sugar (**I**) and MDA (**J**) in the leaves of DJ and *fkbp12* plants at the 3-leaf stage under normal conditions or after 18%PEG stress for 4 d. Data are presented as mean ± SD ($n = 3$, $^*P \le 0.05$; $^{**}P \le 0.01$, Student's t test)

Overall, these results demonstrate that *fkbp12* mutant displayed enhanced adaptability to drought stress by boosting their antioxidative capacity and increasing the accumulation of osmolytes within their cells.

Decreased Drought and Oxidative Tolerance in *OsFKBP12* Overexpression Plants

To further explore the role of *FKBP12* in rice under drought stress conditions, we developed overexpression lines, specifically focusing on two strains, FKBP12-OE-5 and FKBP12-OE-6, for detailed analysis (Fig. S1). After treating these strains with 18% PEG6000, we observed a significant decrease in their survival rates (Fig. 5A, B, C). Both DAB and NBT staining, along with measurements of H_2O_2 content, indicated an increased accumulation of H_2O_2 and superoxide anions in these overexpressing strains following drought stress (Fig. 5D, G).

Before drought treatment, there were no significant differences in MDA levels and proline levels between the wild type and the FKBP12-OE-5 and FKBP12-OE-6 strains. Post-drought stress, however, these overexpression strains exhibited a significant increase in MDA levels compared to the wild type (Fig. 5H), while their proline levels decreased (Fig. 5I). Furthermore, we assessed chlorophyll content following exposure to varying concentrations of hydrogen peroxide. The results demonstrated that, although chlorophyll content decreased progressively with increasing concentrations of the treatment,

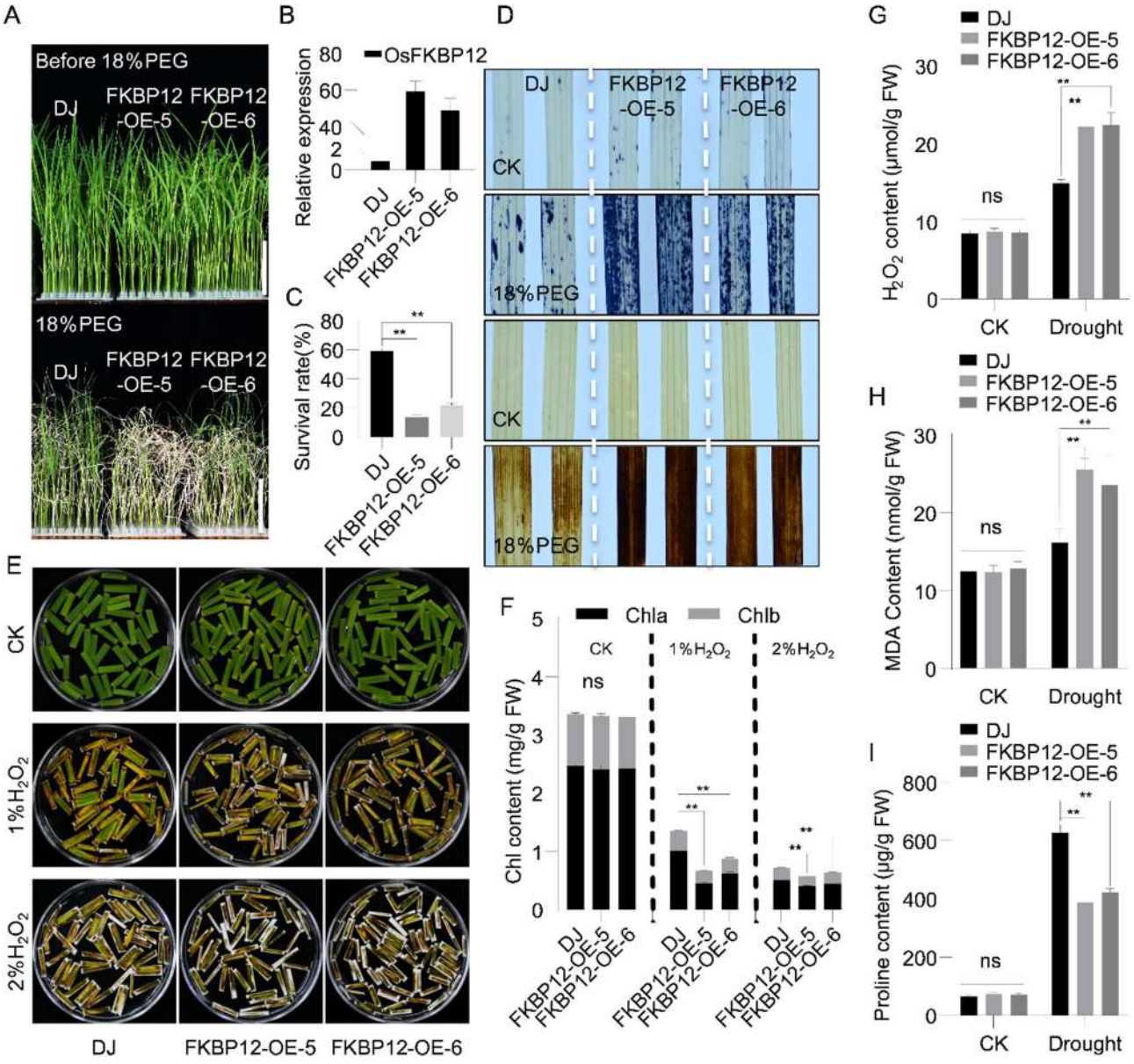

Fig. 5 Overexpression of OsFKBP12 decreased drought and oxidative tolerance in rice at the seedling stage. (**A**) Phenotypic comparison of DJ, FKBP12-OE-5 and FKBP12-OE-6 seedlings under normal conditions or after 18%PEG stress for 8 d. and then allowed to recover for 3 d. Scale bar = 5 cm. (**B**) qRT-PCR analysis of *FKBP12* in FKBP12-OE plants. (**C**) Survival rate of DJ, FKBP12-OE-5 and FKBP12-OE-6 seedlings after 18%PEG stress for 14 d and then allowed to recover for 3 d. (**D**) DAB and NBT staining of the leaves of DJ, FKBP12-OE-5 and FKBP12-OE-6 plants at the 3-leaf stage under normal conditions or after 18%PEG stress for 4 d. (**E**) Leaf phenotype of DJ, FKBP12-OE-5 and FKBP12-OE-6 plants at the 3-leaf stage under normal conditions or after H_2O_2 stress for 4 d. (**F**) Content of Chlorophyll in the leaves of plants at the 3-leaf stage under normal conditions or after H_2O_2 stress for 4 d. G-I. Content of H_2O_2 (**G**), MDA (**H**) and proline (**I**) in the leaves of DJ, FKBP12-OE-5 and FKBP12-OE-6 plants at the 3-leaf stage under normal conditions or after 18%PEG stress for 4 d. Data are presented as mean ± SD ($n = 3$, $^*P \le 0.05$; $^{**}P \le 0.01$, Student's t test)

the decrease was significantly more pronounced in the FKBP12-OE-5 and FKBP12-OE-6 strains than in the wild type (Fig. 5E, F). To validate the drought phenotypes under agriculturally relevant conditions, soil drought assays were performed. FKBP12-OE lines exhibited severe wilting and reduced survival, consistent with PEG-induced stress responses (Fig. S5). The parallel outcomes of PEG and soil drought treatments underscore FKBP12's conserved role in suppressing drought tolerance across stress regimes.

These results underscore FKBP12's involvement in the drought stress response in rice, indicating that overexpression of this gene might compromise the plant's antioxidative defense system, thus heightening drought sensitivity.

Drought Stress-Related Gene Expression Analysis Between the Wild Type and *fkbp12* Mutant

To further elucidate the role of *FKBP12* in rice's response to drought, we analyzed the expression levels of several drought stress-related genes in both wild-type and *fkbp12* mutant lines. These genes include *OsNCED3*, *OsNCED4*, *PP2C09*, *PP2C30*, *OsABA8OX1*, *OsSRO1C*, *OsSNAC1*, *OsABIL2*, *OsNAC5*, *OsNAC6*, *bZIP23*, and *OsDREB2A*. Prior to drought treatment, the expression levels of these genes showed no significant differences between the wild type and mutant. However, upon exposure to drought stress, expression levels in the *fkbp12* mutant were significantly higher than in the wild type (Fig. 6A-L). This indicates that the lack of FKBP12 may enhance the expression of these drought-responsive genes.

This discovery suggests that *fkbp12* mutant is able to respond more swiftly and effectively to drought stress, enhancing their drought adaptability by efficiently transmitting signals to downstream processes. This heightened response likely involves the modulation of key gene expressions within the drought stress signaling pathway, providing valuable insights for further investigations into *FKBP12*'s specific mechanisms in drought stress response.

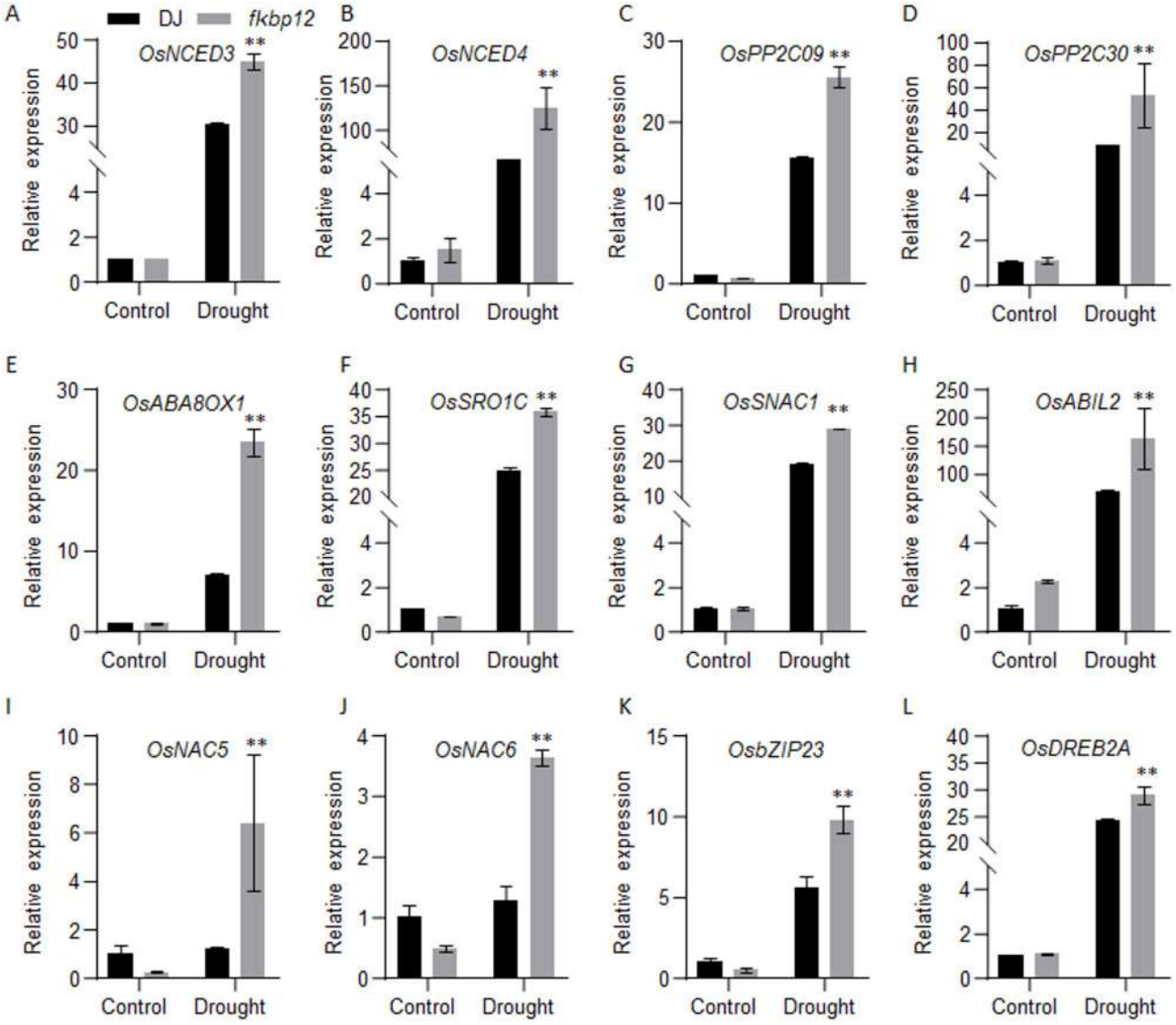

Fig. 6 Relative expression levels of ABA synthesis and response-related genes in *fkbp12* seedlings. **A-B**. Relative expression levels of ABA synthesis genes in 2-week-old *fkbp12* seedlings. **C-L**. Relative expression levels of ABA-responsive and drought stress-related genes in 2-week-old *fkbp12* seedlings. The expression of OsActin1 was used as an internal control. The figure presents the relative expression levels of genes relative to that in WT. Data are presented as mean ± SD ($n = 3$, $^{*}P \le 0.05$; $^{**}P \le 0.01$, Student's t test)

Role of *FKBP12* in ABA-Mediated Drought Response in Rice

Abscisic acid (ABA) plays a critical role in rice's drought tolerance, operating through several mechanisms: it regulates stomatal closure to minimize water loss, promotes deeper root penetration to enhance water uptake, controls the expression of stress-responsive genes, maintains cellular osmotic balance, and participates in complex signaling pathways. To explore the role of FKBP12 in ABA-mediated regulation, we subjected wild-type and *fkbp12* mutant strains to varying concentrations of ABA. Experimental results indicated that both seedling and root lengths decreased with increasing ABA concentrations in both genotypes, with a more marked reduction observed in the *fkbp12* mutant (Fig. 7A-C), suggesting a heightened sensitivity to ABA in the mutant. Furthermore, wild-type, FKBP12-OE-5, and FKBP12-OE-6 overexpression lines were also treated with ABA. The data demonstrated that, in comparison to the wild type, *FKBP12* overexpression lines showed reduced ABA sensitivity (Fig. 7D, E, F). Additionally, germination tests revealed that the *fkbp12* mutant germinated more slowly, whereas the FKBP12-OE-5 and FKBP12-OE-6 overexpression strains germinated faster than the wild type (Fig. 7G, H). These findings underscore the potential involvement of FKBP12 in ABA regulation, suggesting that this protein may influence plant stress responses through modulation of ABA responses.

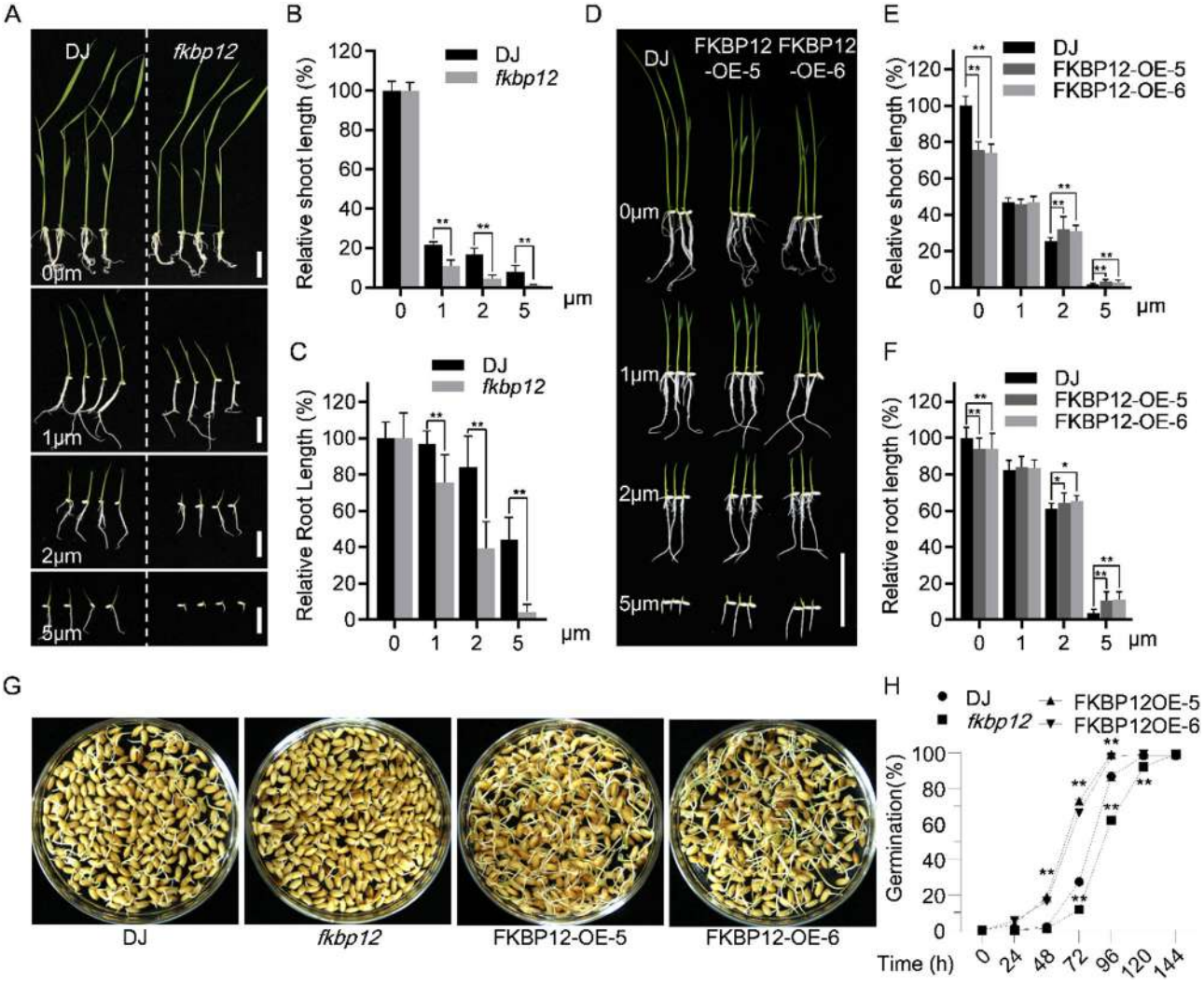

Fig. 7 OsFKBP12 plays a negative role in ABA signaling. (**A**). Seedling growth inhibition of DJ and *fkbp12* by ABA treatment with the indicated concentration for 10 days. Scale bar = 5 cm. B-C. Statistical analysis of relative shoot length (**B**) and relative root length (**C**) of DJ and *fkbp12* in (**A**). (**D**). Seedling growth inhibition of DJ, FKBP12-OE-5 and FKBP12-OE-6 by ABA treatment with the indicated concentration for 7 days. Scale bar = 5 cm. E-F. Statistical analysis of relative shoot length (**E**) and relative root length (**F**) of FKBP12-OE-5 and FKBP12-OE-6 in (D). G. Germination performance of DJ, *fkbp12*, FKBP12-OE-5 and FKBP12-OE-6 at 4d. H. Germination rate of DJ and fkbp12 seeds within seven days. Data are presented as mean ± SD ($n = 3$, $^{*}P \le 0.05$; $^{**}P \le 0.01$, Student's t test)

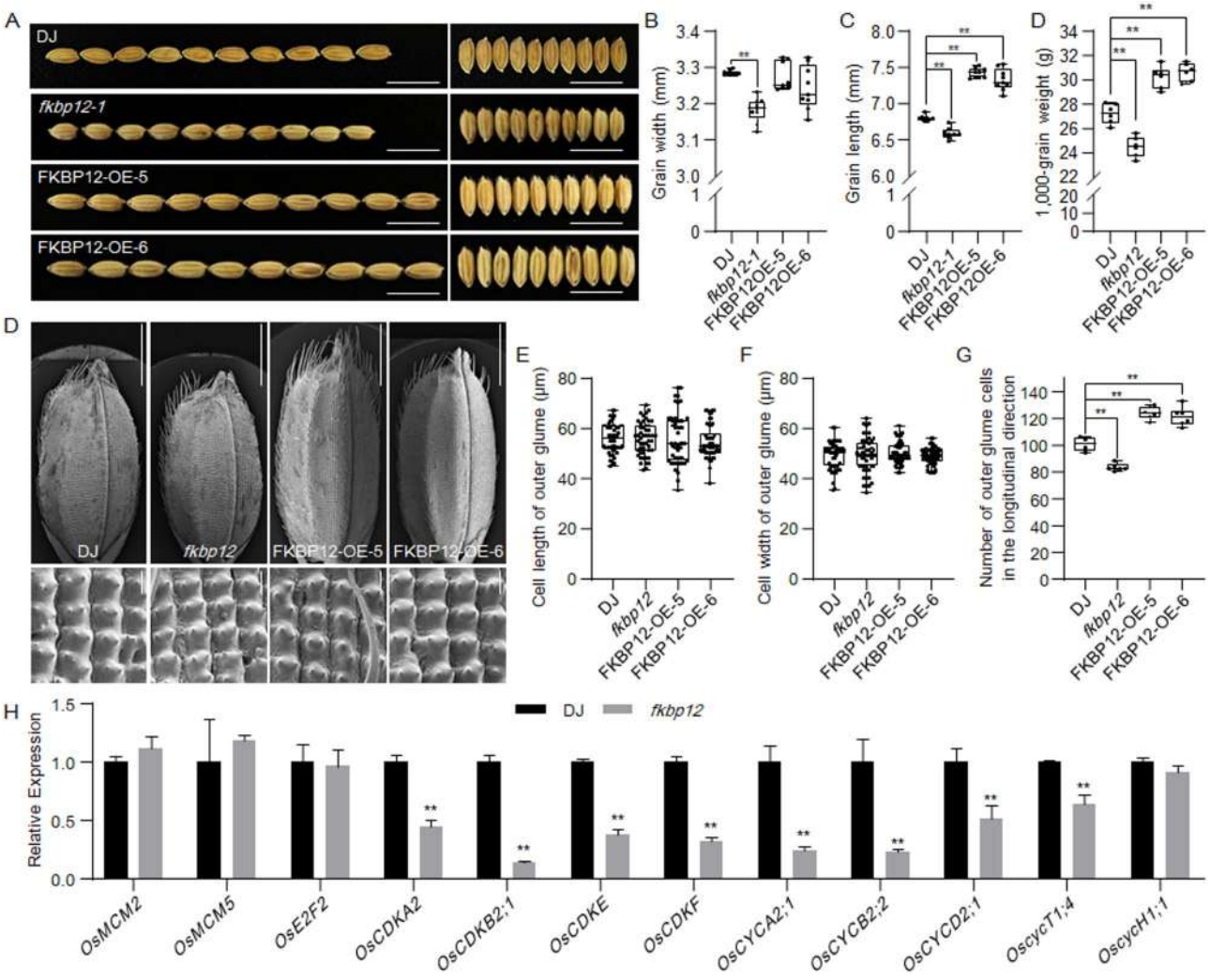

Fig. 8 OsFKBP12 regulates grain size by altering the number of spikelet hull cells. (**A**) Mature grains of DJ, *fkbp12*, FKBP12-OE-5 and FKBP12-OE-6 plants. Scale bar = 1 cm. **B-D**. Comparisons of grain length ($n = 9$) (**B**), grain width ($n = 9$) (**C**), and 1,000-grain weight ($n = 6$) (**D**) between DJ, *fkbp12*, FKBP12-OE-5 and FKBP12-OE-6. D. Scanning electron microscopic analyses of the outer surfaces of DJ, *fkbp12*, FKBP12-OE-5 and FKBP12-OE-6 spikelet hulls. Top scale bar = 2 mm, Bottom scale bar = 50 μm. (**E-F**), cell length ($n = 50$) (**E**), cell width ($n = 50$) (**F**) and Cell number ($n = 6$) (**G**), of the outer epidermal cell as indicated in (**D**). H. Relative transcriptional expression of several cell expansion- and cell cycle-related genes in DJ and *fkbp12* young panicles. Error bars indicate the standard deviation of three independent samples. OsACTIN1 was used as the internal control to normalize expression. Data are presented as mean ± SD ($n = 3$, $^{*}P \le 0.05$; $^{**}P \le 0.01$, Student's t test)

Impact of *FKBP12* on Seed Size and Cell Cycle Regulation in Rice

We conducted size measurements on seeds from wild-type plants, *fkbp12* mutant, complemented line FKBP12-RE-1, FKBP12-RE-2 and the FKBP12-OE-5 and FKBP12-OE-6 overexpression lines. Results indicated that the seeds of the *fkbp12* mutant were shorter and narrower, with a significantly lighter 1000-grain weight compared to the wild type. The complementary lines reverted the short-grain phenotype of the fkbp12 mutant (Fig. S3). In contrast, seeds from the FKBP12-OE-5 and FKBP12-OE-6 lines were longer, with their width remaining unchanged, but their 1000-grain weight increased (Fig. 8A-D). Observations using scanning electron microscopy on the seed husk surfaces revealed that variations in seed length were primarily driven by changes in cell numbers (Fig. 8D-G). Notably, *fkbp12* mutant and overexpression lines showed a slight decrease in plant height at the booting stage compared to the wild type, while other agronomic traits did not show significant differences compared to the wild type (Fig. S2).

Additionally, we performed quantitative expression analyses of cell cycle-related genes in both the wild type and *fkbp12* mutant. The results showed significant downregulation of key cell cycle genes, including *OsCDKA2*, *OsCDKB2;1*, *OsCDKE*, *OsCDKF*, *OsCYCB2;1*, *OsCYCD2;1*, and *OscycT1;4*, in the *fkbp12* mutant (Fig. 8H). These findings suggest that *FKBP12* may

influence the size of rice seeds, particularly seed length, by modulating cell cycle regulation, thus underscoring *FKBP12*'s crucial role in plant growth and development.

Discussion

The examination of the FKBP12 protein in rice unfolds profound insights into the molecular mechanics governing drought tolerance, a critical trait for upholding agricultural output amid increasingly unpredictable climate conditions. The observed disparities between the *fkbp12* mutant and the FKBP12-overexpressing strains under drought stress delineate the intricate role this protein plays in fine-tuning plant stress responses.

Mechanistic Insights from *FKBP12* Gene Modulation

The role of the *FKBP12* gene in rice under drought conditions highlights critical aspects of plant stress physiology and provides insights into the complex regulatory mechanisms that govern plant responses to abiotic stress. The contrasting phenotypes observed between the *fkbp12* mutant and *FKBP12*-overexpressing plants underscore the dualistic nature of this protein's function in stress modulation.

The *fkbp12* mutant demonstrates notable drought tolerance, which is evident from its ability to maintain higher fresh weight, survive longer under drought conditions, and exhibit reduced cellular damage as indicated by lower levels of malondialdehyde (MDA), a byproduct of lipid peroxidation used as a marker for oxidative stress (Figs. 3 and 4). These phenotypic traits suggest a fundamental cellular resilience that may be rooted in a compensatory biological mechanism activated in response to the absence of FKBP12. The enhanced expression of key drought-responsive genes such as *OsNCED3*, *OsSNAC1*, and *OsDREB2A* in the *fkbp12* mutant (Fig. 6) provides a molecular basis for this observation. *OsNCED3* is involved in ABA biosynthesis, a crucial hormone that regulates stomatal closure and other drought response mechanisms (Gao et al. 2022). *OsSNAC1* and *OsDREB2A* are transcription factors that play significant roles in the regulation of a plethora of genes associated with stress tolerance, enhancing the plant's ability to withstand drought conditions (Dubouzet et al. 2003; Hu et al. 2006). The upregulation of these genes likely contributes to a robust ABA-mediated signaling pathway, enhancing the drought response by improving water retention and reducing water loss through transpiration. This compensatory upregulation suggests that FKBP12 might normally act as a modulator, fine-tuning the expression and activity of these stress-related genes to optimize the plant's response to environmental fluctuations. In its absence, the plant may overactivate these pathways, leading to an enhanced, albeit potentially energetically costly, tolerance to drought.

In stark contrast, *FKBP12*-overexpressing lines exhibit increased susceptibility to drought (Fig. 5), which could indicate a negative regulatory role of *FKBP12* within the plant's stress response network. This phenotype could be due to the protein interfering with normal stress response processes. For instance, FKBP12 might bind to and inhibit the function of other crucial proteins in the stress signaling pathway, thereby dampening the activation of defense mechanisms against drought.

Additionally, the modulation of ROS scavenging mechanisms by FKBP12 might also play a role. Under stress conditions, plants generate ROS, which must be detoxified to prevent cellular damage (Kneeshaw et al. 2017). The overexpression of FKBP12 could disrupt these scavenging pathways, as suggested by increased oxidative damage markers and decreased activity of antioxidative enzymes such as SOD and CAT in these plants (Fig. 5). This disruption leads to an accumulation of ROS, exacerbating cellular damage under drought stress and reducing the plant's ability to cope with the adverse conditions.

FKBP12's Role in ABA Signaling and Osmotic Balance

The findings regarding ABA sensitivity in the *fkbp12* mutant and overexpression lines add complexity to FKBP12's functional landscape. The enhanced sensitivity to ABA in the *fkbp12* mutant (Fig. 7) implies that *FKBP12* might typically suppress ABA signaling pathways. This augmented response to ABA could be integral to the mutant's improved drought toleranc (Li et al. 2021a, b), facilitating more rapid stomatal closure and swift activation of ABA-dependent stress-responsive genes. On the contrary, the reduced ABA sensitivity observed in *FKBP12* overexpression lines might delay essential physiological adjustments to drought, thereby diminishing drought tolerance.

The *fkbp12* mutant's higher accumulation levels of osmolytes such as proline and soluble sugars under drought conditions suggest superior osmotic adjustment capabilities, which are essential for maintaining cellular turgor and ensuring survival under drought stress (Verslues et al. 2006). This ability to effectively adjust osmotic potential may be a key factor in the enhanced drought tolerance observed.

Although FKBP12 is a key factor in TOR inhibition in animals, the regulatory network of TOR in plants is more complex. Recent studies have shown that plant TOR may be activated through non-canonical pathways, such as via energy status or sugar signaling, rather than relying on *FKBP12* (Xiong et al. 2013; Shi et al. 2018). In Arabidopsis, the knockout of *FKBP12* did not significantly affect TOR activity, suggesting that *FKBP12* may not be a primary regulator of TOR in plants. Therefore, the enhanced drought tolerance observed in the *fkbp12* mutant may largely result from the activation of other

pathways independent of TOR, such as the ABA signaling pathway and the reinforcement of ROS scavenging systems.

In this study, we found that ABA biosynthesis genes (*OsNCED3*) and downstream responsive genes (*OsSNAC1*, *OsDREB2A*) were significantly upregulated in the *fkbp12* mutant, indicating that the activation of the ABA signaling pathway may be a key driver of enhanced drought tolerance. Although TOR typically inhibits SnRK activity, members of the SnRK2 family can be directly activated by ABA, independent of the TOR-SnRK1 axis (Belda-Palazón et al. 2020). Thus, the *fkbp12* mutant may enhance drought tolerance by strengthening ABA-SnRK2 signaling, thereby bypassing the antagonistic effects of TOR-SnRK1.

Implications for Rice Seed Development and Yield

The impact of *FKBP12* on seed development and size observed in both the *fkbp12* mutant and *FKBP12*-overexpressing lines provides significant insights into the multifaceted roles that this protein may play beyond stress responses, specifically in the regulation of cellular proliferation and growth.

The correlation between *FKBP12* expression and changes in the expression of cell cycle-related genes in the *fkbp12* mutant suggests a profound role for *FKBP12* in the cell cycle regulation. FKBP12 might interact with cell cycle machinery, influencing the progression of the cell cycle and thus affecting cellular growth and division. Cell cycle genes, such as cyclins and cyclin-dependent kinases, are fundamental for the proper progression of the cell cycle, impacting everything from DNA replication to mitosis. The downregulation of these genes in the *fkbp12* mutant (Fig. 8), as suggested by quantitative expression analysis, implies that FKBP12 may normally promote the expression or activity of these critical regulators, facilitating optimal cell cycle progression and cellular proliferation.

The reduction in seed size in the *fkbp12* mutant hints at a complex trade-off between growth and stress tolerance. This trade-off may be due to a reallocation of physiological and molecular resources; instead of fueling growth and development, resources may be redirected towards enhancing mechanisms that confer stress tolerance. In plants, such decisions are often mediated by hormonal signals and transcriptional networks that integrate environmental cues with developmental programs. ABA, for instance, plays a dual role in promoting stress responses and inhibiting growth. In the *fkbp12* mutant, enhanced ABA signaling—indicated by the upregulation of ABA biosynthesis and response genes—could be inhibiting growth pathways, thereby reducing seed size as a side effect of bolstered stress defense mechanisms.

Conversely, the overexpression of *FKBP12* might lead to an overactive cell cycle, resulting in larger cells or more rapid progression through the cell cycle. However, this can also have deleterious effects, as uncontrolled cell proliferation can lead to developmental abnormalities and decreased overall plant fitness. Moreover, if *FKBP12* overexpression negatively impacts stress response pathways by interfering with the normal functioning of stress response proteins, this could result in plants that are larger but less capable of coping with environmental stresses, thus potentially reducing overall yield under suboptimal conditions.

Understanding the role of FKBP12 in the regulation of growth and stress responses provides crucial information for agricultural practices. Manipulating *FKBP12* expression or function could be a strategy to optimize both stress tolerance and yield in rice. For instance, by modulating *FKBP12* levels through genetic engineering or selective breeding, it might be possible to create rice varieties that maintain good yield without sacrificing stress resilience.

The multifaceted role of FKBP12 in abiotic stress response and plant growth necessitates further investigation to elucidate its specific interactions and the pathways it modulates. Future research should focus on detailed protein-protein interaction studies and the exploration of downstream targets of FKBP12 in the ABA signaling pathway. Additionally, understanding the interaction between FKBP12-mediated pathways and other stress-related pathways could unveil new insights into the complex network of plant stress responses.

The study of FKBP12 in rice provides valuable insights into the molecular adaptations of plants to drought stress, emphasizing the potential of genetic engineering strategies to bolster crop resilience. By strategically modulating the expression of such key genes, it might be feasible to develop rice varieties that exhibit enhanced drought tolerance while maintaining optimal growth and yield, aligning with sustainable agricultural practices amid global climate challenge.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Not applicable.

Author Contributions
Y. Y, L. W, and B. R designed the experiments; B. R, Y. J, Y. Q, C. Y, F. C, Y. Z, Y. M, S. W, and L. W performed the experiments; Y. Y, B. R, Y. J. and Y. Q. analyzed the data; B. R wrote the manuscript. L. W. and Y. Y. revised the manuscript. All authors read and approved the manuscript.

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Data Availability
No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate
Not applicable.

Consent for Publication
Not applicable.

Competing Interests
The authors declare no competing interests.

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