



TaDJA-6D, the DnaJ family gene, enhances the regeneration and transformation in wheat (*Triticum aestivum* L.)

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Abstract

Key message *TaDJA-6D* boosts wheat regeneration and transformation efficiency across genotypes, offering a new molecular breeding resource.

Abstract As a key member of the heat shock protein 40 family, the co-chaperone DnaJ proteins play a crucial role in plant growth, development, and stress responses. However, very few studies have focused on the function of DnaJ protein in wheat (*Triticum aestivum* L.). Here, we characterized a DnaJ family gene from wheat, *TaDJA-6D*, (*TraesCS6D02G232600*) and analyzed its spatiotemporal expression pattern. During wheat regeneration, the transcriptional expression level of the *TaDJA-6D* gene was continuously upregulated with the extension of tissue culture time; in situ hybridization revealed that the genes were highly expressed in the vigorously dividing regions of wheat calli. Functional analysis demonstrated that, compared with the control vector, overexpression of *TaDJA-6D* significantly improved the wheat regeneration and transformation efficiency across diverse wheat genotypes, including Fielder, Kenong 199, Yanda 1817 and Xiaoyan 6. Our findings suggest that *TaDJA-6D* plays an important role in the process of wheat regeneration and genetic transformation. This study deepens our understanding of the functions of wheat co-chaperone proteins and provides novel genetic resources for efficient wheat transformation.

Keywords *TaDJA-6D* · Regeneration · Transformation efficiency · Tissue culture · Wheat

1 Introduction

Wheat (*Triticum aestivum* L.) is a vital global food crop, providing approximately 20% of dietary calories and proteins for humans (FAO 2024). High-yield and stable-yield are crucial for global food security. While traditional biological breeding technologies have significantly contributed to wheat variety improvement, they alone are insufficient to meet the challenges posed by complex planting environments and the growing demand for high-quality wheat.

Consequently, the integration of biotechnological breeding with traditional methods is becoming a crucial direction for future wheat breeding (Hua et al. 2019; Chen et al. 2024).

Genetic transformation remains a critical bottleneck in modern crop genetic improvement. Recent advances have shown that the rational utilization of regeneration-related genes such as *GRF4-GIF1*, *TaWOX5*, *TaDOFs*, and *TaLAX1* can effectively improve both shoot regeneration ability and genetic transformation efficiency in wheat (Debernardi et al. 2020; Qiu et al. 2022; Wang et al. 2022; Liu et al. 2023a, b; Yu et al. 2024; Yang et al. 2024). These findings have established these genes as promising molecular tools for improving wheat genetic transformation protocols. Nevertheless, the limited repertoire of currently identified regeneration-related genes, coupled with persistent genotype-dependent transformation limitations, continues to constrain the broader application and accelerated development of wheat genetic improvement programs.

DnaJ proteins, also called J proteins or heat shock protein 40 (HSP40), exist in archaea, bacteria, and various eukaryotes (Georgopoulos et al. 1979; Park et al. 2015; Jacob et al.

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2017; Zhao et al. 2022). Generally, DnaJ proteins contain three conserved domains: N-terminal DnaJ domain, Cxx-CxGxG zinc-finger domain, and C-terminal domain (Chiu et al. 2013; Kampinga et al. 2019). According to the presence of these domains, DnaJ proteins are classified into three types: Type I includes three domains; Type II contains the DnaJ domain and C-terminal domain; Type III only has the DnaJ domain (Walsh et al. 2004; Verma et al. 2019).

As classical chaperone proteins, DnaJ proteins play pivotal roles in regulating plant growth and stress responses throughout the plant life cycle (Richter and Buchner 2010; Faust et al. 2020; Carvalho et al. 2023). In *Arabidopsis*, two DnaJ family members, AtDJA5 and AtDJA6, can bind iron through the conserved cysteine residues and facilitate iron incorporation into Fe-S clusters by interactions with the sulfur utilization factor apparatus through their DnaJ domains, which is essential for plant viability and chloroplast development (Zhang et al. 2021). Functional characterization studies have revealed the importance of DnaJ proteins in stress responses. *AtDjB1* knockout mutant displayed greater sensitivity to heat stress than wild-type plants in *Arabidopsis*, suggesting that reduced expression of *AtDjB1* gene impaired thermotolerance (Zhou et al. 2012). Similarly, the *atdja3* mutant had increased sensitivity to salt and osmotic stress in the germination and post-germination development comparison to parental Col-0 (Salas-Muñoz et al. 2016). Conversely, overexpression of the *GmDnaJA6* gene in soybean (*Glycine max* L.) enhanced tolerance to saline and alkaline stress (Zhang et al. 2023). In alfalfa (*Medicago sativa* L.), *MsDjB4* enhanced aluminum stress tolerance by facilitating protein synthesis and improving antioxidant capacity in hairy roots (Liu et al. 2023a, b). A comprehensive analysis of the ZmDnaJ family in maize further revealed a correlation between heat stress tolerance and the regulation of gene transcriptional expression (Li et al. 2024). Beyond abiotic stress, DnaJ proteins are also implicated in plant disease resistance. Transgenic tomato plants with *SLDnaJ* knocked out exhibited significantly enhanced resistance to tomato spotted wilt virus compared to wild-type plants (Qi et al. 2025). In rice, OsDjA6 was identified as a negative regulator of innate immunity against the blast fungus *Magnaporthe oryzae* (Zhong et al. 2018). Despite these advances, research on the molecular functions of DnaJ proteins in wheat remains limited, highlighting the need for further investigation into their roles in this important crop species.

Notably, plant tissue culture and genetic transformation processes are accompanied by severe abiotic stresses, including mechanical wounding, *Agrobacterium* infection, osmotic pressure changes, and oxidative stress (Robertson et al. 1995; Skrzypek et al. 2008; Kumar et al. 2019; Li et al. 2022). These stresses often cause protein misfolding, cell damage, and reduced cell viability, which directly restrict callus proliferation, shoot differentiation, and final

regeneration efficiency (Yusof et al. 2023). As key molecular chaperones, DnaJ family proteins play vital roles in maintaining protein homeostasis and stress responses. Based on our previous study, *TaDJA-6D*, a member of the DnaJ family, was found to be significantly upregulated during wheat regeneration, suggesting that this gene may play an important regulatory role in plant regeneration and genetic transformation (Liu et al. 2023a, b). However, whether DnaJ proteins participate in regulating wheat tissue culture and regeneration processes remains largely unknown. In this study, we identified and characterized a novel DnaJ protein from wheat, designated TaDJA-6D. Through integrated bioinformatics and molecular biology approaches, we clarified the structural features and subcellular localization of the TaDJA-6D protein. Furthermore, we analyzed the spatiotemporal expression pattern of the *TaDJA-6D* gene during wheat regeneration process, and functionally validated its role through wheat genetic transformation. Our findings not only expand the functional spectrum of DnaJ proteins but also provide a novel genetic resource for improving wheat regeneration and genetic transformation efficiency.

2 Materials and methods

2.1 Plant materials and growth condition

The experimental materials included four wheat cultivars, Fielder, Kenong 199 (KN199), Yanda 1817 (YD1817), and Xiaoyan 6 (XY6), as well as *Nicotiana benthamiana*. Grains of the spring wheat cultivar Fielder were directly sown in pots. In contrast, the winter wheat cultivar KN199 required vernalization treatment, and seedlings at 3–5 days post-germination were transferred to a growth chamber at 6 °C for 35 days. For *N. benthamiana*, seedlings were transplanted into pots after germination. All plants were grown under the controlled conditions: 24 °C (16 h light)/18 °C (8 h dark), with a light intensity of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a relative humidity of 40%.

2.2 Plasmid construction

Total RNA was extracted from wheat calli using an RNA extraction kit (Cowin Biotech, Jiangsu). First-strand cDNA was synthesized from 1 μg of total RNA using a high-efficiency reverse transcription kit for qPCR (Vazyme, Nanjing). Gene-specific primers were designed based on the target gene sequence to amplify the full-length CDS using the synthesized cDNA as a template. The PCR product was ligated into the pEASY®-Blunt3 Cloning Vector (TransGen Biotech, Beijing) following the manufacturer's instructions. After confirmation by sequencing, the overexpression vector pUbi110-TaDJA-6D containing the *TaDJA-6D* gene (Fig.

S1), and the reporter vector pROKII-TaDJA-6D-GFP were constructed using the one-step cloning method (Meng et al. 2017). Primers for vector constructs are listed in Table S1.

2.3 In situ hybridization

Wheat callus tissues were fixed in 0.1 M phosphate buffer saline (pH 7.0) containing 4% (v/v) paraformaldehyde (Sigma, USA). After dehydration with gradient concentrations of ethanol and clearing with xylene, the samples were embedded in paraffin (Leica, Germany) and sectioned into 8.0 µm-thick wax ribbons using a Leica microtome (Leica Microsystems, Germany), followed by probe hybridization and signal observation. In situ hybridization probes were synthesized using the DIG RNA Labeling Kit (SP6/T7) (Roche, Switzerland) (Yao et al. 2024). Primers used are listed in Table S1.

2.4 RT-qPCR experiment

Total RNA was isolated using the Trizol reagent (Covin Biotech, Jiangsu, China), and its purity and integrity were validated. After genomic DNA contamination removal, first-strand cDNA was synthesized with a reverse transcription kit (Vazyme, Nanjing, China). Specific primer pairs targeting the target genes and the reference gene TaActin were designed for subsequent RT-qPCR analysis. Each sample was amplified with three technical replicates. The RT-qPCR program included an initial denaturation at 95 °C, followed by 45 cycles of denaturation and annealing; melting curve analysis was performed to confirm primer specificity. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Primers used are listed in Table S1.

2.5 RNA-seq experiment

Calli transformed with *TaDJA-6D* and the *GUS* control were collected at the 9 days after induction (DAI9) of the Fielder cultivar for RNA sequencing. Total RNA was extracted using the Ultra-Pure RNA Kit (CWBIO, CW0581M) in accordance with the manufacturer's instructions. Three biological replicates were set up for each treatment to ensure the reliability and reproducibility of the experimental results, and RNA sequencing was performed by Gene Denovo Biotechnology Co., Ltd (Guangzhou, China).

2.6 Subcellular localization analysis

The full-length coding region of *TaDJA-6D* was cloned into pROKII-GFP to generate the TaDJA-6D-GFP construct, with the empty pROKII-GFP vector as a negative control; both constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, which was then cultured,

resuspended in infiltration buffer, and infiltrated into 4-week-old *Nicotiana benthamiana* leaves (Meng et al. 2017). The infiltrated leaves were incubated in a growth chamber for 48–72 h. For localization validation, *Agrobacterium* carrying the plasma membrane marker p35S-CBL1-RFP was prepared and mixed with that containing TaDJA-6D-GFP prior to infiltration (Ma et al. 2021). Subsequently, GFP and RFP fluorescence images were captured using an LSM880 confocal laser scanning microscope (Carl Zeiss, Germany).

2.7 Wheat genetic transformation

Wheat immature embryos were collected at 14–15 days after pollination and infected via the *Agrobacterium*-mediated method following the protocol of previous study (Ishida et al. 2015). Briefly, the immature embryos underwent induction, selection, and differentiation culture to generate putative transgenic plantlets. Genetic transformation was confirmed by PCR identification and the primers used are listed in Table S1. The number of immature embryos used as explants, the number of embryonic callus, the number of differentiation callus, and the number of callus with at least one positive plantlet were counted to calculate the callus induction efficiency, callus differentiation efficiency, and transformation efficiency (Bie et al. 2026). The relevant data are shown in Table S3.

Callus induction efficiency

$$= \frac{\text{the number of embryonic callus}}{\text{number of explants}} \times 100\% \quad (1)$$

Callus differentiation efficiency

$$= \frac{\text{the number of differentiation callus}}{\text{number of explants}} \times 100\% \quad (2)$$

Transformation efficiency

$$= \frac{\text{the number of callus with at least one positive plantlet}}{\text{number of explants}} \times 100\%. \quad (3)$$

3 Results

3.1 Phylogenetic analysis of DnaJ proteins from Poaceae species

Using the TaDJA protein sequence as a query, homologous proteins were identified from NCBI (www.ncbi.nlm.nih.gov/gorf/gorf.html). A total of 33 full-length amino acid sequences of DnaJ proteins derived from 19 representative

Poaceae species and 11 species belonging to other families were first aligned using ClustalW, followed by the construction of a phylogenetic tree with MEGA6.0 software.

Results displayed that the TaDJA proteins from common hexaploid wheat showed high homology with those from its closely related species, including *Triticum dicoccoides* (100%), *Triticum urartu* (99.76%) and *Aegilops tauschii* (99.76%). These species clustered in the same clade, indicating a close evolutionary relationship and conserved protein structure. Sequence homology of TaDJA6 was further compared with functionally characterized DnaJ proteins from *Arabidopsis thaliana* (Pulido et al., 2018), *Zea mays*

(Dong et al. 2022), and *Oryza sativa* (Zhong et al. 2018). Phylogenetic analysis revealed that TaDJA proteins was evolutionarily distant from AtDJA6 and ZmDnaJ, but closely related to OsDJA6 (Fig. 1). These findings suggest that functional divergence may have occurred between TaDJA and the known functional proteins in *Arabidopsis* and maize. Within Poaceae, TaDJA is evolutionarily closer to the rice homolog OsDJA6, implying conserved biological functions with OsDJA6. The DnaJ protein family exhibits high sequence conservation among closely related species, thereby providing a molecular basis for the maintenance of their conserved functions.

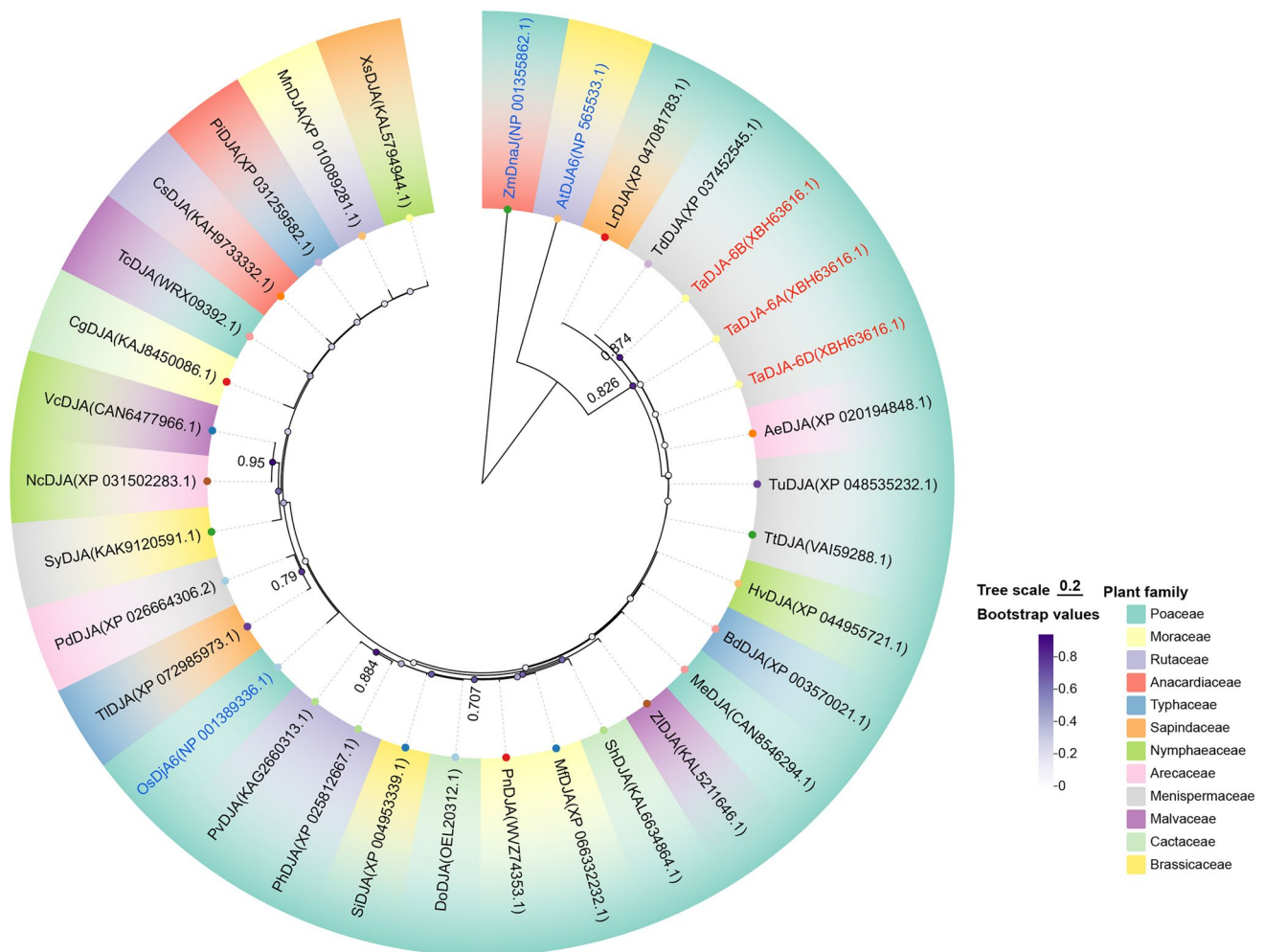


Fig. 1 Phylogenetic analysis of DnaJ proteins in *Triticum aestivum* (Ta), *Zea mays* (Zm), *Aegilops tauschii* (At), *Lolium rigidum* (Lr), *Triticum dicoccoides* (Td), *Aegilops tauschii* (Ae), *Triticum urartu* (Tu), *Triticum turgidum* (Tt), *Hordeum vulgare* (Hv), *Brachypodium distachyon* (Bd), *Melica nutans* (Me), *Zizania latifolia* (Zl), *Stipagrostis hirtigluma* (Sh), *Miscanthus floridulus* (Mf), *Paspalum notatum* (Pn), *Dichanthelium oligosanthes* (Do), *Setaria italica* (Si), *Panicum hallii* (Ph), *Panicum virgatum* (Pv), *Oryza sativa* (Os), *Typha latifolia* (Tl), *Phoenix dactylifera* (Pd), *Stephania yunnan-*

ensis (Sy), *Nymphaea colorata* (Nc), *Victoria cruziana* (Vc), *Carnegieia gigantea* (Cg), *Theobroma cacao* (Tc), *Citrus sinensis* (Cs), *Pistacia vera* (Pi), *Morus notabilis* (Mn) and *Xanthoceras sorbifolium* (Xs) from 19 representative Poaceae species and 11 other plant families. A phylogenetic tree was constructed in MEGA6.0 using the neighbor-joining method with 1000 bootstrap replicates based on the full-length amino acid sequences aligned with ClustalW. The Chiplot website was used for the visualization and beautification of the constructed phylogenetic tree (Xie et al. 2023)

3.2 Analysis of cis-acting elements, gene structure and characteristic domains in TaDJAs

To gain a further understanding of the regulatory and structural characteristics of the *TaDJA* genes, we analyzed their promoter regions (3 kb upstream of the translation start site) and exon–intron architectures. Using PLANTCARE for cis-acting elements prediction and TBtools for visualization, we generated schematic representations. Our findings indicated that the promoter of *TaDJA-6A*, *TaDJA-6B* and *TaDJA-6D* encompass a variety of elements, including auxin-responsive elements, meristem expression elements, and wound-responsive elements (Fig. 2a). Notably, the distribution patterns

of these *cis*-regulatory elements exhibit notable differences among the A, B, and D sub-genomes, suggesting potential sub-genome specific regulation mechanisms that may contribute to divergent response to environmental and developmental cues. In addition, gene structure provides crucial insights into functional analyses. *TaDJA-6A*, *TaDJA-6B*, and *TaDJA-6D* genes share a conserved architecture, consist of a 5'-UTR, seven exons, six introns, and a 3'-UTR (Fig. 2b). Specifically, we observed a significant structural variation: the third intron of *TaDJA-6B* is considerably longer than those of *TaDJA-6A* and *TaDJA-6D*. This intron length polymorphism may influence gene expression regulation through mechanisms such as alternative splicing or transcriptional efficiency.

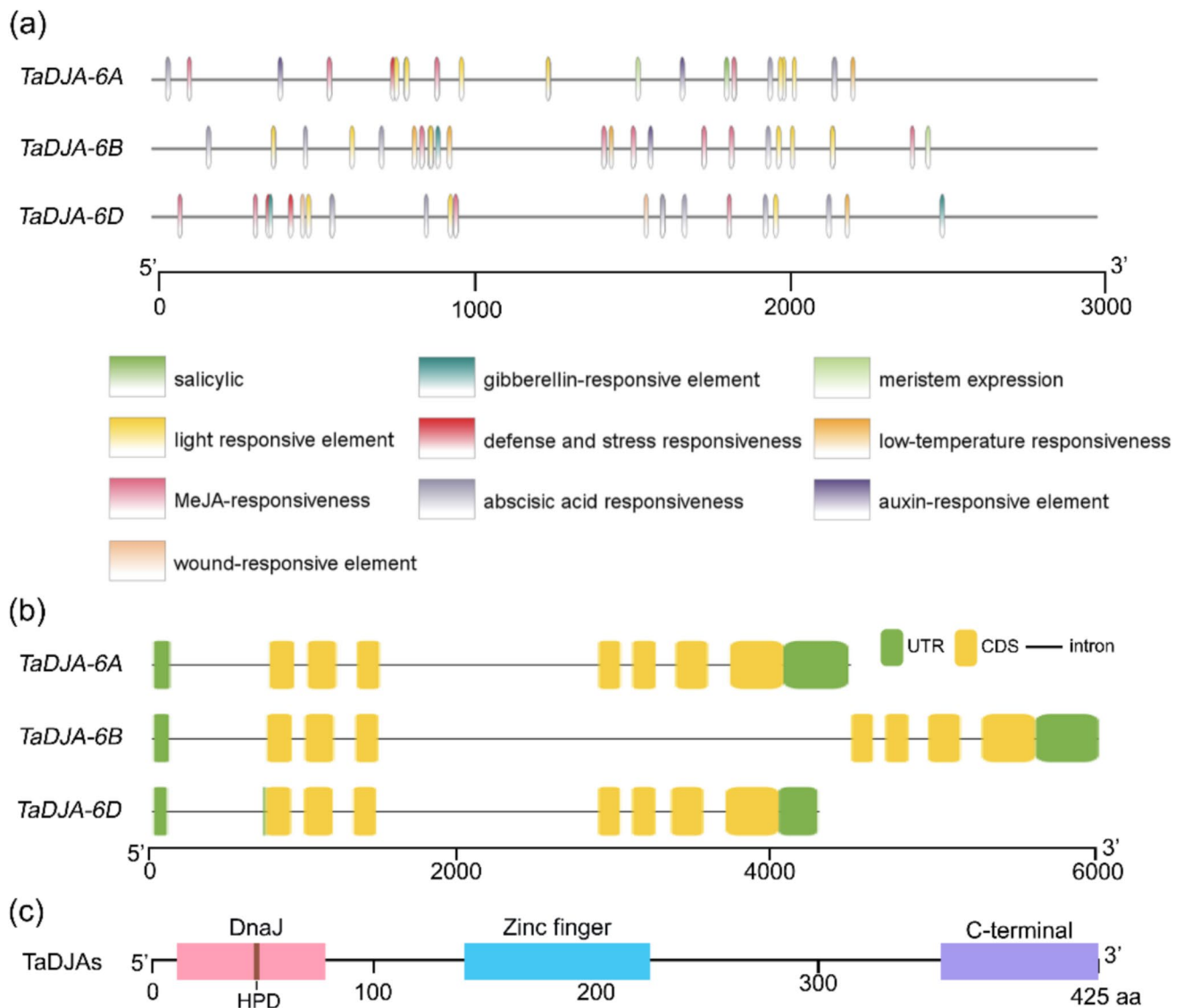


Fig. 2 Schematic diagrams of cis-acting elements, gene structures and characteristic domains of *TaDJA* genes. **a** Prediction of *TaDJA* genes promoter sequence elements. **b** Structural schematic of *TaDJA* genes. Green squares, yellow squares and black line indicate UTR,

CDS, and intron, respectively. **c** Conserved domains of *TaDJA* proteins. Pink square, blue square and purple square indicate DnaJ domain, zinc finger domain, and C-terminal domain, respectively. The schematic diagrams were mapped by TBtools

DnaJ proteins typically possess conserved functional domains, including the DnaJ domain, zinc-finger domain, and C-terminal domains (Chiu et al. 2013; Kampinga et al. 2019). Domain organization indicated that TaDJA-6A, TaDJA-6B, and TaDJA-6D each contain complete domain architecture, classifying them as type I DnaJ proteins (Fig. 2c). The DnaJ domain, which is essential for HSP70 co-chaperone activity, contains a highly conserved histidine-proline-aspartic acid (HPD) tripeptide motif. This motif mediates the interaction between TaDJAs and the nucleotide-binding domain of HSP70 proteins. The conservation of these domains across wheat homeologs suggests functional redundancy and evolutionary conservation of DnaJ-mediated protein quality control mechanisms in hexaploid wheat.

3.3 Spatiotemporal expression pattern of TaDJAs in wheat regeneration

To investigate the dynamic changes in the expression level of the *TaDJA* genes during wheat regeneration, we performed a systematic analysis of the spatiotemporal expression pattern using the wheat cultivar Fielder. In situ hybridization showed that the *TaDJA* genes had a relatively strong signal in the root apical meristem of wheat immature embryos (Fig. 3a). At 3 days after induction (DAI3), *TaDJAs* was highly expressed mainly in the epidermal cells of calli and the vascular tissues (Fig. 3b). At DAI6, the hybridization signal was mainly concentrated in the callus regions with vigorous cell division (Fig. 3c). At DAI9, the expression signal of *TaDJA* genes were highly expressed at the initiation site of shoot primordia (Fig. 3d).

Furthermore, RT-qPCR analysis was performed to detect the relative expression levels of *TaDJAs* at five callus tissue culture stages, including DAI0, DAI3, DAI6, DAI9, and DAI12. The results revealed that the expression of *TaDJAs* was upregulated in wheat regeneration process (Fig. 4a). Analysis of RNA-seq data from Fielder regeneration showed that *TaDJA-6A*, *TaDJA-6B*, and *TaDJA-6D* exhibited a consistent upregulation trend throughout the regeneration process (Fig. 4b; Table S2) (Liu et al. 2023a, b). It suggests that the *TaDJA* genes play a key regulatory role in the initial stages of wheat regeneration. In particular, the *TaDJA-6D* gene displayed the most significant response to tissue culture induction, with higher expression levels compared than the other two subgenome homeologs at various stages. Based on the comprehensive analysis of the spatiotemporal expression pattern, we speculate that the sustained high-level expression of *TaDJA* genes promote the in vitro regeneration process of wheat. Furthermore, considering the transcriptional expression level of the *TaDJA* genes, further research will focus on the functional analysis of the *TaDJA-6D* gene.

3.4 Subcellular localization of TaDJA-6D

The subcellular localization of proteins is closely associated with their biological function. To determine the subcellular localization of TaDJA-6D, a fusion reporter vector pROKII-TaDJA-6D-GFP was constructed and utilized to infiltrate tobacco leaves of approximately 4-week-old plants with the empty pROKII-GFP vector used as a control. In contrast to the control vector, distinct green fluorescent signals of TaDJA-6D were concentrated at the cell membrane and

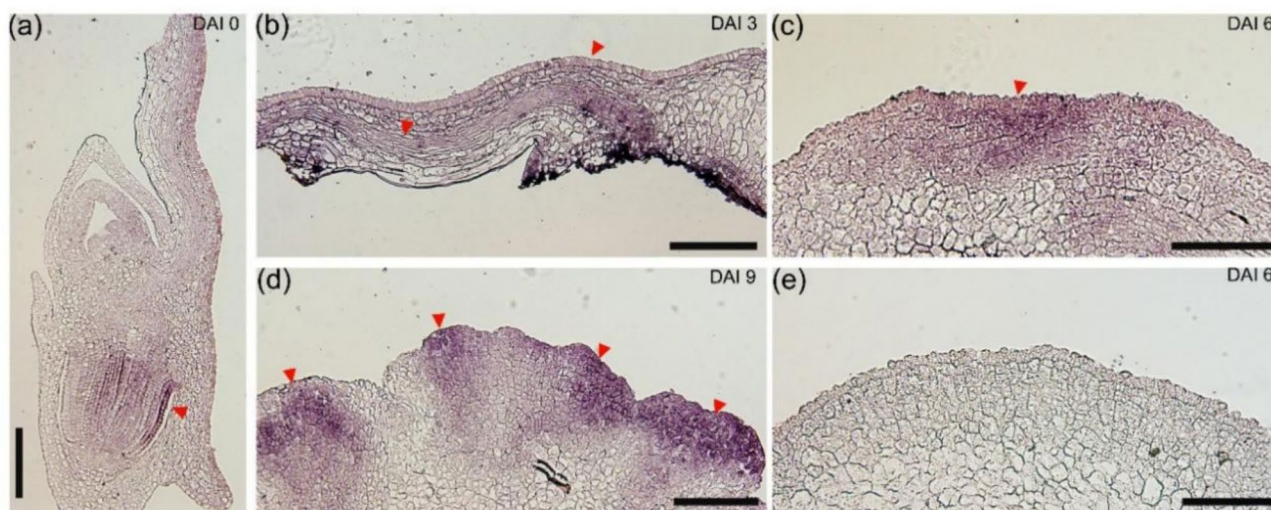


Fig. 3 In situ hybridization of *TaDJA* genes in wheat embryo and callus at different tissue culture stages. **a** The immature embryo of wheat at 0 day after induction, **b** The callus at 3 days after induction, **c** The

callus at 6 days after induction, **d** The callus at 9 days after induction, **e** The hybridization result of sense probe. Scale bars: 250 μ m

nucleus of tobacco cells using an LMS880 laser confocal microscope (Fig. 5). To further verify the localization patterns, nuclear staining with DAPI and co-expression of the plasma membrane marker CBL1-RFP were performed. Merged images clearly showed that the green fluorescence of TaDJA-6D co-localized with both the DAPI-labeled nucleus and the CBL1-RFP-labeled plasma membrane (Fig. 5a, b; Fig. S2a, b). These results indicate that the TaDJA-6D protein exhibits a dual localization pattern, targeting both the plasma membrane and the nucleus in plant cells.

3.5 Functional validation of TaDJA-6D in wheat and statistical analysis of agronomic traits

To gain insight into the biological function of TaDJA-6D in wheat regeneration and genetic transformation, we constructed the overexpression vector pUbi110-TaDJA-6D and transformed into wheat immature embryos via *Agrobacterium*-mediated method, with the *GUS* construct as a control. Statistical analysis found that, in the Fielder cultivar, *TaDJA-6D* overexpression (*TaDJA-6D*-OE) increased the callus induction efficiency from 90.38% to 94.48%, callus

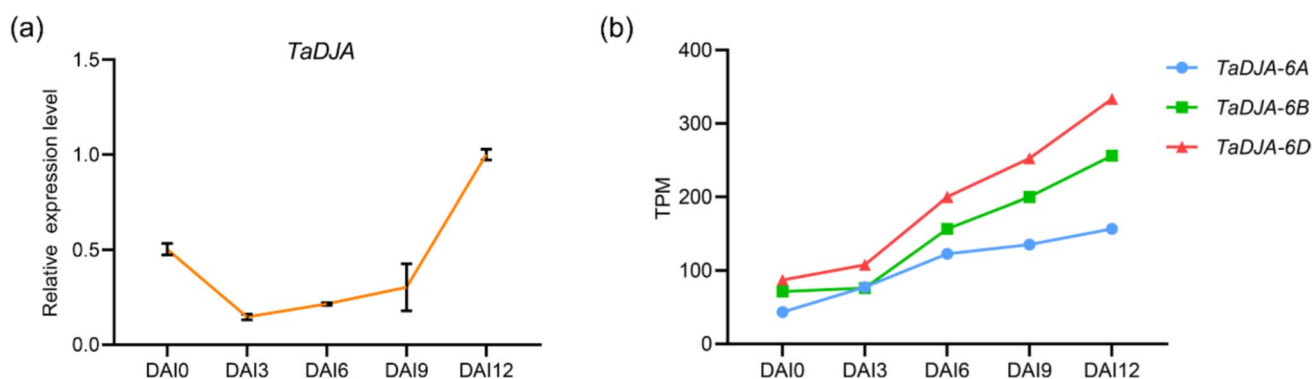


Fig. 4 Expression patterns of *TaDJA* genes in wheat regeneration process. **a** Relative *TaDJA* expression levels in the callus of Fielder at DAI0, DAI3, DAI6, DAI9, and DAI12 were determined by RT-qPCR,

with expression levels normalized to *TaActin* (n=3). **b** Transcript levels (presented as TPM, Transcripts Per Million) of *TaDJA-6A*, *TaDJA-6B* and *TaDJA-6D* at DAI0, DAI3, DAI6, DAI9 and DAI12

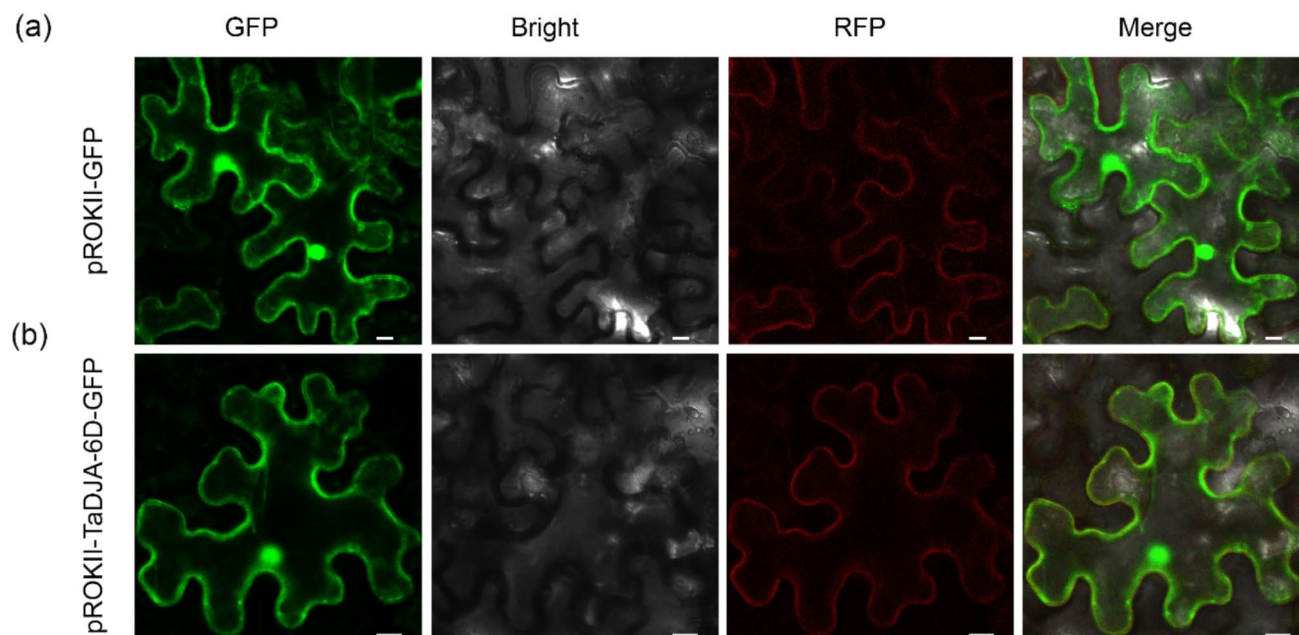


Fig. 5 Green fluorescent signals in tobacco leaves of pROKII-TaDJA-6D-GFP. **a** The control vector, pROKII-GFP. **b** The fusion reporter vector, pROKII-TaDJA-6D-GFP. Scale bars: 10 μ m

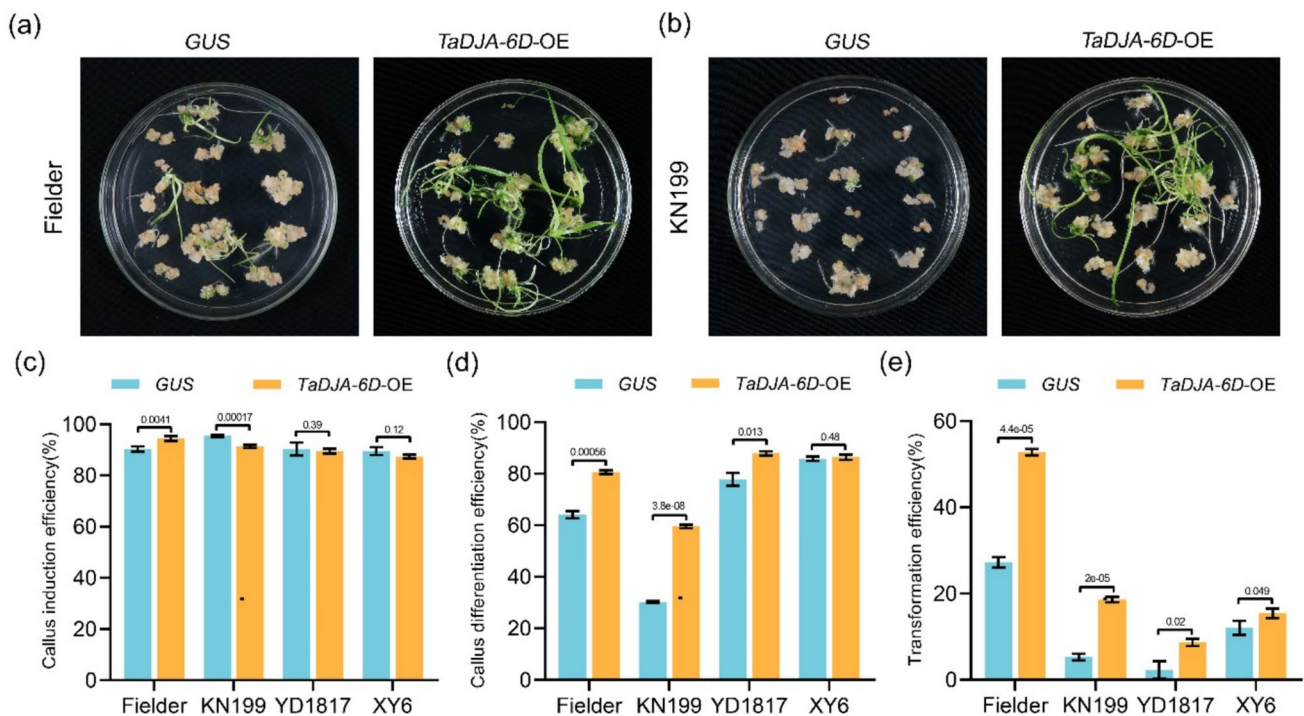


Fig. 6 Overexpression of *TaDJA-6D* gene improved the transformation efficiency in Fielder, KN199, YD1817 and XY6. **a, b** Regeneration phenotypes of Fielder (**a**) and KN199 (**b**) immature embryos transformed with *GUS* (control) or *TaDJA-6D-OE*. **c–e** Comparison of callus induction efficiency (**c**) callus differentiation efficiency (**d**)

and callus transformation efficiency (**e**) between callus transformed *GUS* and *TaDJA-6D-OE* vectors in Fielder, KN199, YD1817 and XY6 ($n=3$; Fielder-*TaDJA-6D-OE* and KN199-*TaDJA-6D-OE*, $n=4$). Data are presented as mean $\pm$ standard error (SE). p values in panels (**c–e**) were calculated using Student's t -test

differentiation efficiency from 64.10 to 80.59%, and significantly elevated the transformation efficiency from 27.24% to 52.80% (Fig. 6a, c–e; Table S3). In KN199, *TaDJA-6D-OE* significantly improved callus differentiation efficiency and transformation efficiency, increasing them from 30.28 to 59.56% and from 5.26% to 18.59%, respectively ($p < 0.01$), representing a more than threefold enhancement in the overexpression group (Fig. 6b, c–e; Table S3).

To verify whether *TaDJA-6D-OE* can overcome the genotype dependence of wheat transformation, we further performed functional validation in YD1817 and XY6. In YD1817, *TaDJA-6D-OE* increased the callus differentiation efficiency from 77.81% to 87.84% and the transformation efficiency from 2.26 to 8.66%, with no significant difference in the callus induction efficiency. In XY6, the transformation efficiency increased from 12.03 to 15.42%, whereas the callus induction efficiency and the differentiation efficiency showed no significant differences (Fig. 6c–e; Table S3). Taken together, these results demonstrate that overexpression of *TaDJA-6D* significantly boosts genetic transformation efficiency in Fielder, KN199, YD1817 and XY6. The findings suggest that genes from DnaJ protein family in wheat may possess the potential to enhance regeneration capacity. Subsequently, positive transgenic plants were verified by PCR identification (Fig. S3).

To investigate whether *TaDJA-6D* influences key agronomic traits in wheat, we compared plant height, tiller number, spikelet number, and grain morphology between transgenic and wild-type (WT) lines. Statistical analysis demonstrated that overexpression of *TaDJA-6D* had no significant effects on tiller number, spikelet number, grain length, or grain width compared with WT plants (Fig. 7a, c–g). Plant height and thousand-grain weight were significantly reduced in the *TaDJA-6D-OE-1* line ($p < 0.05$), but showed no significant differences in the *TaDJA-6D-OE-4* line (Fig. 7b, h). Overall, these results indicate that overexpression of *TaDJA-6D* exerts no obvious adverse effects on major agronomic traits of wheat.

3.6 Transcriptomic profiling of *TaDJA-6D*-overexpressing across wheat tissue culture

To analyze the transcriptional expression profile of *TaDJA-6D* during wheat regeneration, we collected tissue-cultured samples at DAI9 and performed RNA-seq. Principal component analysis (PCA) revealed a clear separation between *TaDJA-6D-OE* and *GUS* control samples. The first two principal components (PC1 and PC2) accounted for 81.46% and 9.25% of the total variance, respectively (Fig. 8a). This

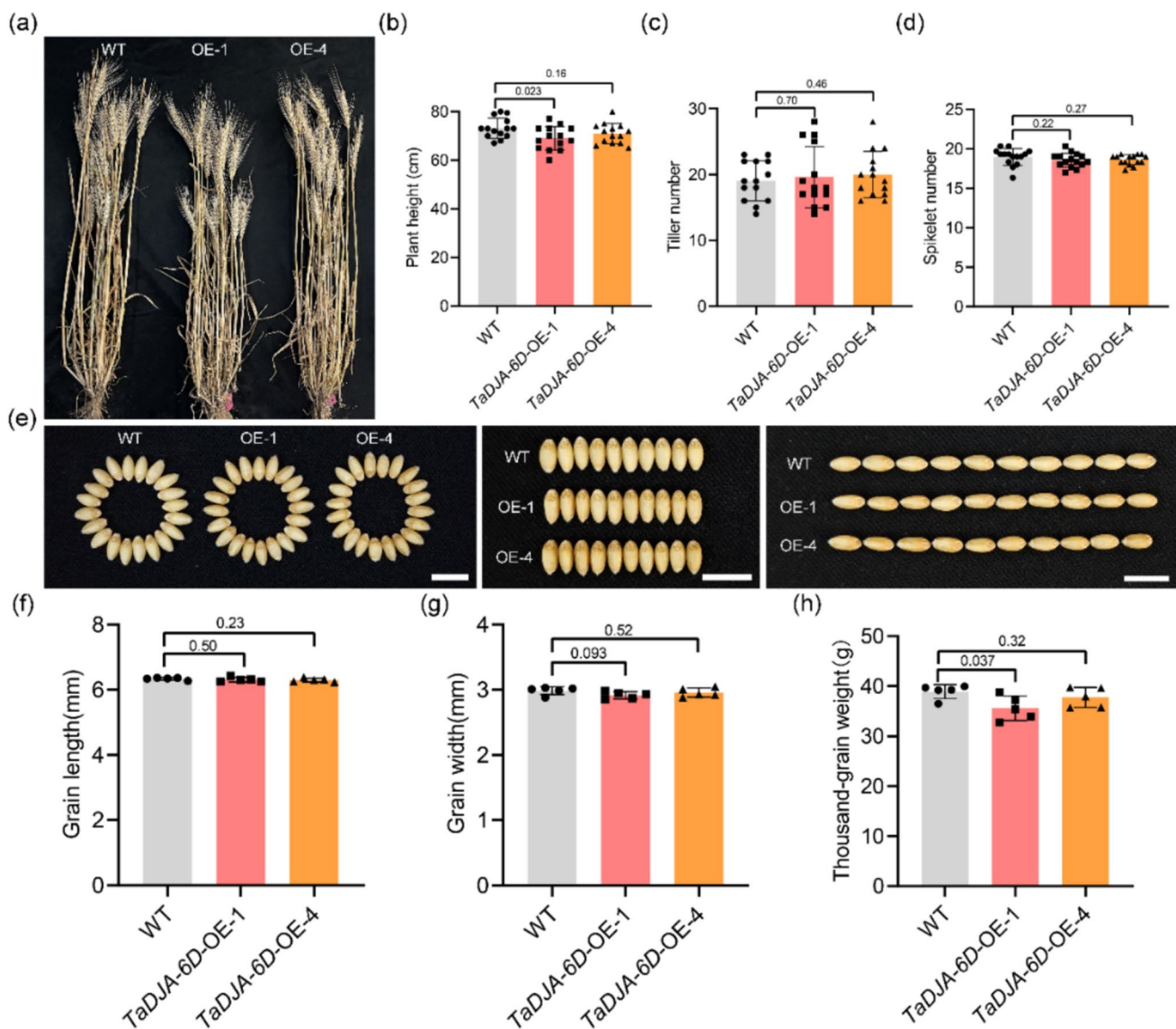


Fig. 7 Phenotypes and agronomic trait analysis of *TaDJA-6D*-OE transgenic plants. **a** Phenotypic comparison of WT and *TaDJA-6D*-OE plants (OE-1 and OE-4). **b–d** Statistical analysis of plant height (**b**), tiller number (**c**), and spikelet number (**d**) in transgenic plants, **e** Grain morphology of WT and *TaDJA-6D*-OE plants. Scale

bars, 1 cm. **f–h** Statistical analysis of grain length (**f**), grain width (**g**), and thousand-grain weight (**h**) in transgenic plants and WT. Data are presented as mean $\pm$ standard error (SE). p values in panels (**b–d**) and (**f–h**) were calculated using Student's t-test

indicated that overexpression of *TaDJA-6D* triggered pronounced transcriptomic reprogramming at DAI9 in wheat.

Differential expression analysis identified 1,738 differentially expressed genes (DEGs) under the criteria of $\log_2\text{FCI} > 1$ and adjusted p value < 0.05 . Compared with the *GUS* control group, the *TaDJA-6D*-OE line exhibited 1,343 upregulated and 395 downregulated genes (Fig. 8b; Table S4). GO enrichment analysis indicated that DEGs were significantly enriched in biological processes including DNA metabolism and replication, cell cycle regulation, protein folding, meristem development and stress response (Fig. S4). KEGG pathway enrichment analysis further

demonstrated that these DEGs were mainly enriched in pathways related to protein processing in the endoplasmic reticulum and DNA replication and repair; plant hormone signal transduction was also significantly enriched.

Heatmap analysis of DEGs revealed distinct expression profiles of key functional genes, including hormone signaling-related genes such as *TaPYL10*, *TaPIN1A* and *TaERF4* (Yang et al. 2005; Petrášek et al. 2006; Park et al. 2009; Wentink et al. 2026), stress-responsive genes such as *TaWRKY24* (Wang et al. 2025), and developmental regulators such as *TaLBD15* (Ohashi-Ito et al. 2018) (Fig. 8d). Based on our RNA-seq data, we examined

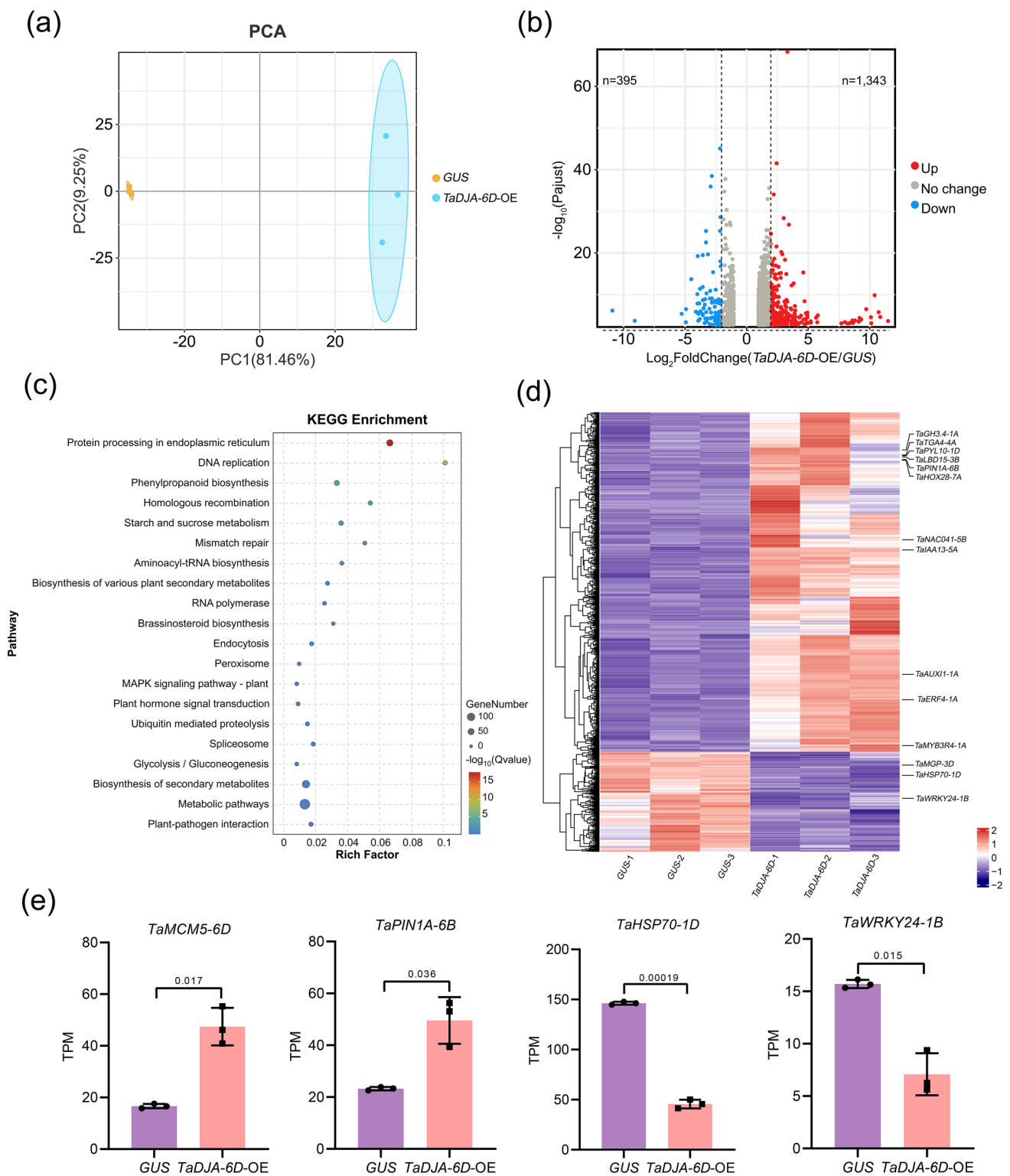


Fig. 8 Transcriptomic analysis of *GUS* and *TaDJA-6D-OE* at DA19. **a** Principal component analysis (PCA) plot of transcriptomic profiles, **b** Volcano plot showing DEGs between *GUS* and *TaDJA-6D-OE*, **c** KEGG enrichment scatter plot of significantly enriched pathways

among DEGs, **d** Hierarchical clustering heatmap of DEGs, **e** TPM values of representative genes involved in key pathways in *GUS* and *TaDJA-6D-OE* wheat calli. Data are presented as mean $\pm$ standard error (SE). p values in panels **e** was calculated using Student's t-test

the TPM values of selected genes involved in TaDJA-6D-mediated biological processes, including *TaMCM5*, *TaPIN1A*, *TaHSP70*, and *TaWRKY24* (Petrásek et al. 2006; Shultz et al. 2009; Wang et al. 2025; Wentink et al. 2026). Collectively, these results demonstrate that TaDJA-6D promotes wheat regeneration by coordinating multiple biological processes, including maintenance of protein homeostasis, cell cycle regulation, hormone signal transduction, and enhancement of stress tolerance (Fig. 8e; Table S4).

4 Discussion

In recent years, advancement in gene editing and genetic transformation have accelerated research on plant gene functions and crop improvement. However, genetic transformation in crops still faces many challenges (Chen et al. 2022). The efficient regeneration ability of wheat is the basis for genetic transformation and gene editing. Therefore, in-depth exploration of wheat regeneration-related genes is essential to break the bottlenecks of genetic transformation.

Previous studies have found that the effective utilization of regeneration-related factors can, to a certain extent, mitigate limitations in wheat regeneration and genetic transformation (Debernardi et al. 2020; Qiu et al. 2022; Wang et al. 2022; Liu et al. 2023a, b; Yu et al. 2024; Bie et al. 2026). Building upon our preliminary work, we found that *TaDJAs* was mainly expressed in the cell clusters with vigorous division activities in the wheat calli and shoot primordia initiation sites via in situ hybridization (Fig. 3). Meanwhile, RNA-seq data and RT-qPCR analyses consistently indicated that the transcriptional level of the *TaDJA* genes were significantly upregulated after tissue culture induction (Fig. 4). Spatiotemporal expression pattern analysis preliminarily suggested that TaDJAs may regulate wheat regeneration. Further functional validation demonstrated that overexpression of *TaDJA-6D* significantly enhanced genetic transformation efficiency in Fielder, KN199, YD1817, and XY6 cultivars, highlighting its potential as a valuable regeneration-related gene (Fig. 6).

Notably, unlike most known regeneration-related genes, which are transcription factors, TaDJA is a type of functionally conserved molecular chaperone. It contains a DnaJ domain, zinc finger domain and C-terminal domains (Fig. 2c) (Kampinga et al. 2019; Verma et al. 2019). Moreover, TaDJA has high homology among gramineous plants, indicating that the function maybe conserved (Fig. 1). The DnaJ domain is a crucial domain for the function of DnaJ proteins, as it contains a conserved histidine/proline/aspartic acid tripeptide (HPD). HPD is essential for the interaction between DnaJ proteins and HSP70, which can activate the

ATPase activity of HSP70, thereby playing a key role in protein folding, transport, and degradation (Tsai and Douglas., 1996; Pulido et al., 2018). We found that TaDJA contains an HPD site and speculate that it may also exert function through interaction with HSP70 in wheat (Fig. 2c). In addition, the gene function is closely related to subcellular localization. To date, studies on DnaJ proteins in plants has primarily focused on those localized in chloroplasts, which are involved in processes such as chloroplast development, photosynthesis, and protein transport (De Luna-Valdez et al. 2019; Zhao et al. 2022), and extensively regulate plant stress responses and immune reactions (Craig and Marszalek 2017; Rosenzweig et al. 2019). However, we found that the TaDJA-6D protein is localized on the nucleus and cell membrane (Fig. 5), indicating that its function in wheat may be different from previously studies, thus providing clues for the functional diversity of DnaJ proteins.

Previous studies have demonstrated that the GRF-GIF complex upregulates the expression of core cell cycle-related genes (e.g., *CYCD3;1* and *CDKB2;1*), thereby promoting explant cell proliferation, significantly enhancing cereal crop regeneration capacity, and reducing genotype dependence (Kowalik et al. 2026). As a key regulator of the plant root stem cell niche, WOX5 participates in auxin and cytokinin signaling pathways and exerts a positive effect on improving wheat regeneration efficiency (Drisch et al., 2015; Wang et al. 2022; Kowalik et al. 2026). As a DnaJ/Hsp40 molecular chaperone, TaDJA-6D plays a pivotal role in regulating protein homeostasis (Carvalho et al. 2023; Jiang et al. 2025). Transcriptome analysis revealed that *TaDJA-6D* overexpression promotes the expression of genes involved in pathways such as protein folding, meristem development, and plant hormone signal transduction (Fig. 8 and Fig. S4). We hypothesize that TaDJA-6D enhances regeneration and genetic transformation efficiency by modulating these biological processes. In future research, combinatorial application of TaDJA-6D with known regeneration factors could be explored to facilitate the establishment of highly efficient genetic transformation systems in wheat and other cereal crops.

5 Conclusions

In this study, a gene localized to both the cell nucleus and membrane, *TaDJA-6D*, was identified. Its transcriptional expression level gradually increased with the prolongation of tissue culture duration, with high expression predominantly localized to callus regions characterized by vigorous cell division. Overexpression of *TaDJA-6D* in wheat cultivars Fielder, KN199, YD1817 and XY6 significantly enhanced

genetic transformation efficiency. Furthermore, core biological processes including protein homeostasis, cell cycle regulation, phytohormone signal transduction, and stress tolerance are closely involved in the regulatory mechanism of TaDJA. Collectively, these findings delineate the functional role of TaDJA in wheat regeneration progress and provide a novel candidate gene resource for improving the regeneration capacity of wheat and other gramineous crops, thereby offering valuable insights for optimizing the genetic transformation systems of related crops.

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Author contributions Xiao Min Bie and Xian Sheng Zhang designed and supervised the research. Xiao Min Bie and Menglu Li wrote the manuscript. Menglu Li performed plasmid constructions, subcellular localization and in situ hybridization. Yanyan Chang and Xiaoting Shi produced the phylogenetic tree and gene structure analysis diagram. Xiao Min Bie, Ying Song and Mengyi Zhou performed wheat genetic transformation and data analysis. Menglu Li and Xiao Min Bie performed RNA-seq experiments and prepared all the figures. All authors discussed the results and commented on the manuscript.

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

Declarations

Conflict of interest Author Xian Sheng Zhang is the Editor-in-Chief of WheatOmics and he was not involved in the journal's review of, or decisions related to, this manuscript. The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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