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Heterologous expression of physic nut *JcHDZ25* confers tolerance to drought stress in transgenic rice

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Abstract

Background The HD-Zip family of plant-specific transcription factors coordinates developmental processes and abiotic stress adaptation, including drought tolerance in bioenergy crops such as physic nut. Although HD-Zip proteins are known regulators of stress responses, the functional roles of physic nut HD-Zip genes in drought adaptation remain uncharacterized.

Results In this study, we functionally characterized *JcHDZ25*, a drought-inducible HD-Zip I gene from physic nut, which is predominantly expressed in roots and upregulated by ABA and drought. Subcellular localization and transcriptional activity assays confirmed that JcHDZ25 localized to the nucleus and exhibited intrinsic transcriptional activation. Transgenic rice overexpressing *JcHDZ25* displayed enhanced drought tolerance and ABA sensitivity compared to wild-type plants. Under drought stress, *JcHDZ25*-overexpressing lines showed significantly higher proline content, elevated SOD and CAT activities, and reduced electrolyte leakage and MDA accumulation relative to wild-type controls. Furthermore, transgenic plants showed higher expression of abiotic stress-responsive genes (*OsAPX2*, *OsCATA*, *OsLEA3*, *OsP5 CS*, *OsDREB2 A*, *OsADC1*) and ABA pathway-related genes (*OsNCED3*, *OsRD29 A*) under drought stress compared to wild-type plants.

Conclusions *JcHDZ25* positively regulates drought tolerance in rice possibly through an ABA-dependent transcriptional regulation, providing mechanistic insights into physic nut's drought adaptation and highlighting its potential as a genetic resource for engineering stress-resilient crops.

Keywords HD-Zip, *JcHDZ25*, Drought stress, Physic nut, ABA

Background

Drought represents one of the most pervasive abiotic stressors, severely limiting crop productivity by disrupting physiological and biochemical processes during plant growth. It accelerates organ and tissue senescence, ultimately leading to plant mortality [1]. To mitigate these effects, plants activate intrinsic response mechanisms, regulating physiological and biochemical pathways to adapt to adverse conditions [1]. Among these mechanisms, transcription factors serve as pivotal regulators of stress responses, as demonstrated by extensive studies [2]. Key transcription factor families involved in stress

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regulation include MYB, bZIP, bHLH, HD-Zip, and WRKY, which modulate gene expression by binding to specific cis-elements in target promoters [3–7].

The HD-Zip family represents a unique class of plant-specific transcription factors characterized by a conserved homeodomain-leucine zipper structure that integrates plant development and stress adaptation. These HD-Zip proteins contain two functional domains: an N-terminal homeodomain (HD) with 60 amino acids for sequence-specific DNA binding, and a C-terminal leucine zipper (LZ) domain facilitating dimerization to modulate transcriptional regulatory activity [4]. Based on sequence variation and DNA-binding preferences, HD-Zip proteins are phylogenetically classified into four subfamilies (I–IV) [8].

Members of the HD-Zip subfamily I primarily function in mediating plant stress responses and regulating senescence processes. Notably, drought stress upregulates *Zmhdz9* expression, and transgenic maize lines with enhanced *Zmhdz9* expression exhibit improved drought tolerance [9]. Similarly, *GhHB4*-like transgenic Arabidopsis demonstrates enhanced salt stress resistance [10], while *PeHDZ72*-overexpressing rice plants show increased drought tolerance [11]. Within this subfamily, both *Zmhdz10* and *CaHDZ12* improve abiotic stress tolerance in transgenic plants through ABA-dependent pathways [12, 13]. *Oshox22* positively regulates the salt stress response through ABA-mediated signaling [14], and *AtHB6* modulates drought stress responses downstream of ABI1 [15]. The HD-Zip I homologs *HaHB1* (sunflower) and *AtHB13* (Arabidopsis) improve plant cold tolerance [16, 17]. Conversely, *SlHB2* from tomato negatively regulates salt stress tolerance in transgenic plants [18], and *LpHOX22* and *LpHOX24* act as negative regulators of osmotic and heat stress responses [19]. HD-Zip II subfamily proteins primarily regulate auxin signaling and light stress responses during developmental processes. For example, Arabidopsis overexpressing *ATHB2* (a subfamily II member) displays characteristic shade avoidance responses [20]. The subfamily II gene *HAT2* is auxin-inducible, and its overexpression leads to auxin-overproduction phenotypes including elongated hypocotyls, extended petioles, and reduced leaf size [21]. Subfamily III members are predominantly involved in lateral organ initiation and meristem differentiation. In soybean, two subfamily III genes (*GmHB15-L- 1* and *GmHB14-L- 2*) exhibit distinct functions: *GmHB15-L- 1* participates in vascular cambium formation/maintenance, while *GmHB14-L- 2* regulates xylem differentiation [22]. Meanwhile, class IV HD-Zip proteins primarily govern stomatal development, epidermal cell differentiation, and root hair formation. The Arabidopsis *ANTHOCYANINLESS2* (*AtANL2*) gene orchestrates anthocyanin accumulation, root growth, and epidermal cell proliferation [23]. Current genomic analyses reveal 42 HD-Zip family genes in Arabidopsis, including 17 subfamily I members [8], while 32 HD-Zip genes (12 in subfamily I) have been identified in physic nut (*Jatropha curcas* L.) [24]. Despite the established regulatory role of subfamily I genes in abiotic stress responses and the availability of whole-genome HD-Zip analyses in physic nut, functional characterization of HD-Zip genes in drought stress adaptation remains unexplored in this oilseed crop.

As a bioenergy crop, physic nut thrives in nutrient-poor soils and exhibits exceptional drought resilience, reducing irrigation demands while maintaining productivity under water-limited conditions [25]. Its deep root system and high water-use efficiency make it a valuable genetic resource for identifying drought-tolerant genes applicable to both sustainable biofuel production and climate-resilient agriculture. Previous studies have documented its unique physiological adaptations, such as rapid stomatal regulation and osmolyte accumulation under drought [26], underscoring its potential as a system for discovering novel stress-responsive genes. Based on previous expression analysis of HD-Zip genes in physic nut [24], we identified *JcHDZ25*, a drought-inducible member of subfamily I, as a prime candidate for functional characterization.

In this study, we characterized *JcHDZ25*, a novel HD-Zip I transcription factor from physic nut. Using molecular and transgenic approaches, we investigated its expression patterns under drought and ABA treatments, subcellular localization, and transcriptional activity. To evaluate its agronomic potential, *JcHDZ25* was overexpressed in rice, a model crop system. Our findings provide foundational insights into *JcHDZ25*'s role in drought adaptation and highlight its utility for engineering stress-resilient crops.

Methods

Plant material and stress treatments

The inbred physic nut (*Jatropha curcas* L.) variety GZQX0401, whose genome has been sequenced [27], and wild-type (WT) rice (*Oryza sativa* L. japonica cv. Zhonghua 11, ZH11) were used. For tissue-specific expression analysis, roots, stem cortex, leaves (sixth leaf stage), flowers, and seeds (35 days post-pollination) were harvested from physic nut. For drought stress assay, six-leaf-stage seedlings were grown in a 3:1 (v/v) sand-nutrient soil matrix under controlled greenhouse conditions (30 °C, 60% relative humidity, 16 h photoperiod, 300 μmol m^{-2} s^{-1} light intensity). Soil moisture was monitored daily using a TDR- 350 sensor (Spectrum Technologies). During drought stress, soil moisture decreased linearly from 80 to 15% over 7 days, with visible wilting symptoms

observed at < 20% moisture content. Drought stress was imposed by withholding irrigation for 0 (control), 2, 4, and 7 days (n = 15 plants/group), with fully expanded fourth leaves were sampled for RNA extraction. For ABA treatment, A 100 μM ABA solution (Sigma-Aldrich) was foliar-sprayed (5 mL/plant) onto six-leaf-stage seedlings using a handheld atomizer, ensuring complete coverage of both adaxial and abaxial leaf surfaces. Leaves were sampled at 0, 2, 6, and 12 h post-treatment. Three biological replicates were analyzed.

Bioinformatics analysis of JcHDZ25 protein

Multiple sequence alignment of JcHDZ25 homologs was performed using DNAMAN 9.0. Conserved domains were identified through SMART (v9.0; http://smart.embl.de) [28], and physicochemical properties (molecular weight, isoelectric point) were predicted using ExPASy ProtParam (https://web.expasy.org/protparam/) [29]. Potential transmembrane helices were analyzed via TMHMM 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) [30].

Subcellular localization assay

The *JcHDZ25* coding sequence (excluding stop codon) was amplified from physic nut root cDNA using RT-PCR with primers listed in Additional file 1. The PCR product was cloned into pDONR™/Zeo (Thermo Fisher) via BP recombination (Gateway® BP Clonase™ II) and sequence-verified (Sangon Biotech). An LR reaction transferred the correct sequence into the destination vector pBWA(V) HS-GLosgfp. The 35S::*JcHDZ25-GFP* fusion construct and empty 35S::*GFP* control were transformed into Arabidopsis mesophyll protoplasts via PEG-mediated transfection [31]. After 16 h of dark incubation (22 °C), GFP fluorescence was visualized using a Leica TCS SP8 confocal microscope (488 nm excitation; 500–550 nm emission). Chloroplast autofluorescence was detected at 630–680 nm [31].

Transcriptional activity assay

The coding sequence (CDS) of *JcHDZ25* was cloned into pGBKT7 vector to generate pGBKT7-*JcHDZ25*. The pGBKT7-*JcHDZ25*, pGBKT7 and pGBKT7-GLA4 plasmids were transformed into yeast strain AH109 (TaKaRa Bio) using the lithium acetate method [32]. Transformants were cultured on the SD/-Trp medium, and then transferred to SD/-Trp/-His and SD/-Trp/-His/-Ade plates containing 40 μg/mL X-α-Gal. Plates were incubated at 30 °C for 3–5 days. Transcriptional activation was confirmed by blue colony formation due to α-galactosidase activity.

JcHDZ25 cloning and transgenic plant construction

The *JcHDZ25* coding sequence was PCR-amplified from physic nut root cDNA using primers designed against the reference sequence (GenBank: XP_012083130.1). PCR reactions (50 μL) contained 2 μL cDNA template, 25 μL 2× Phanta® Max Master Mix (Takara Bio), 1 μM each primer, 21 μL nuclease-free water. PCR conditions: initial denaturation at 95 °C for 3 min; 32 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 1 min; final extension at 72 °C for 10 min. The purified PCR product was ligated into pCAMBIA1301 (driven by the CaMV 35S promoter) through *Kpn* I/*Xba* I restriction sites and transformed into DH5α competent cells (Thermo Fisher). Recombinant plasmids were verified by colony PCR and restriction digestion.

The 35S::*JcHDZ25* construct were electroporated into *Agrobacterium tumefaciens* EHA105 prepared via the $CaCl_2$ freeze–thaw method [33]. A schematic diagram of the pCAMBIA1301-*JcHDZ25* vector was displayed in Additional file 2. Rice calli (cv. ZH11) were transformed through co-cultivation [34] and selected on hygromycin-supplemented medium (50 mg/L). Callus induction and regeneration followed established protocols [34]. The transformation efficiency was calculated as 15% (15 hygromycin-resistant calli per 100 co-cultivated calli). Homozygous T3 lines (OE1, OE2, OE3) were validated by GUS histochemical staining and RT-PCR (confirmed transgene expression levels). For GUS analysis, seedlings were incubated in staining buffer (1 mM X-Gluc, 50 mM sodium phosphate pH 7.0, 0.1% Triton X- 100) at 37 °C for 12 h, followed by chlorophyll removal in 70% ethanol.

Drought and ABA stress treatments

For drought assays, surface-sterilized *JcHDZ25*-OE and wild-type seeds (1% NaClO, 1 min) were germinated on moist filter paper (28 °C, 48 h dark). Uniform seedlings were transferred to vermiculite: nutrient soil (3:1 v/v) and grown under 28 ± 2 °C, 16/8 h light/dark cycle. Drought stress was initiated at 14 days after germination by water withholding. For ABA response assays, germinated seedlings (0.5 cm height) were cultured on Yoshida's medium with or without 5 μM ABA (4 days, 28 °C). Root and shoot lengths were measured (n = 40/group) with three biological replicates.

Physiological parameter quantification

Leaf samples from drought-treated (14 days) and control rice plants were collected for biochemical analyses (n = 3 biological replicates). Electrolyte leakage assay, leaf segments (0.2 × 0.2 cm) were excised from both wild-type (WT) and transgenic rice plants. The tissue samples were immersed in 20 mL deionized water (H_2O) and subjected

to vacuum infiltration until complete tissue submergence. Following incubation at 25 °C with continuous shaking (100 rpm for 1.5 h), the initial conductivity (S1) was quantified using a DDSJ- 308 A conductivity meter. Samples were subsequently boiled at 100 °C for 10 min, allowed to cool to 25 °C, and the final conductivity (S2) was recorded. Relative electrolyte leakage was calculated as (S1/S2) ×100%. SOD activity, proline content, and MDA concentration were measured as described [31]. CAT activity was assayed according to published protocols [7].

RNA isolation and qRT-PCR analysis

Total RNA was extracted using RNeasy Plant Mini Kit (Magen), with integrity verified by agarose gel electrophoresis (28S/18S rRNA) and Nanodrop ND- 1000 (A260/A280 ≥1.8). First-strand cDNA was synthesized with PrimeScript™ Reverse Transcriptase (Takara Bio; 1 μg RNA). qPCR reactions (20 μL) contained 10 μL SYBR Premix Ex Taq™ II (2 ×, Takara Bio), 2 μL cDNA (5 ×diluted), 0.4 μM each primer and 7.2 μL nuclease-free H_2O. Cycling conditions (Roche Light Cycler 96): 95 °C for 15 min; 40 cycles of 95 °C for 5 s, 60 °C for 30 s; melt curve 65–95 °C (0.5 °C/s). Gene expression was normalized to reference genes using $2^{-\Delta\Delta CT}$ method (three technical replicates). All the primers used in this study were listed in Additional file 1.

Statistical analysis

All data were analyzed using one-way ANOVA with Tukey's post-hoc test (multi-group comparisons) or Student's t-test (two-group comparisons). Significance thresholds ($P < 0.05$, $P < 0.01$) are explicitly indicated in figures.

Results

Isolation and bioinformatics characterization of *JcHDZ25*

Using cDNA templates from physic nut root tissues, we amplified the *JcHDZ25* gene through RT-PCR with gene-specific primers. BLAST alignment against NCBI databases confirmed 100% sequence identity with the annotated HD-Zip I protein XP_012083130.1 (designated *JcHDZ25*). The open reading frame (ORF) of *JcHDZ25* was 876 bp, encoding a 291-amino acid protein with a predicted molecular weight of 33.74 kDa and an isoelectric point (pI) of 5.45. Transmembrane domain analysis using TMHMM revealed no transmembrane regions in *JcHDZ25*.

We further used DNAMAN software to perform an amino acid sequence alignment analysis of JcHDZ25 protein with functionally characterized HD-Zip I homologs from other species. The results indicated that JcHDZ25, like the HD-Zip family proteins reported in different species, also contained the conserved HD and LZ domains and belonged to the HD-Zip I subgroup (Fig. 1). Additionally, *JcHDZ25* had the highest sequence similarity with maize *Zmhdz9* (70.59%) and Arabidopsis *ATHB12* (58.37%). These results suggest that *JcHDZ25* may have similar functions as *Zmhdz9*, and require further study.

Expression profile of *JcHDZ25*

To clarify the expression characteristics of *JcHDZ25* in different tissues of physic nut, the expression profile of *JcHDZ25* was analyzed by qRT-PCR. The results reveled that tissue-specific expression of *JcHDZ25* in physic nut, with highest transcript levels in roots, followed by leaves, stem cortex, flowers, and seeds (Fig. 2A).

We further investigated the expression of *JcHDZ25* in the leaves of physic nut in response to drought and ABA using qRT-PCR. Under drought stress, *JcHDZ25* expression in leaves was significantly upregulated at 4 and 7 days post-treatment compared to controls (Fig. 2B). Similarly, exogenous ABA treatment induced a rapid increase in *JcHDZ25* transcript levels, peaking at 6 h post-application (Fig. 2C). These results indicate that *JcHDZ25* is transcriptionally responsive to both drought and ABA, implicating its role in ABA-mediated drought adaptation.

Subcellular localization of JcHDZ25

To determine the subcellular localization of JcHDZ25 protein, we transiently expressed a 35S::JcHDZ25-GFP fusion construct in Arabidopsis protoplasts via PEG-mediated transformation. After 16 h of dark, the green fluorescence signal was observed using laser confocal microscopy. The results indicated that the GFP signal from the empty 35S::GFP control was distributed throughout the cell, whereas the 35S::JcHDZ25-GFP signal was exclusively localized to the nucleus (Fig. 3). These results confirm that JcHDZ25 is a nuclear-localized protein.

Analysis of JcHDZ25 transcriptional activity

To assess the transcriptional activation capacity of JcHDZ25, the coding sequence was cloned into the yeast expression vector pGBKT7 (containing the GAL4 DNA-binding domain), generating the recombinant plasmid pGBKT7-JcHDZ25. The pGBKT7-JcHDZ25, empty pGBKT7 vector, and positive control pGBKT7-GAL4 were transformed into yeast strain AH109. All transformants grew normally on SD/-Trp medium, confirming successful plasmid transformation. On SD/-Trp/-His and SD/-Trp/-His/-Ade media supplemented with X-α-Gal, yeast harboring pGBKT7-JcHDZ25 and pGBKT7-GAL4 formed blue colonies, while those transformed with empty pGBKT7 failed to grow (Fig. 4). These findings demonstrate that JcHDZ25 possesses intrinsic

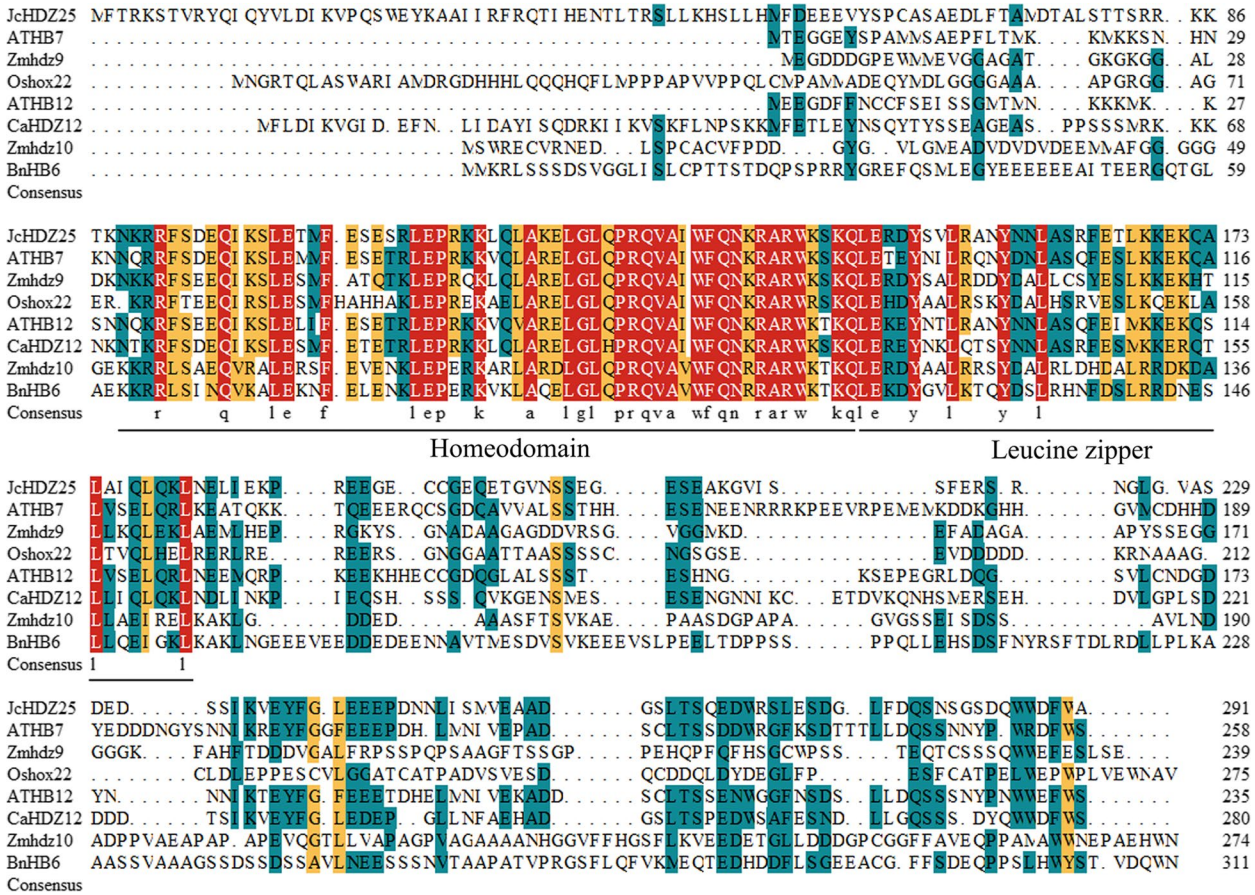

Fig. 1 Amino acid sequence alignment analysis of *JcHDZ25* in plant HD-Zip I proteins. Multiple sequence alignment of *JcHDZ25* (physic nut; XP_012083130.1) with homologs: *Zmhdz9* (maize; GRMZM2G041462), *Zmhdz10* (maize; NP_001132844.1), *Oshox22* (rice; NP_001389333.1), *CaHDZ12* (chickpea; NP_001352145), *BnHB6* (rapeseed; AAR04932.1), *ATHB7* (Arabidopsis; AT2G46680), and *ATHB12* (Arabidopsis; AT3G61890). Conserved HD and LZ domains are boxed. Alignment performed using DNAMAN 9.0

transcriptional activation activity, enabling yeast survival on triple-deficient media through reporter gene activation. These results confirm that JcHDZ25 has transcription factor activity.

Phenotypic analysis of *JcHDZ25* transgenic rice plants

To assess *JcHDZ25*'s functional potential and evaluate cross-species transferability of physic nut HD-Zip genes for crop improvement, we generated rice overexpression lines (35S::*JcHDZ25*) using *Agrobacterium*-mediated transformation. Transgenic rice plants (OE1, OE2 and OE3) were selected through hygromycin resistance screening combined with GUS histochemical staining. RT-PCR confirmed *JcHDZ25* expression in transgenic lines but not in wild-type (Fig. 5A). Under normal growth conditions, *JcHDZ25* transgenic plants displayed no morphological differences compared to wild-type plants (Fig. 5B and C). No significant variations were observed in plant height, seed-setting rate, or yield per plant between wild-type and transgenic plants (Fig. 5D, E and F). These results indicate that *JcHDZ25* overexpression does not compromise agronomic traits under non-stress conditions.

JcHDZ25 positively regulates drought stress responses in rice

Given the drought stress significantly up-regulated the expression of *JcHDZ25*, we hypothesized its role as a positive regulator of drought tolerance. To test this, we further investigated the effect of *JcHDZ25* overexpression on rice drought tolerance. Two-week-old wild-type and *JcHDZ25* transgenic plants were subjected to water withholding for 25 days. Both transgenic and wild-type lines exhibited leaf curling and whitening under drought, but symptoms were markedly more severe in wild-type plants (Fig. 6A). After 4 days of rehydration, transgenic rice plants showed a significantly higher survival rate (59.4%) compared to wild-type (9.6%) (Fig. 6B). Additionally, transgenic leaves exhibited lower relative electrolyte leakage than wild-type under drought stress (Fig. 6C),

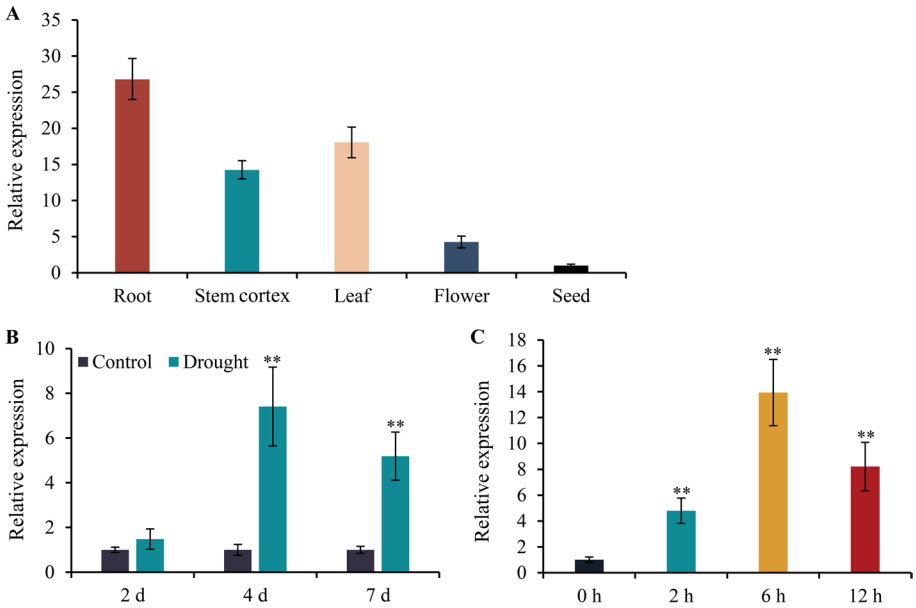

Fig. 2 Expression pattern of *JcHDZ25* gene. **A** Tissue-specific expression in roots, stem cortex, leaves, flowers, and seeds of physic nut. **B** Drought-induced expression in leaves at 0, 2, 4, and 7 days (d) post-water withholding. **C** ABA-responsive expression at 0, 2, 6, and 12 h post-treatment. Data are presented as mean ± standard deviation (SD) from three biological replicates. **P < 0.01 (Student's t-test)

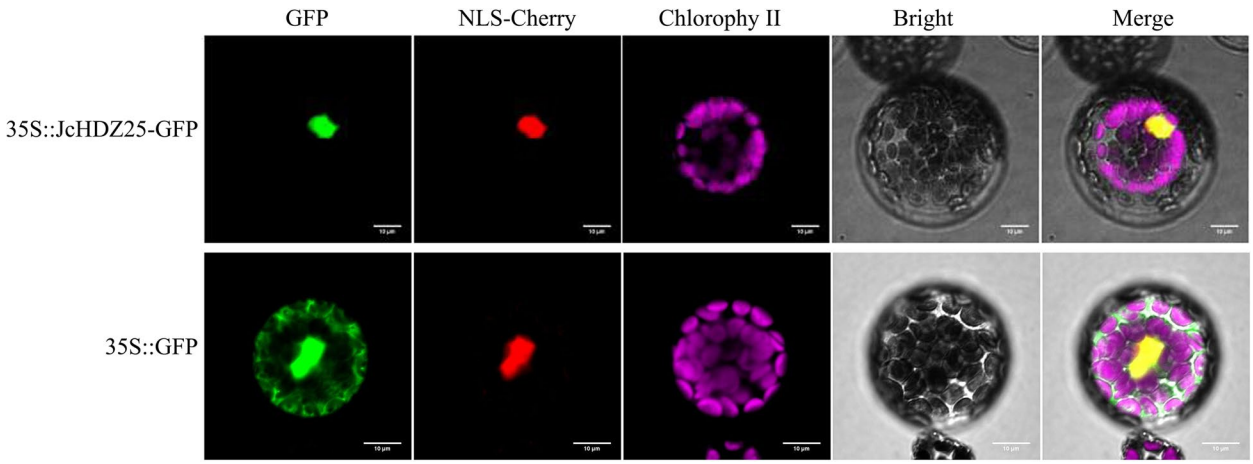

Fig. 3 Subcellular localization of JcHDZ25-GFP in Arabidopsis protoplasts. Transient expression of 35S::GFP (control) and 35S::JcHDZ25-GFP fusion constructs. GFP signals were captured via confocal microscopy. Scale bar = 10 μm

indicating enhanced membrane stability. Collectively, these result display that *JcHDZ25* confers drought tolerance in rice.

Drought-induced physiological changes

Proline accumulation serves as a key indicator of plant drought resilience [35]. To further evaluate drought adaptation mechanisms, we analyzed physiological changes in transgenic and wild-type rice plants. Under non-stress conditions, no significant differences in proline content, MDA levels, or SOD and CAT activities were observed between *JcHDZ25*-overexpressing lines and wild-type plants. However, under drought stress, transgenic lines accumulated significantly higher proline levels than wild-type controls (Fig. 7A), indicating activation of osmoprotective mechanisms. Although MDA content (a marker of membrane lipid peroxidation) increased in both transgenic and wild-type plants under drought, transgenic plants exhibited marginally lower MDA accumulation than wild-type (Fig. 7B). Furthermore,

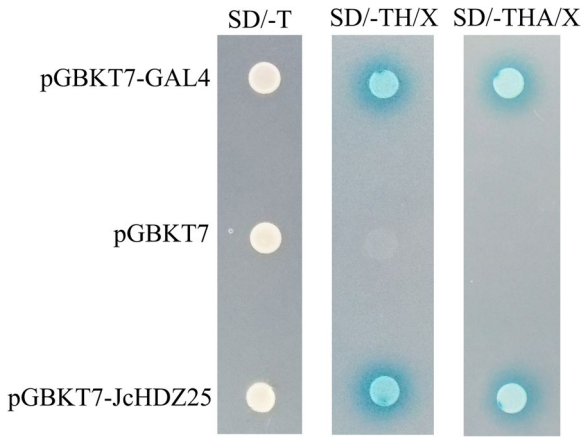

Fig. 4 Transcriptional activation assay of JcHDZ25 in yeast. The left panel depicts pGBKT7-GAL4, pGBKT7, and pGBKT7-JcHDZ25 grown on SD/-Trp plates for 4 days. The middle panel presents pGBKT7-GAL4, pGBKT7, and pGBKT7-JcHDZ25 grown on SD/-Trp/-His/X-a-gal plates for 4 days. The right panel exhibits pGBKT7-GAL4, pGBKT7, and pGBKT7-JcHDZ25 grown on SD/-Trp/-His/-Ade/X-a-gal plates for 4 days. Blue colonies indicate α-galactosidase activity

JcHDZ25-overexpressing plants displayed elevated SOD and CAT activities compared to wild-type under drought stress (Fig. 7C and 7D), demonstrating enhanced reactive oxygen species scavenging capacity.

JcHDZ25 enhances ABA sensitivity in rice

Given the central role of ABA in drought responses [36, 37], we evaluated ABA sensitivity in transgenic rice plants. Wild-type and *JcHDZ25* transgenic rice seedlings were growth in Yoshida's nutrient solution with or without 5 μM ABA. While both wild-type and transgenic plants showed growth inhibition under ABA, transgenic lines exhibited significantly greater suppression of shoot and root elongation (Fig. 8). These results suggest that *JcHDZ25* enhances ABA sensitivity, likely contributing to drought tolerance through ABA-mediated signaling.

Expression of stress-responsive genes

To elucidate the molecular mechanism of *JcHDZ25*-mediated drought tolerance, we analyzed transcript levels of key stress-responsive genes (*OsAPX2*, *OsCATA*, *OsLEA3*, *OsP5 CS*, *OsDREB2 A*, *OsADC1*, *OsNCED3*, and *OsRD29 A*) involved in abiotic stress or the ABA pathway by qRT-PCR (Fig. 9). Under non-stress conditions, the expression levels of these genes in *JcHDZ25* transgenic rice were not significantly different from those in wild-type. However, drought stress induced pronounced upregulation of all tested genes, with significantly higher transcript levels in *JcHDZ25*-overexpressing plants than wild-type. These findings indicate that *JcHDZ25* coordinates multiple stress-adaptive pathways, including reactive oxygen species scavenging, osmolyte synthesis, and ABA signaling, to enhance drought tolerance.

Discussion

HD-Zip transcription factors are pivotal regulators of plant stress responses, yet their functional characterization in non-model species, particularly bioenergy crops like physic nut, remains limited [4, 24]. As a drought-tolerant species with deep root systems and high water-use efficiency, physic nut provides unique insights into molecular adaptations to arid environments [25]. In this study, we characterize *JcHDZ25*, an HD-Zip I transcription factor from physic nut, and demonstrate its ability to enhance drought tolerance in transgenic rice, likely through ABA-mediated pathways. Our findings expand the functional understanding of HD-Zip proteins in drought adaptation and underscore the translational potential of cross-species gene transfer for crop improvement.

The nuclear localization of JcHDZ25 (Fig. 3) and its transcriptional activation in yeast assays (Fig. 4) confirm its role as a functional transcription factor. Similar nuclear localization patterns have been observed in other HD-Zip I members, such as maize *Zmhdz10* and bamboo *Phehdz1*, which directly bind drought-responsive gene promoters to regulate stress adaptation [12, 38]. The conserved HD and LZ domains in *JcHDZ25* likely facilitate dimerization and sequence-specific DNA binding, key mechanisms for transcriptional regulation under stress [4, 8]. These structural and functional parallels align *JcHDZ25* with known stress-responsive HD-Zip I genes, reinforcing its role as a central regulator of abiotic stress signaling.

Proline acts as an osmoprotectant to stabilize cellular structures, while SOD and CAT scavenge reactive oxygen species, thereby reducing membrane lipid peroxidation [35, 39]. Notably, under drought stress, the lower MDA content and higher SOD and CAT activities in transgenic rice plants suggest reduced oxidative damage, a phenomenon consistent with findings in *MsHDZ23*-overexpressing Arabidopsis [40]. These results collectively demonstrate *JcHDZ25*'s role in activating stress-responsive pathways to maintain cellular homeostasis. Intriguingly, *JcHDZ25* exhibits 70.59% amino acid sequence identity with maize *Zmhdz9* (Fig. 1), a homolog known to enhance drought tolerance through lignin biosynthesis [9]. However, unlike *Zmhdz9*, which primarily upregulates lignin-related pathways under drought [9], *JcHDZ25* activates osmoprotectant synthesis (e.g., proline) and antioxidant defenses (Fig. 7), reflecting lineage-specific adaptations to arid conditions. This functional divergence underscores the importance of studying HD-Zip proteins

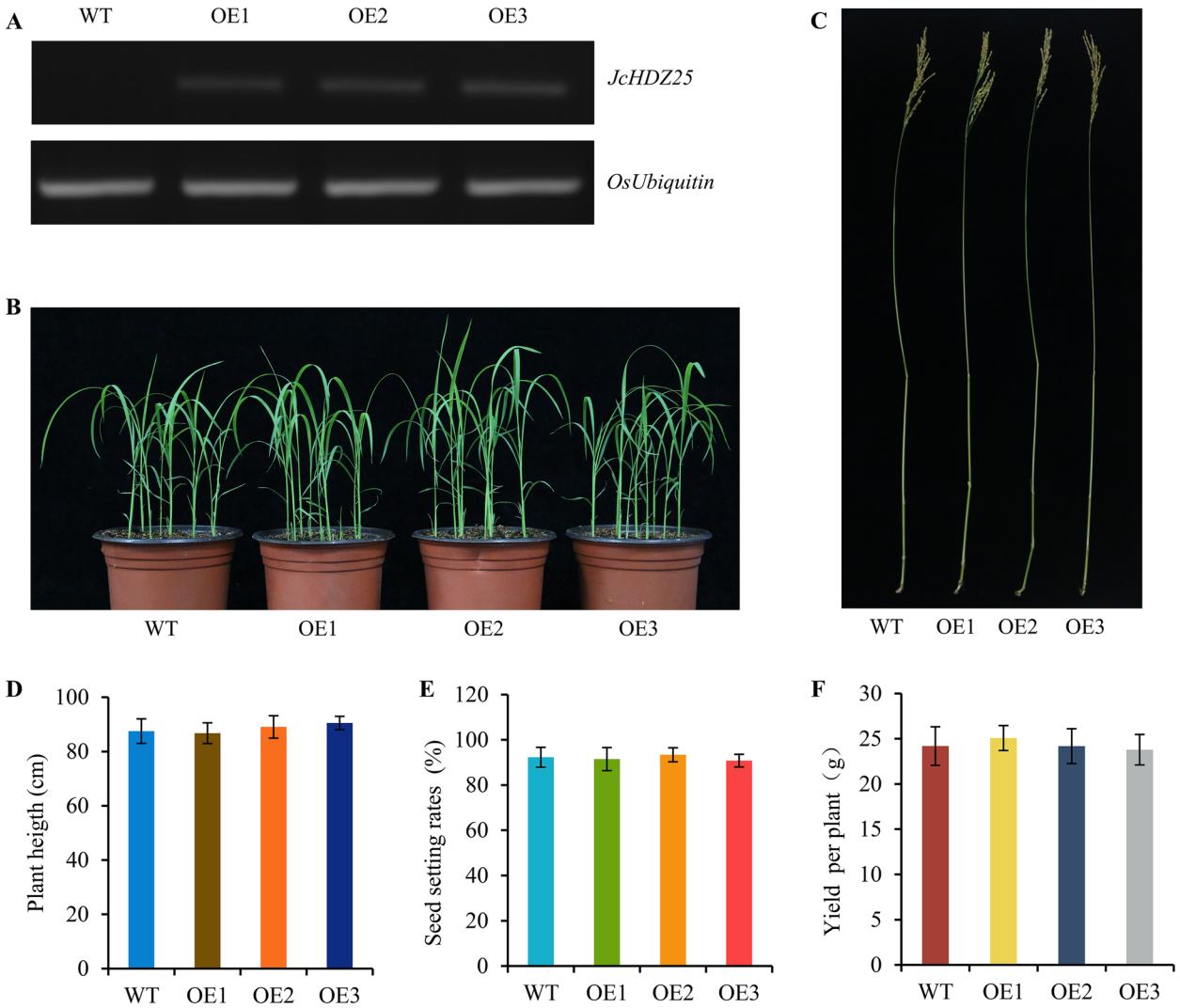

Fig. 5 Agronomic characterization of *JcHDZ25* transgenic rice lines (OE1, OE2, and OE3). **A** RT-PCR validation of transgene expression. **B** Vegetative growth of 18-day-old seedlings. **C** Phenotypic of plant height of wild type and *JcHDZ25* transgenic lines at maturity. **D** Plant height of wild type and *JcHDZ25* transgenic lines at maturity: means of n = 40 ± SD from three independent biological replicates. **E** Seed setting rates of wild type and *JcHDZ25* transgenic lines: means of n = 40 ± SD from three independent biological replicates. **F** Yield per plant of wild type and *JcHDZ25* transgenic lines: means of n = 40 ± SD from three independent biological replicates

across taxonomically diverse species. As a pioneer species in extreme arid environments, physic nut may have prioritized the evolution of HD-Zip I genes linked to osmotic adjustment and ROS scavenging to combat prolonged water scarcity [25]. In contrast, maize, as a high-yield crop, likely favors HD-Zip I genes that reinforce cell wall structures to maintain tissue integrity. Such functional diversity implies that the evolutionary trajectories of HD-Zip I genes are profoundly shaped by species' ecological niches and stress-driven selective pressures.

Molecular analysis revealed that *JcHDZ25* overexpression upregulated expression of key antioxidant genes, including *OsCATA* and *OsAPX2*, which are critical for abiotic stress responses [41]. This transcriptional activation provides a molecular basis for the observed enhancement of SOD and CAT activities in transgenic plants. Furthermore, drought-stressed transgenic lines showed elevated expression of stress-responsive genes such as *OsLEA3* (encoding a late embryogenesis abundant protein) [42], *OsADC1* (involved in polyamine biosynthesis) [43], *OsP5 CS1* (a proline biosynthesis regulator) [44], and *OsDREB2 A* (a drought-responsive transcription factor) [45] (Fig. 9). These findings suggest that *JcHDZ25* coordinates multiple stress-adaptive pathways to enhance drought tolerance.

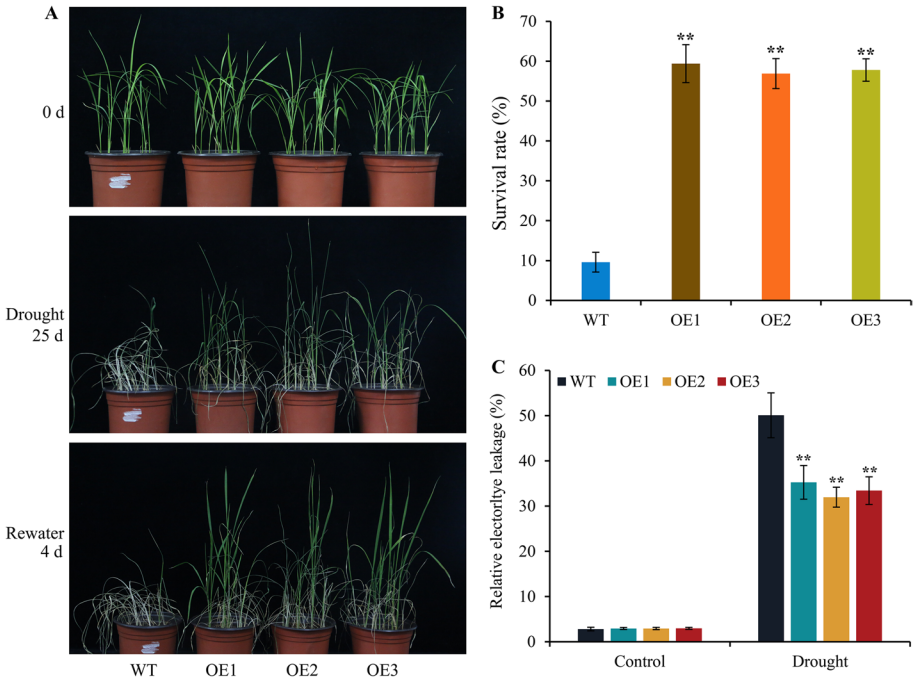

Fig. 6 Drought tolerance phenotypes of *JcHDZ25* transgenic rice lines. **A** Phenotypes of *JcHDZ25* transgenic and wild-type lines under drought stress. **B** Survival rate of wild-type and *JcHDZ25* transgenic lines under drought stress (n = 3 ± SD, **P < 0.01). **C** Relative electrolyte leakage of wild-type and *JcHDZ25* transgenic lines subjected to drought stress (n = 3 ± SD, asterisks above the bars indicate significant differences from wild-type lines at p < 0.01)

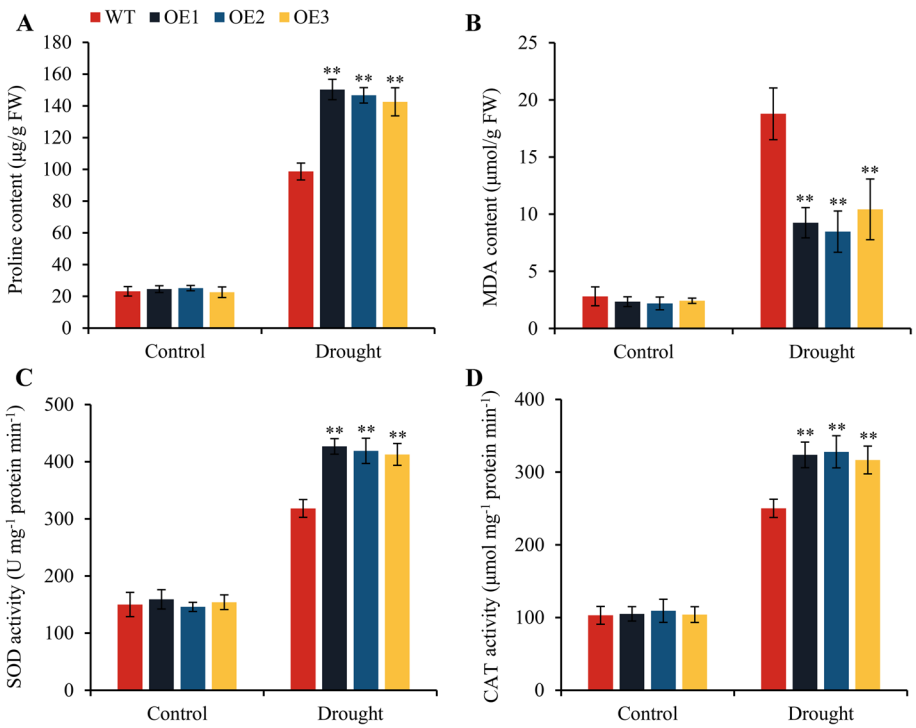

Fig. 7 Physiological indexes and enzyme activities of *JcHDZ25* transgenic and wild-type rice plants under normal and drought-stressed conditions. **A** Changes of proline content in leaves of wild-type and transgenic plants. **B** Changes of MDA content in leaves of wild-type and transgenic plants. **C** Changes in SOD activities in leaves of wild-type and transgenic plants. **D** Changes in CAT activities in leaves of wild-type and transgenic plants. Data are derived from three independent biological replicates, and significant differences are indicated by asterisks (p < 0.01)

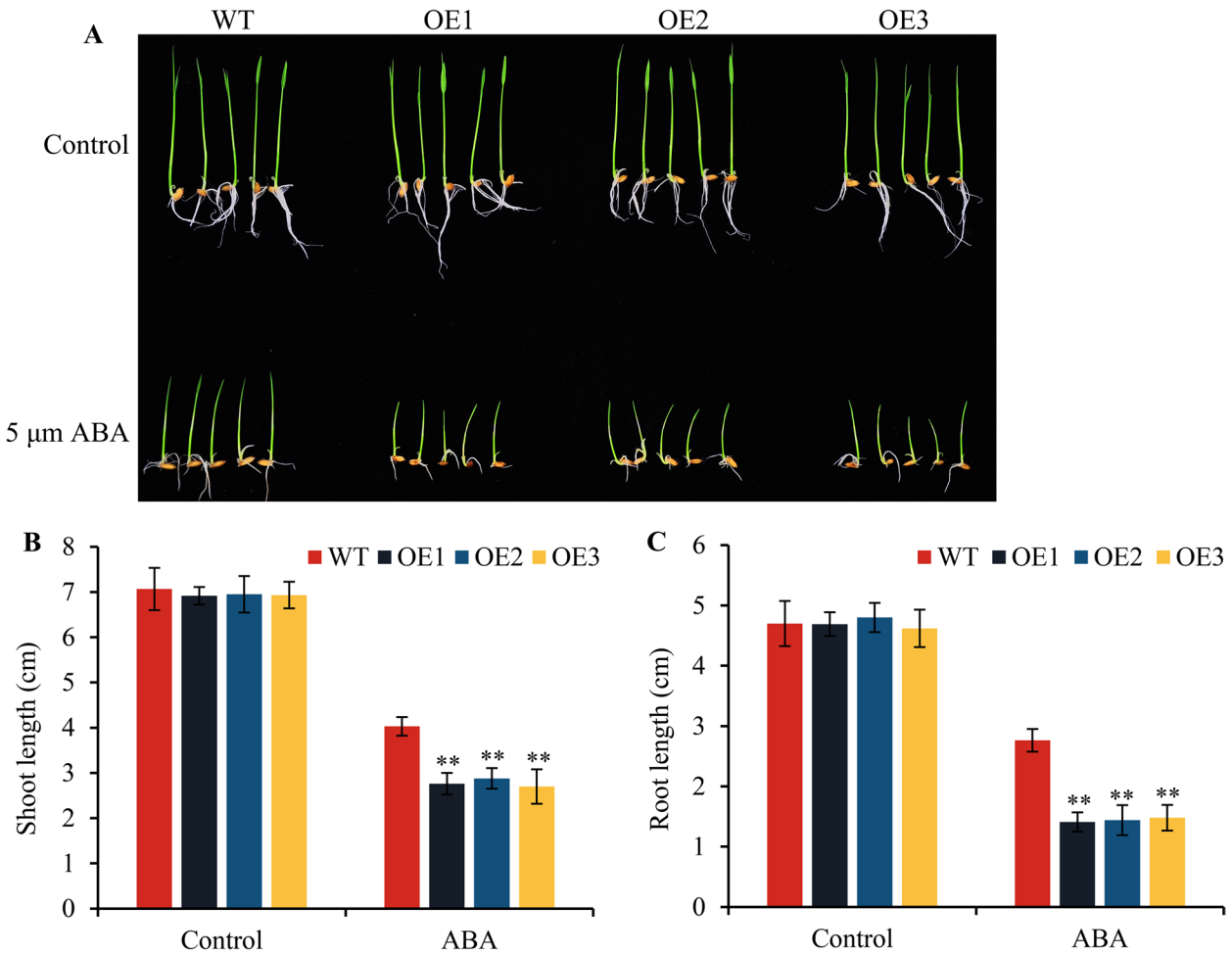

Fig. 8 Analysis of ABA sensitivity of wild-type and *JcHDZ25* transgenic rice lines. **A** Phenotypes of wild-type and transgenic lines grown on Yoshida's medium with or without 5 μM ABA. Shoot (**B**) and Root (**C**) growth of transgenic and wild-type lines after four days of growth on Yoshida's medium with or without 5 μM ABA: means of n = 40 ± SD from three independent biological replicates. Asterisks above the bars indicate significant differences from wild-type lines at $p < 0.01$

Notably, *JcHDZ25* transgenic plants exhibited heightened ABA sensitivity (Fig. 8) and upregulated expression of ABA biosynthesis (*OsNCED3*) [46] and signaling (*OsRD29 A*) genes [47] under drought (Fig. 9). ABA serves as a central regulator of stomatal closure and stress-responsive gene networks during water deficit [36]. The elevated *OsNCED3* expression aligns with its role in boosting endogenous ABA levels to improve drought tolerance [46], mirroring mechanisms reported for *Zmhdz10* and *CafHDZ12* [12, 13]. These parallels position *JcHDZ25* as a key mediator linking ABA signaling to drought adaptation. However, the precise mechanistic relationship between *JcHDZ25* and ABA biosynthesis requires further validation, such as complementation assays in *osnced3* mutants or fluridone-mediated pharmacological inhibition, to conclusively establish ABA dependency.

The successful transfer of *JcHDZ25* from physic nut to rice demonstrates the feasibility of leveraging stress-tolerant genes from wild species for crop improvement. Physic nut's inherent drought resistance makes it a valuable genetic resource for enhancing staple crops like rice, particularly in arid regions [25]. Notably, *JcHDZ25* overexpression does not compromise agronomic traits under non-stress conditions, ensuring minimal fitness costs in transgenic lines during breeding. This gene likely establishes a sustainable mechanism to maintain metabolic activities under drought stress by coordinating the activation of both osmoprotectant biosynthesis pathways and antioxidant systems. To further explore its potential, future work will investigate *JcHDZ25*'s role in enhancing drought tolerance and resilience to other abiotic stresses across multiple crop species. Field trials under controlled drought conditions will assess its agronomic

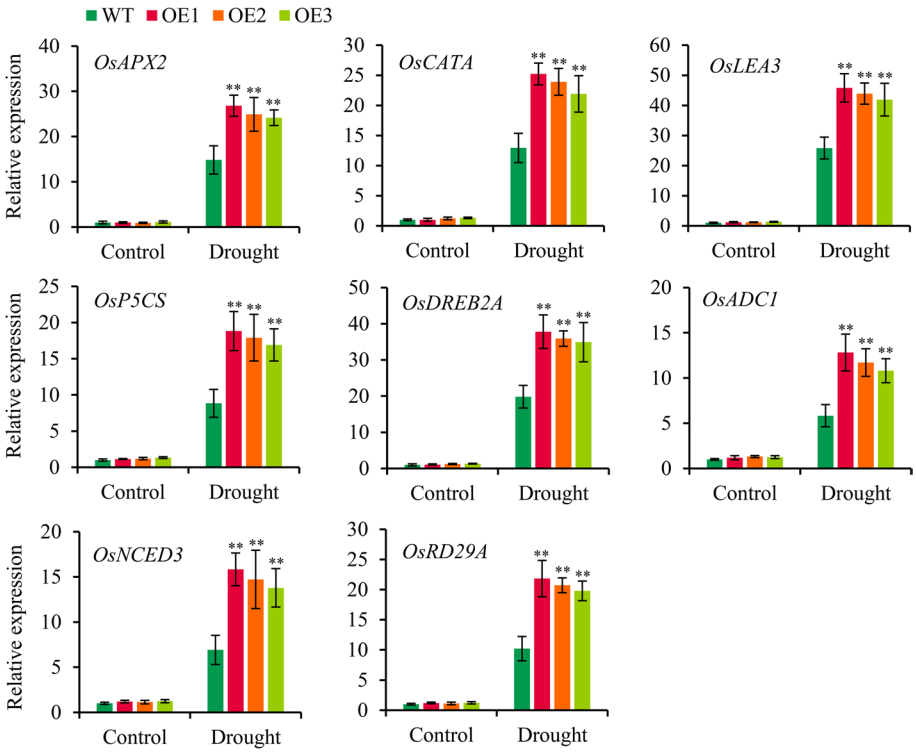

Fig. 9 Expression of abiotic stress-related genes and ABA-related genes in transgenic and wild-type rice plants under normal and drought stress conditions. Data are derived from three independent biological replicates, and asterisks represent a significant difference ($P < 0.01$)

performance, while pharmacological inhibition or mutant complementation will confirm ABA dependency. Mechanistically, ChIP-seq and yeast one-hybrid assays will identify direct downstream targets (e.g., *OsNCED3*, *OsRD29 A*) to elucidate its regulatory network. Collectively, this study establishes *JcHDZ25* as a promising candidate for engineering drought tolerance in crops. Our findings advance the functional characterization of HD-Zip transcription factors in non-model species and highlight their translational potential in climate-resilient agriculture.

Conclusions

We functionally characterized *JcHDZ25*, a drought-responsive HD-Zip transcription factor from physic nut. *JcHDZ25* exhibits root-predominant expression and is strongly induced by drought and ABA. Subcellular localization and transcriptional activation assays confirmed its nuclear localization and intrinsic transcriptional activity, positioning it as a central regulator of stress signaling. Overexpression of *JcHDZ25* in rice enhances drought tolerance, likely through ABA-mediated pathways, as evidenced by activation of antioxidant defense systems (SOD, CAT), induction of osmoprotectant biosynthesis (proline) and upregulation of ABA-responsive genes (*OsNCED3*, *OsRD29 A*). Collectively, this work positions *JcHDZ25* as a key candidate for elucidating drought adaptation mechanisms in physic nut and engineering stress-resilient crops via translational genomics.

Abbreviations

HD-ZIP	Homeodomain-leucine zipper
HD	Homeodomain
LZ	Leucine zipper
REL	Relative electrolytic leakage
MDA	Malondiaklehyde
CAT	Catalase
SOD	Superoxide dismutase
ABA	Abscisic acid
RT	Reverse transcription
ChIP-seq	Chromatin immunoprecipitation sequencing
PEG	Polyethylene glycol

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11566-1.

Additional file 1. Primers used in this study.

Additional file 2. Schematic diagram of the pCAMBIA1301-JcHDZ25 vector. GUS: β-Glucuronide reporter gene; NOS: nopaline synthase gene; Hpg: Hygromycin phosphotransferase gene; rbcs: small subunit gene of ribulose-1,5-bisphosphate carboxylase/oxygenase.

Additional file 3.

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Authors' contributions
The research was conceived and designed by XW and YT. The experiments were performed by YW, JX, RZ, TL and SJ, and the data were analyzed by YW, JX, SJ and XB. The manuscript was written and revised by YT. All the authors read and approved the final manuscript.

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Data availability
The relevant data analyzed during the research are included in this published article and its supplementary information files.

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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