

MILD PEG-INDUCED OSMOTIC STRESS AFFECTS THE REGULATION OF THE DEHYDRATION-RESPONSIVE ELEMENT-BINDING (*DREB*) FAMILY GENES IN RICE CV. INPAGO 6 AND INPAGO 9 BASED ON TRANSCRIPTOME AND GENE EXPRESSION ANALYSIS

Miftahul Huda Fendiyanto ^{1,*}, Mentari Putri Pratami ², Elsa Gafira Cahyani¹, Ifan Rizky Kurniyanto ³, Eko Setiawan ⁴, Fandri Sofiana Fastanti ², Syatrianty A. Syaiful ⁵, Achmad Kautsar Baharuddin ⁶ and Muhammad Fuad Anshori ^{5,6*}

¹Department of Biology, Faculty of Military Mathematics and Natural Sciences, Indonesia Defense University, Kompleks Indonesia Peace and Security Center (IPSC) Kampus Universitas Pertahanan-Sentul, Bogor 16680, Indonesia

²Research Center for Biosystematics and Evolution, National Research and Innovation Agency of Indonesia. Jl. Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, West Java, Indonesia

³Department of Agricultural Science and Technology, Faculty of Agriculture, University of Trunojoyo Madura, Bangkalan 69162, Indonesia

⁴Natural Resource Management Study Program, Faculty of Graduate, University of Trunojoyo Madura, Bangkalan 69162, Indonesia

⁵Department of Agronomy, Hasanuddin University, Makassar, 90245, Indonesia

⁶Rice Research Group, Faculty of Agriculture, Hasanuddin University, Makassar, 90245, Indonesia

*Corresponding author: fuad.anshori@unhas.ac.id (MFA), miftahul.fendiyanto@idu.ac.id (MHF)

ABSTRACT

Drought stress is a major constraint to rice (*Oryza sativa* L.) production, while mild PEG-6000-induced osmotic stress can be used under controlled conditions to simulate reduced water availability. Members of the Dehydration-Responsive Element-Binding (*DREB*) family play important roles in regulating plant responses to osmotic and drought-related stress. This study aimed to investigate *DREB* responses to mild PEG-6000-induced osmotic stress through public RNA-seq re-analysis and qRT-PCR analysis of the selected *DREB* gene in rice cultivars Inpago 6, Inpago 9, and IR64. Publicly available RNA-seq data from rice cv. Nipponbare were re-analyzed to identify drought-responsive genes, followed by independent qRT-PCR analysis of the selected *DREB* gene in rice cultivars Inpago 6, Inpago 9, and IR64. The RNA-Seq transcriptomic analysis revealed that members of the *DREB* gene family, particularly *OsDREB2A*, play important roles in rice drought response. In addition, we identified 1,793 upregulated and 1,793 downregulated genes, with a threshold of $|\log_2 \text{Fold Change}| \geq 2$ and an adjusted p-value (FDR) < 0.05 . Meanwhile, qRT-PCR showed that *DREB4* expression under low drought stress was highest in Inpago 9 (250-fold), followed by IR64 (7.5-fold) and Inpago 6 (2.5-fold). These findings indicate that *DREB4* plays an active role in drought tolerance under mild PEG-6000-induced osmotic stress and may serve as a candidate gene for developing drought-tolerant rice varieties, particularly Inpago 9.

Keywords: *DREB4*, Drought stress, Inpago 6, Inpago 9, Transcriptomics.

Article History: ABR-26-279 || Received: 13-Jul-2026 || Revised: 01-Sep-2026 || Accepted: 08-Sep-2026 || Published Online: 01-Oct-2026

This is an open-access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. INTRODUCTION

Rice (*Oryza sativa* L.) is a major food crop cultivated extensively worldwide and is particularly important for food security in Indonesia (Miftahudin et al., 2021; Sari et al., 2026). However, climate variability and abiotic stresses, particularly drought, increasingly threaten rice production and can adversely affect plant growth, development, and grain yield (Lathif & Listyorini, 2018; Aslam et al., 2022; Roy et al., 2026). Water scarcity is especially challenging in regions with irregular or low rainfall, highlighting the need to develop and use rice cultivars better adapted to water-limited environments (Wang et al., 2022; Azzahroh et al., 2024). In Indonesia, dry-season drought episodes can affect agricultural productivity, including rice cultivation, and therefore pose an important challenge for sustainable rice production (Kumar et al., 2021; Cahyani et al., 2024). Understanding the molecular mechanisms underlying rice responses to water limitation is therefore important for developing drought-resilient rice cultivars (Giri et al., 2021).

Citation: Fendiyanto, M.H., Pratami, M.P., Cahyani, E.G., Kurniyanto, I.R., Setiawan, E., Fastanti, F.S., Syaiful, S.A., Baharuddin, A.K., & Anshori, M.F. (2026). Mild PEG-induced osmotic stress affects the regulation of the dehydration-responsive element-binding (*DREB*) family genes in rice cv. Inpago 6 and Inpago 9 based on transcriptome and gene expression analysis. *Agrobiological Records*, 26, 1-18. <https://doi.org/10.47278/journal.abr/2026.079>

Several rice cultivars have been developed for cultivation under water-limited or suboptimal conditions (Miftahudin et al., 2021). Among them, Inpago 6 and Inpago 9 are upland rice cultivars reported to have characteristics associated with drought adaptation and suitability for suboptimal environments (Azzahroh et al., 2024; Cahyani et al., 2024). Although these cultivars have potential for cultivation under water-limited conditions, their molecular responses to mild water-deficit conditions remain insufficiently characterized. Understanding the molecular responses of Inpago cultivars is important because drought adaptation is a complex trait involving physiological, biochemical, and molecular processes. Physiologically, rice plants respond to water limitation by regulating water use, adjusting osmotically, accumulating compatible solutes, and protecting cellular structures (Henry et al., 2016). At the molecular level, these responses involve coordinated changes in stress-responsive gene and transcription factor expression (Guo et al., 2022; Singh et al., 2024; Ramadhan et al., 2024).

Among the transcription factors involved in plant responses to drought and other abiotic stresses, the APETALA2/ethylene-responsive factor (AP2/ERF) family has an important regulatory function (Ma et al., 2024; Wang et al., 2024). A major subgroup within this family is the dehydration-responsive element-binding (*DREB*) transcription factors (Wei et al., 2024). *DREB* proteins recognize dehydration-responsive element/C-repeat (DRE/CRT) cis-elements, generally characterized by the core motif A/GCCGAC, in the promoters of downstream stress-responsive genes (Zhang & Xia, 2023; Singh et al., 2024). Through this interaction, *DREB* transcription factors regulate the expression of genes involved in plant responses to dehydration, salinity, and low-temperature stress (Sakuma et al., 2002; Aslam et al., 2022; Chakraborty et al., 2022). Thus, changes in *DREB* expression can provide molecular information about how plants perceive and respond to osmotic or water-deficit conditions.

Researchers have identified and characterized several *DREB* genes in rice. Within the *DREB4* subgroup, *OsDREB4-1* and *OsDREB4-2* have been reported as distinct genes with different expression characteristics (Tian et al., 2005; Fendiyanto et al., 2025a). *OsDREB4-1* is inducible by dehydration and high salinity, whereas *OsDREB4-2* has been reported to be constitutively expressed. These findings indicate that *DREB4* family members may have distinct regulatory functions and should not be considered interchangeable with other *DREB* subgroups. In particular, *DREB4* genes should be distinguished from *DREB2* family members, such as *OsDREB2A*, which represents a separate *DREB* subgroup associated with abiotic stress responses (Hu et al., 2022). Therefore, accurate gene identification and nomenclature are essential when integrating transcriptomic and targeted gene-expression analyses. Regulation of *DREB* transcription factors may contribute to activating downstream stress-responsive genes and maintaining cellular homeostasis under water-limited conditions (Huang et al., 2020; Fendiyanto et al., 2025a).

For controlled molecular studies, polyethylene glycol (PEG)-induced osmotic stress is commonly used to simulate reduced water availability without directly imposing the heterogeneous physical and environmental effects associated with soil drying. PEG-6000 reduces the water potential of the growth medium and consequently creates an osmotic environment in which plants experience limited water availability. Mild PEG-induced osmotic stress can therefore be used as an experimental model to investigate early molecular responses associated with water-deficit conditions under controlled environments. In the present study, the 1% PEG-6000 treatment is referred to as mild PEG-6000-induced osmotic stress, rather than a direct equivalent of field drought stress (Sobahan et al., 2022). This distinction is important because a PEG treatment represents a controlled osmotic challenge and does not reproduce all physiological and environmental components of natural drought (Sobahan et al., 2022).

Transcriptomic approaches, particularly RNA sequencing (RNA-seq), provide powerful tools for investigating complex molecular responses to abiotic stress. RNA-seq enables the simultaneous detection and quantification of thousands of transcripts, allowing differentially expressed genes and biological pathways associated with stress responses to be identified. Transcriptomic analyses have therefore been widely applied to characterize drought-responsive mechanisms in rice and other crops (Zaffar et al., 2025). Publicly available RNA-seq datasets also provide valuable resources for identifying candidate genes that can subsequently be evaluated using targeted expression analysis. However, transcriptomic discovery and experimental qRT-PCR assessment represent complementary but distinct analytical approaches. A gene identified in a transcriptomic dataset should be clearly distinguished from a different candidate gene selected independently for targeted expression analysis.

In this context, publicly available transcriptomic data from rice cv. Nipponbare provides an opportunity to identify genes associated with osmotic or drought-related responses. In the present study, re-analysis of the public RNA-seq dataset identified *OsDREB2A* (*Os01t0165000*) as a *DREB*-family transcript associated with the stress response. Importantly, *OsDREB2A* is distinct from *OsDREB4-2*. Therefore, *OsDREB4-2* was subsequently selected as an additional *DREB*-family candidate for independent qRT-PCR analysis based on its biological relevance to stress-responsive transcriptional regulation. The qRT-PCR analysis was performed in the Indonesian rice cultivars Inpago 6 and Inpago 9, with IR64 included as a comparative cultivar, under mild PEG-6000-induced osmotic stress.

Despite the importance of *DREB* transcription factors in abiotic stress responses, information concerning the

expression response of specific *DREB* genes in Inpago cultivars remains limited. In particular, the response of *OsDREB4-2* in Inpago 6 and Inpago 9 under controlled mild osmotic stress has not been sufficiently characterized. Addressing this gap may provide additional information on the molecular responses of these cultivars to reduced water availability, although gene expression alone cannot be interpreted as direct evidence of drought tolerance. Therefore, integrating public transcriptomic re-analysis with targeted qRT-PCR provides a complementary approach for connecting genome-wide stress-responsive patterns with cultivar-level gene expression.

Accordingly, this study had two objectives: (i) to re-analyse publicly available RNA-seq data from rice cv. Nipponbare to identify differentially expressed genes associated with the response to osmotic stress, including *DREB*-family candidates; and (ii) to experimentally assess the expression response of the selected *OsDREB4-2* gene in rice cultivars Inpago 6; Inpago 9, and IR64 under mild 1% PEG-6000-induced osmotic stress using quantitative real-time PCR (qRT-PCR). We hypothesized that mild osmotic stress would differentially regulate *OsDREB4-2* expression among the evaluated rice cultivars, providing molecular evidence of cultivar-specific transcriptional responses to water-limiting conditions.

2. MATERIALS AND METHODS

2.1 Experimental Design

The materials needed in this research are rice seeds cv. Inpago 6, Inpago 9, and IR64, plant growth medium, PEG 6000 drought stress solution, RNA extraction reagent, *DREB* and *Actin* gene primers, qRT-PCR kit, liquid nitrogen, Trizol reagent, Chlorofoam, Isopropanol, ethanol, ddH₂O, TBE buffer, DN Master Mix, Green Master Mix, Forward and Reverse primers, cDNA template, Tris HCl, RevertAid RT, RiboLock RNase Inhibitor 250 mM Tris-HCl (pH 8.3) 250 mM, KCl 20 mM MgCl₂ 50 mM DTT, 10 mM dNTP Mix, Oligo(dT)18 primer 100 µM, Random Hexamer primer 100 µM, Forward GAPDH primer 10 µM, Reverse GAPDH primer 10 µM. The experiment was conducted under controlled hydroponic conditions at the Laboratory of Plant Physiology and Molecular Biology, the Republic of Indonesia Defense University (RIDU). Rice seedlings of Inpago 6, Inpago 9, and IR64 were assigned randomly to control and 1% PEG-6000 treatment groups, with 15 biological replicates per treatment. Plants were maintained at 25°C, 70% relative humidity (RH), under a 16 h light/8 h dark regime with a light intensity of 150–250 µmol m⁻² s⁻¹. Samples were collected at 96 h after PEG-6000 treatment.

2.2 Research Procedure

2.2.1. Seed Preparation, Germination, and PEG-Induced Drought Stress Treatment in Rice Cultivars: Rice cultivar seeds of cv. Inpago 6, Inpago 9, and IR64 were selected to ensure their quality using a water-soaking method, selecting seeds that sank as an indication of good viability (Fendiyanto et al., 2025b). The rice seeds were germinated on moist cotton for 5 days. The germination process began with sterilization of the Petri dishes and other equipment to prevent contamination. The Petri dishes were lined with cotton that had been evenly moistened with water, but not submerged. The rice seeds were placed on the moist cotton, with sufficient spacing to prevent mild growth due to excess moisture. The petri dishes were then tightly closed to maintain humidity and prevent contamination from outside air. The environmental conditions were then adjusted to darkness to support germination. The germination process lasted for 7 days before the seeds were transferred to a liquid medium. Germinated rice seeds were planted using Hoagland medium with a standard solution of 2 liters: 10 mL of 1M Ca(NO₃)₂, 1 mL of KNO₃, 4 mL of 1M MgSO₄, 2 mL of 1M KH₂PO₄, and 2 mL of 1M FeEDTA. In the stress treatment group, PEG 6000 1% (w/v) was added to the medium to induce an initial plant response without causing severe stress, thus triggering an increase in resistance to subsequent osmotic stress (Arnon & Hoagland, 1944). A 1% PEG-6000 treatment was used to impose a mild osmotic stress corresponding to an estimated water potential of approximately −0.13 MPa at 25°C (Michel & Kaufmann, 1973; Chakraborty et al., 2022). During the growth process, the water in the medium was changed every two days to prevent fungal growth (Susilawati et al., 2022). Each rice cultivar was allowed to grow under normal conditions for three days before being subjected to drought stress. Initially, all plants showed normal growth. The osmotic stress treatment was then administered for four days, with plant conditions monitored periodically to ensure the mild-PEG osmotic stress had produced physiological effects, such as wilting or changes in leaf colour. After the treatment period, plants from both groups were harvested for further analysis. After the treatment, leaf tissue samples from the control and treatment plants were carefully taken using sterile scissors. Samples were taken from different plants to ensure biological variation, with five plants per group. Data on leaf and root length from each cultivar were then statistically evaluated using analysis of variance (ANOVA). Data were first tested for normality using the Shapiro-Wilk test, and if the data were normally distributed, then tested for homogeneity using Levene's test.

2.2.2. RNA Isolation: RNA isolation was performed using the TRIzol method. Rice plant leaves were ground using liquid nitrogen until they became powder. A total of 1 mL of TRIzol Reagent was added to a 1.5 mL Eppendorf tube containing the ground rice, then vortexed and left for 5 minutes at room temperature. After that, 0.2 mL of chloroform per 1 mL of

TRIzol was added, then the mixture was inverted for 15 seconds and incubated for 2-3 minutes at room temperature.

The mixture was then centrifuged at $12,000 \times g$ for 15 minutes at 4°C , forming three phases: the upper phase (containing RNA), the interphase (containing DNA), and the lower phase (containing protein). Carefully transfer the upper phase containing RNA to a new tube. Add 0.5 mL of isopropanol to the RNA solution, mix gently, and centrifuge again at $12,000 \times g$ for 10 minutes at 4°C . The supernatant was discarded, and a transparent RNA pellet was visible at the bottom of the tube. After that, 0.5 mL of 75% ethanol was added, incubated at room temperature for 10 minutes, and then centrifuged again at the same speed. After that, the supernatant was discarded, and the RNA pellet was dried for 5-10 minutes. The RNA was then dissolved by adding 20 μL of ddH₂O. The dissolution process was carried out using a tapping technique (gently tapping the tube) to prevent damage to the RNA. The RNA solution was then incubated in a water bath at $55-60^{\circ}\text{C}$ for 10 minutes to dissolve completely. After that, the RNA was ready to use or stored at -70°C for long-term storage.

RNA purity was analyzed using a Nanodrop spectrophotometer by measuring absorbance at a wavelength of 260 nm, and determined based on the A260/A280 ratio. The isolated RNA was tested using electrophoresis on a 1% agarose gel with 1x TBE buffer, run at 48 Volts for 30 minutes. The electrophoresis results were observed using a UV transilluminator (Rio et al., 2010).

2.2.3. cDNA Synthesis from Isolated RNA: The cDNA synthesis process begins with preparing an RNA template that has been confirmed free of DNA contamination through DNase I treatment. The RNA template is then mixed with an oligo(dT) primer, which serves as the primer for cDNA synthesis initiation. This mixture is heated at 65°C for 5 minutes, then cooled to 4°C to allow the primer to bind optimally to the RNA template. Next, the cDNA synthesis reaction is carried out by adding reaction buffer, dNTP mix, RNase inhibitor, and reverse transcriptase enzyme (ReverAid RT 200 U/ μL) to the RNA-primer mixture. The reaction is incubated at 42°C for 60 minutes to allow the reverse transcriptase enzyme to efficiently synthesize cDNA from the RNA template (Fendiyanto et al., 2021).

The reaction is then stopped by heating at 70°C for 5 minutes to inactivate the enzyme. Once synthesis is complete, the resulting cDNA can be diluted and used for PCR or RT-qPCR amplification. To avoid degradation, cDNA is stored at -20°C or -70°C for long-term storage. Verification of cDNA products is carried out through electrophoresis of PCR products using Actin gene primers and DREB4 gene primers, as listed in Table 1. The amplification process is carried out using a PCR machine, with a temperature setting of 95°C for 10 minutes, followed by 50 cycles.

Table 1: DREB4 (Os04g46400) and ACTIN (Os03g0718100) primers for gene expression studies

Primer	Sequence (5'-3')	Locus ID	Amplicon size
DREB_F	ATGGAAGCAGACGCGAGCCATA	Os04g46400	230 bp
DREB_R	GGCTACTCGGCCACAAGAGT		
Actin_F	GAAGGATGCCTATGTTGGTGA	Os03g0718100	260 bp
Actin_R	CTTCATAGATTGGCACGGTGT		

2.3. Data Observation and Analysis

RNA-seq data were processed using the Galaxy bioinformatics platform for transcript mapping. This analysis enabled the identification of genes involved in rice's response to drought stress, including DREB4 (GeneBank: AY196209, genome rice ID: LOC_Os04g46400, synonym OsDREB4-2) genes and other regulatory pathways. OsDREB4-2 (DREB4) was analyzed independently by qRT-PCR to assess its transcriptional response to mild PEG-induced osmotic stress. The qRT-PCR analysis was not intended as direct validation of the OsDREB2A transcript identified from the RNA-seq dataset.

We then analyzed RT-qPCR data to determine the relative expression levels of selected target genes. Relative expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Schmittgen & Livak, 2008; Fendiyanto et al., 2021; Sindiya et al., 2024), as follows:

$$DREB4 \text{ relative gene expression} = 2^{-\Delta\Delta\text{Ct}}$$

Where ΔCt represents the difference between the cycle threshold (Ct) of the target gene and that of the Actin reference gene, and $\Delta\Delta\text{Ct}$ represents the difference between the ΔCt of the treatment sample and the ΔCt of the control sample.

ACTIN or OsACT1 (Os03g0718100) was used as the reference gene for normalization of DREB4 transcript abundance. The OsACT1-specific primer pair, Actin-F (5'-GAAGGATGCCTATGTTGGTGA-3') and Actin-R (5'-CTTCATAGATTGGCACGGTGT-3'), generated an expected amplicon of 260 bp (Table 1). The target gene was DREB4 (Os04g46400), which was amplified using DREB4-F (5'-ATGGAAGCAGACGCGAGCCATA-3') and DREB4-R (5'-GGCTACTCGGCCACAAGAGT-3'), generating an expected amplicon of approximately 230 bp in the Inpago rice cultivars (Table 1).

Citation: Fendiyanto, M.H., Pratami, M.P., Cahyani, E.G., Kurniyanto, I.R., Setiawan, E., Fastanti, F.S., Syaiful, S.A., Baharuddin, A.K., & Anshori, M.F. (2026). Mild PEG-induced osmotic stress affects the regulation of the dehydration-responsive element-binding (DREB) family genes in rice cv. Inpago 6 and Inpago 9 based on transcriptome and gene expression analysis. *Agrobiological Records*, 26, 1-18. <https://doi.org/10.47278/journal.abr/2026.079>

2.4. In silico Transcriptomic Data Retrieval and RNA-seq Analysis

Publicly available RNA-seq data of (*Oryza sativa* L.) Japonica Group cv. Nipponbare were retrieved from NCBI BioProject PRJNA922103, comprising plants subjected to 8 h of drought treatment at 10 days after germination, with corresponding control samples. The dataset was generated using the Illumina MiSeq platform and consisted of 3 biological replicates per condition. Raw sequencing reads were analysed using the Galaxy platform Initial read quality was assessed using FastQC and summarized using MultiQC, followed by adapter and low-quality base removal using Cutadapt. The filtered reads were aligned to the *O. sativa* reference genome IRGSP-1.0 using RNA-STAR. The reference genome was obtained from the International Rice Genome Sequencing Project using the file IRGSP-1.0_genome.fasta.gz, together with IRGSP-1.0_representative_annotation_2024-07-12.tsv.gz. We assessed library strandedness using the Galaxy tool and incorporated the resulting strand information into the read-counting procedure. Gene-level read counts were generated using featureCounts with the corresponding IRGSP-1.0 annotation. The feature Counts strandedness parameter (-s) was set according to the library orientation determined by the Infer Experiment analysis, and read configuration was specified accordingly.

Differential gene expression analysis was performed using DESeq2 in Galaxy, with the experimental design specified as the treatment/ mild osmotic condition, where control and drought-stressed samples represented the two experimental conditions. DESeq2 normalized the raw gene-count matrix and modelled count data using a negative binomial generalized linear model. Gene-wise dispersion estimates were calculated and fitted to the mean-expression trend before statistical testing. Low-count genes were subjected to DESeq2 independent filtering to remove features with insufficient information for reliable differential testing. Differential expression was assessed using the Wald test, and the resulting *p*-values were adjusted for multiple testing using the Benjamini–Hochberg procedure to control the false discovery rate (FDR). Genes were considered significantly differentially expressed when they met both predefined criteria of adjusted *P*-value (*padj*) < 0.05 and $|\log_2$ fold change ($\log_2FC$) ≥ 2 . Genes with $\log_2FC \geq 2$ were classified as upregulated, whereas genes with $\log_2FC \leq -2$ were classified as downregulated. Principal component analysis (PCA), intersample-distance heatmap, dispersion estimation, *p*-value distribution, and MA-plot were subsequently generated to evaluate sample relationships, data structure, dispersion, and differential expression patterns.

3. RESULTS

3.1. Growth Responses of Inpago Rice Cultivars to Mild PEG-Induced Osmotic Stress

Rice plants were grown until 10 days after germination, after which half of the plants from each cultivar were subjected to 1% (w/v) polyethylene glycol (PEG) 6000 added to Hoagland's solution. Following 4 days of treatment, plants were evaluated for leaf and root growth. At 14 days after germination, visible differences in plant morphology were observed between the control and PEG-treated plants (Fig. 1). Inpago 6 and IR64 generally showed shorter leaves under PEG treatment than under control conditions, whereas Inpago 9 showed relatively similar leaf growth between the two treatments. Some IR64 plants did not survive the PEG treatment. Root responses also varied among cultivars, with differences in root length observed between treatments (Fig. 1).

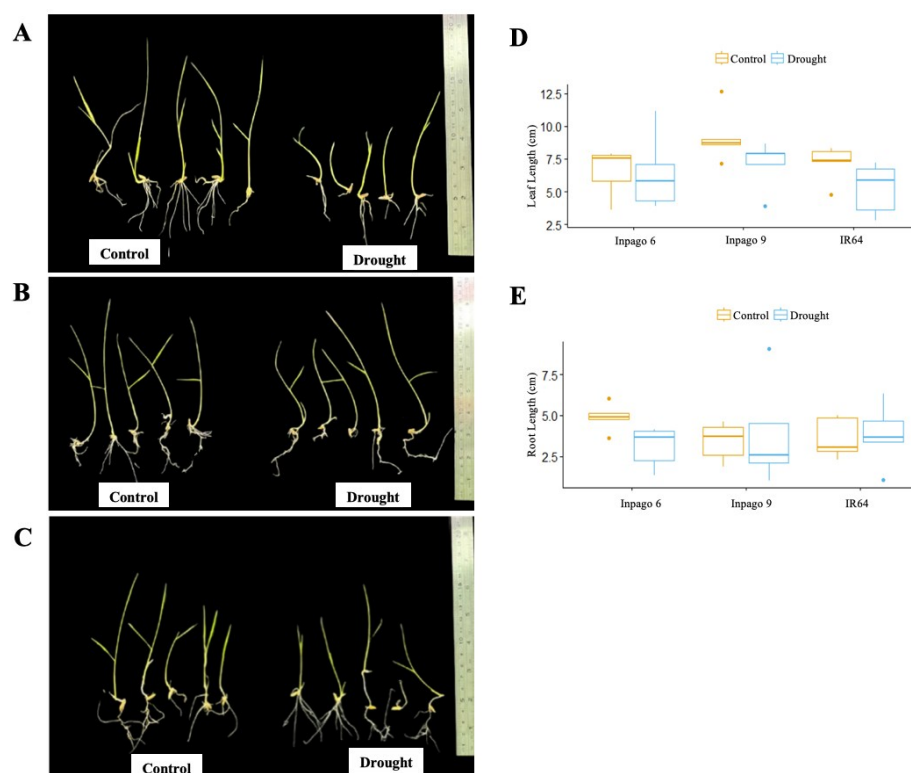
We then analyzed leaf and root length statistically. The Shapiro–Wilk test indicated no significant deviation from normality for root length (*P*=0.1522) or leaf length (*P*=0.1488). Levene's test also indicated homogeneous variances for leaf length (*P*=0.3719) and root length (*P*=0.2451). Therefore, the data met the assumptions for parametric analysis. ANOVA showed that neither treatment nor cultivar significantly affected leaf length (*P*>0.05). Similarly, treatment and cultivar had no significant effect on root length (*P*>0.05). Fig. 1D and Fig. 1E present the distributions of leaf and root length, respectively. Although some cultivars showed reduced median leaf or root length under PEG treatment, these differences were not statistically significant. Therefore, the observed morphological trends should not be interpreted as evidence of differences in drought tolerance among the cultivars.

3.2. Transcriptomic Analysis of Gene Expression Patterns in Response to Mild PEG-Induced Osmotic Stress

The transcriptomic data in this study were obtained from Bioproject PRJNA922103, which contains a gene expression dataset from *Oryza sativa* L. Japonica Group cv. Nipponbare plants subjected to 8 hours of drought stress at 10 days after germination. This bioProject was selected because it provided relevant RNA-Seq data for analyzing plant responses to water-limited environmental conditions. The sequencing instrument used was Illumina MiSeq. The dataset consisted of samples categorized into control and drought-stressed conditions, allowing for differential gene expression (DGE) analysis.

RNA-Seq data quality was assessed using FastQC and summarized using MultiQC. The analysis results showed that all samples had high-quality values, with an average Phred Score >30, GC content consistent with rice genome characteristics (~49-50%), and duplication levels within reasonable limits (37.9% - 47.1%). Based on these results, the sequencing data was suitable for further differential gene expression analysis (Conesa et al., 2016). After analyzing

the data quality, preprocessing was performed using Cutadapt to remove adapters or low-quality sequences. This process aims to ensure that only high-quality data is used in the analysis, resulting in more accurate and standardized results (Martin, 2011).



After trimming, we aligned reads to the rice reference genome using RNA-STAR. The reference genome used in this analysis was obtained from the International Rice Genome Sequencing Project (IRGSP), with the reference genome file IRGSP-1.0_genome.fasta.gz and the annotation IRGSP-1.0_representative_annotation_2024-07-12.tsv.gz. The alignment results showed a high mapping rate (>90%), indicating that the data was of good quality and suitable for further analysis. Read orientation validation analysis was performed using the Infer Experiment tool. The Infer Experiment results showed that the read orientation was predominantly distributed in one direction (++/--) or (+/-/+), depending on the sample, with values ranging from 0.31 to 0.76. This variation corresponds to the type of stranded library used (Appendix 2). No failures in determining orientation were found (0.0000 failed), so the data were deemed valid for expression quantification analysis.

Next, gene expression was quantified using Feature Counts, which counts the number of reads mapped to each gene in the dataset. These quantification results were then used in differential expression analysis with DESeq2, which aimed to identify genes differentially expressed between drought-stressed and control samples. The analysis revealed that 1,793 genes were identified as upregulated and 1,793 genes as downregulated, with a threshold of $|\log_2$ Fold Change ≥ 2 and an adjusted p-value (FDR) <0.05. We further analysed genes with significant expression changes to understand drought-stress response mechanisms in the studied rice cultivars. This analysis resulted in a gene expression dataset ready for further exploration of rice drought resistance mechanisms, including the identification of candidate genes with potential roles in regulating plant adaptation to environmental stress conditions.

Differentially Expressed Genes (DEG) analysis is a crucial step in transcriptomic studies to identify genes that experience significant changes in expression levels due to a specific treatment, in this case, drought stress (Table S1). This analysis generally uses a negative binomial distribution model to detect differences in gene expression between two groups: control and drought stress (Love et al., 2014). Before evaluating significantly differentially expressed genes, data quality analysis and global visualization of gene expression are performed to ensure the reliability of the results. Initial visualizations such as Principal Component Analysis (PCA) and heatmaps of intersample distances are used to assess data quality, intersample relationships, and verify separation between treatment groups. The results of this DEG analysis provide the basis for understanding the molecular response of rice plants to mild drought stress.

Table S1: Raw Ct values and relative expression calculations for the *DREB4* gene in rice cultivars Inpago 6, Inpago 9, and IR64 under control and drought stress treatments

Sample	Treatment	Biological replicate	Technical replicate	Ct (<i>DREB4</i>)	Ct (<i>Actin</i>)	ΔCt	Mean ΔCt	$\Delta\Delta Ct$	Relative expression ($2^{-\Delta\Delta Ct}$)
IP6	Control	UB1	UT1	20.16	20.14	0.02	—	—	—
IP6	Control	UB1	UT2	20.20	20.22	-0.02	—	—	—
IP6	Control	UB1	UT3	20.19	20.13	0.06	—	—	—
IP6	Control	UB1	UT4	20.15	20.13	0.02	—	—	—
IP6	Control	UB1	UT5	20.10	20.16	-0.06	—	—	—
IP6	Control	UB2	UT1	19.73	19.81	-0.08	—	—	—
IP6	Control	UB2	UT2	19.73	19.84	-0.11	—	—	—
IP6	Control	UB2	UT3	19.83	19.77	0.06	—	—	—
IP6	Control	UB2	UT4	19.77	19.82	-0.05	—	—	—
IP6	Control	UB2	UT5	19.75	19.77	-0.02	—	—	—
IP6	Control	UB3	UT1	20.06	19.97	0.09	—	—	—
IP6	Control	UB3	UT2	20.04	19.99	0.05	—	—	—
IP6	Control	UB3	UT3	20.12	19.99	0.13	—	—	—
IP6	Control	UB3	UT4	20.01	20.01	0.00	—	—	—
IP6	Control	UB3	UT5	20.00	20.04	-0.04	0.00	0.00	1.00
IP6	Drought	UB1	UT1	18.85	20.05	-1.20	—	—	—
IP6	Drought	UB1	UT2	18.83	20.02	-1.19	—	—	—
...	...	...	...	...	...	...	...	...	...
IP6	Drought	UB3	UT5	18.68	20.00	-1.32	-1.33	-1.33	2.51
IP9	Control	UB1	UT1	19.92	19.89	0.03	—	—	—
...	...	...	...	...	...	...	...	...	...
IP9	Control	UB3	UT5	19.89	19.91	-0.02	0.01	0.00	1.00
IP9	Drought	UB1	UT1	12.24	20.04	-7.80	—	—	—
...	...	...	...	...	...	...	...	...	...
IP9	Drought	UB3	UT5	11.89	19.89	-8.00	-7.97	-7.98	252.13
IR64	Control	UB1	UT1	20.11	20.09	0.02	—	—	—
...	...	...	...	...	...	...	...	...	...
IR64	Control	UB3	UT5	19.76	20.10	-0.34	-0.02	0.00	1.00
IR64	Drought	UB1	UT1	17.29	20.00	-2.71	—	—	—
...	...	...	...	...	...	...	...	...	...
IR64	Drought	UB3	UT5	16.99	19.98	-2.99	-2.92	-2.90	7.46

Ct, cycle threshold; $\Delta Ct = Ct (DREB4) - Ct (Actin)$; $\Delta\Delta Ct = \Delta Ct (drought) - \text{mean } \Delta Ct (control)$; relative expression = $2^{-\Delta\Delta Ct}$. UB, biological replicate; UT, technical replicate.

The first visualization performed is Principal Component Analysis (PCA), which aims to reduce the dimensionality of transcriptomic data and observe global variation in gene expression between samples. PCA results are displayed as a two-dimensional plot that maps each sample based on its first (PC1) and second (PC2) principal components. In this analysis, PC1 explained 95% of the total variation in the data, while PC2 accounted for 3% of the variation, indicating that most of the variation in gene expression between samples can be explained by the PC1 axis (Fig. 2A). This separation indicates significant changes in gene expression in response to the drought treatment.

The PCA plot shows a very clear separation between samples from the Control and Drought Stress groups. Control samples are located on the left side (negative PC1 values), while samples from the drought treatment group are clustered on the right side (positive PC1 values). This pattern indicates that the drought treatment has a consistent and significant effect on overall gene expression. Furthermore, the close clustering within each group indicates that the biological replicates within each condition have good consistency. These results are consistent with the literature stating that PCA can be used to identify key variables influencing gene expression and validate data quality and structure before further differential analysis (Pereira et al., 2018). Thus, PCA successfully demonstrated that the drought treatment has a significant transcriptomic effect distinct from the control condition.

After PCA visualization demonstrated a clear separation between the control and drought-stressed groups based on global gene expression, the analysis continued with a heatmap of intersample distances to evaluate the expression proximity between replicates within each group. The heatmap results showed that samples from the control and drought-stressed treatment groups formed two clearly separated clusters, reflecting that treatment conditions were the primary factor differentiating gene expression patterns (Fig. 2B). The distances between samples within each group were relatively small, indicating a high degree of consistency between replicates. Conversely, the distances between samples from the control and treatment groups were quite large, indicating significant changes in gene expression patterns due to drought stress.

The heatmap visualization of intersample distances (Fig. 2B) shows the pattern of gene expression proximity between rice plants under control and mild drought stress conditions. Based on the distance analysis, samples from the drought-stressed group formed a dense cluster with a low interreplicate distance, indicated by the dark blue color on the diagonal of the cluster. This indicates that replicates in the drought-stressed group exhibit consistent

gene expression patterns. In contrast, samples from the control group showed greater variation between replicates, reflected by the lighter color (white to light blue) in the interaction areas between control samples. This indicates a relatively larger distance between controls samples compared to the drought-stressed group, although still within reasonable limits.

Furthermore, the distance between biological replicates within a single group (both control and drought-stressed) showed good uniformity, indicating consistent data quality and low technical variation between replicates. Meanwhile, the higher distance values between the control and drought-stressed groups strengthen evidence that the drought treatment affected changes in gene expression patterns. These results support the sustainability of the differential gene expression (DEG) analysis, as the data structure showed cluster separation consistent with the treatment design, and no outliers were found among the samples.

DESeq2 models RNA-Seq count data using a Negative Binomial (NB) distribution with mean μ and dispersion parameter α (Love et al., 2014). With NB, variance is calculated as $\text{var} = \mu + \alpha \cdot \mu^2$, so that genes with relatively low expression have high variance (dispersion), and genes with high expression are relatively stable. DESeq2 estimates dispersion per gene separately (gene-wise), then fits a dispersion trend curve to the mean expression (Love et al., 2014). A dispersion estimation plot is then generated, showing the estimated dispersion for each gene (black dots) and the fitted/predicted dispersion value (red curve).

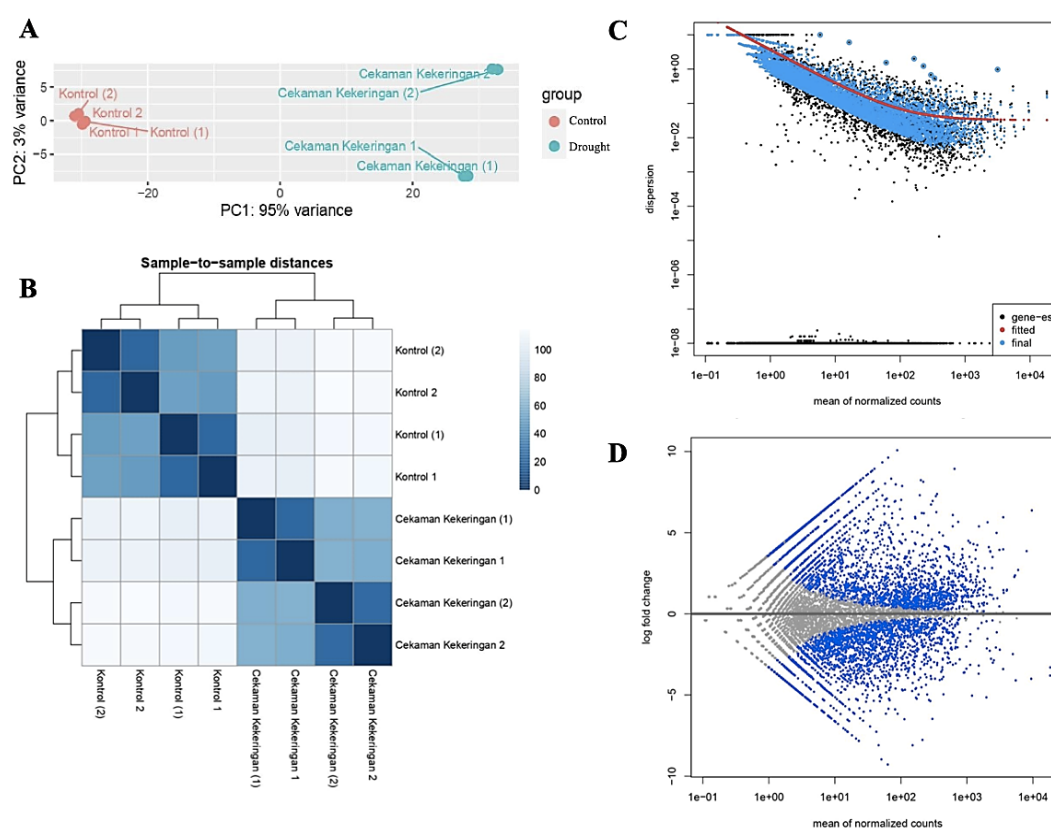


Fig. 2: Clustering study, quality assessment, and differential gene expression analysis. Principal Component Analysis (PCA) plot showing the global expression profile of rice (*Oryza sativa* L.) transcriptomes under control and osmotic stress treatment conditions (n=2 biological replicates per group; total n=4 samples) (A). Heatmap of sample-to-sample Euclidean distances calculated from variance-stabilized transformation (VST) data across all 6 samples, where colour intensity reflects pairwise similarity (darker blue indicates higher correlation) (B). Gene dispersion estimates across mean normalized counts modelled using a negative binomial distribution in DESeq2 (black dots represent per-gene estimates, red line indicates fitted trend, and blue dots represent final shrunken dispersion values) (C). MA plot illustrating log₂ fold change versus mean of normalized counts for all evaluated genes significantly differentially expressed genes (DEGs) are highlighted in red (adjusted p-value<0.05 and log₂ FC>2.0), while non-significant genes are shown in gray (D). Abbreviations: PCA, Principal Component Analysis; DEG, Differentially Expressed Gene; MA, Minus by Add; FC, Fold Change; FDR, False Discovery Rate.

Based on the resulting plot (Fig. 2C), most genes are distributed around the fitted curve (red), indicating that the NB model fits the data well (Petrany et al., 2024). According to Nowicka & Robinson (2016), a characteristic of good RNA-Seq is a downward trend between mean expression and dispersion. Genes with low expression have relatively high dispersion, while genes with high expression have low dispersion. The negative mean-dispersion pattern in the

plot (Fig. 2C) indicates that the RNA-Seq data have been well normalized and fit the assumptions of the NB model, and the statistical model fits the data.

Conversely, the distribution of p-values from the DESeq2 differential gene expression analysis between drought stress and control treatments shows a high-frequency peak near $p=0$ (Fig. 2D). This pattern is consistent with the expectation that p-value histograms in high-resolution experiments will be a combination of a uniform distribution (for genes with no differential expression) and a distribution dominated by low p-values (for genes with significant differential expression) (Ereful et al., 2021). A peak near 0 indicates that most genes reject the null hypothesis (many genes differ between conditions) (Ereful et al., 2021). Thus, the observed p-histogram pattern (a clustering of low p-values) adds confidence that the DESeq2 analysis results are valid and reflect a real biological signal, not a statistical artifact. The DESeq2 method itself models count data using a negative binomial GLM and performs a Wald test to obtain p-values, which are then adjusted (FDR adjustment) using the Benjamini–Hochberg procedure (Love et al., 2014). A total of 1,793 genes were upregulated and 1,793 genes were downregulated (a total of 3,586 genes significant with adjusted $P<0.05$ and $\log_2FC>2$), which was found to be consistent with the p-value distribution of a real biological effect under drought stress conditions.

In the MA plot, the X-axis represents the mean expression (mean normalized counts) and the Y-axis represents the $\log_2$ fold change (LFC) between the drought stress and control groups (Love et al., 2014). Colored gene dots (e.g., blue) indicate significant genes ($p_{adj}<0.1$ or other threshold). A general characteristic of the MA plot is symmetry of the dots around the zero horizontal line (no change in expression), with some genes scattered above/below it for positive/negative $\log_2FC$. The $\log_2FC$ value itself indicates the magnitude of the biological effect; for example, a $\log_2FC$ of 1.5 represents an approximately $2^{1.5}$ -fold increase in expression under stress conditions (Wang et al., 2025).

Biologically, genes that are differentially expressed under drought stress play a role in the water stress response mechanism. Many studies have reported that under drought stress conditions, high gene expression is involved in the accumulation of osmoregulatory substances (e.g., proline and soluble sugars) for antioxidant defence in dry plants. The accumulation of proline and sugars is a crucial strategy for resisting cellular osmotic stress during drought. Antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD) increase to mitigate oxidative damage, reflecting the activation of cellular defense genes (Wang et al., 2025). Conversely, photosynthesis and primary metabolism genes are often repressed (negative $\log_2FC$) during drought (Ma et al., 2022). Metabolites sometimes correlate to the gene expression in other plants like *Jatropha* (Fendiyanto et al., 2024). Thus, the results of this analysis demonstrate an expression profile consistent with plant adaptive mechanisms to drought. DEG identification revealed 1,793 genes with increased expression and 1,793 with decreased expression ($p_{adj}<0.05$ and $\log_2FC>2$). This knowledge is important for understanding specific molecular pathways and can serve as a starting point for further functional analysis, such as gene annotation or co-expression module analysis in the context of drought tolerance.

Analysis of various plots demonstrated clear differences in gene expression between control and drought-stressed samples. The heat map indicates clustering that differentiates the two conditions, while the dispersion estimation plot demonstrates the stability of the data for differential analysis. The histogram of p-values and MA-plot revealed that most genes did not experience significant changes, but several genes showed significant upregulation or downregulation. These results confirm that drought stress affects the expression of specific genes, which could be the focus of further research in understanding plant adaptation mechanisms.

In this study, differential gene expression data was visualized using a volcano plot, a common graphical representation method in RNA-seq analysis. Volcano plots map the relationship between statistical significance (p-value) and the magnitude of gene expression change ($\log_2$ fold change) (O'Connell, 2025). This approach allows genes experiencing the most significant changes in expression, both biologically and statistically, to be visually identified more easily. Significantly upregulated genes are displayed on the right side of the graph, while significantly downregulated genes are displayed on the left side. Genes with the highest level of statistical significance are displayed at the top (O'Connell, 2025).

The volcano plot results in this study were obtained from differential expression data analysis using the DESeq2 package (Fig. 3), which has proven reliable in identifying genes involved in responses to environmental stresses, including drought stress (Love et al., 2014). One of the genes that appeared in the significantly up-expressed group was the *DREB* (Dehydration Responsive Element Binding) gene, which is known to function as an important transcription factor in the regulation of gene expression related to abiotic stress, including drought.

In this analysis, several genes from the *DREB* (Dehydration Responsive Element Binding) family were successfully detected in the differential expression results (Fig. 3C), including the *Os01t0165000-01/02* and *Os08t0545500-01* genes. The *Os01t0165000-01/02* gene showed a very high expression level with a $\log_2$ fold change ($\log_2FC$) value of 21.03 and an adjusted p-value (p_{adj}) of 2.52×10^{-7} ($P<0.05$). This value shows very strong

significance, indicating that the expression of this gene experienced a dramatic increase under drought stress conditions. This gene is suspected to be a homolog of *OsDREB2A*, which is known to be an important transcription factor in the regulation of abiotic stress response genes, including drought (Yavuz, 2025).

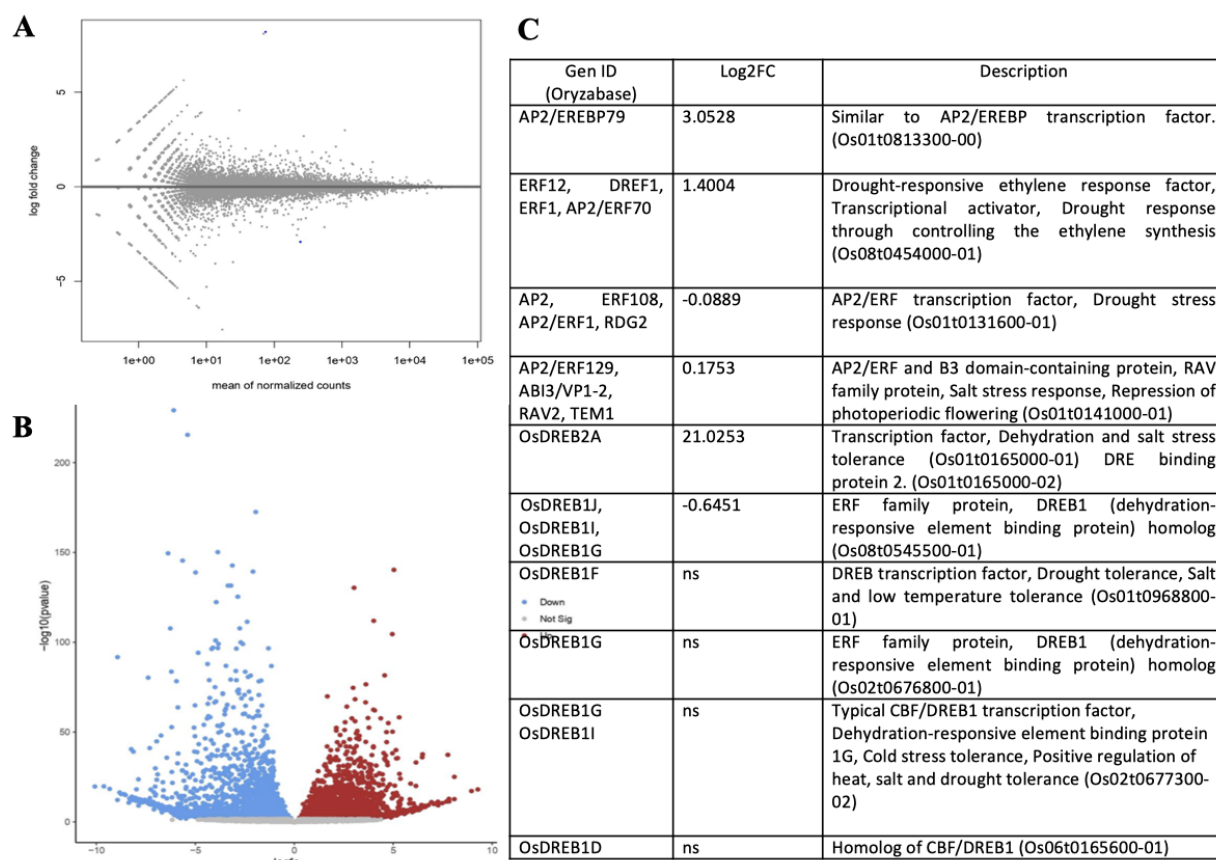


Fig. 3: Significant expression levels of *DREB* genes based on transcriptome studies. MA plot of Differential Gene Expression Analysis (A). Volcano plot of Differential Gene Expression Analysis (DEGs) with $\log_2FC > 2$ and $P\text{-adj} < 0.05$ between Drought Stress and Control Groups (B). Gene List of Differentially Expressed Genes (DEGs) Analysis Results using DESeq2 in the Galaxy Application (C).

In contrast, the *Os08t0545500-01* gene had a $\log_2FC$ value of -0.65 and a padj value of 0.772. These values are well above the general significance threshold ($P < 0.05$), so this gene was not categorized as significantly differentially expressed. In other words, the expression of the *Os08t0545500-01* gene showed no significant changes between control and drought stress conditions. In addition to these two genes, several other *DREB* genes were also detected in the dataset, but had a base Mean value of 0, meaning there were no gene expression readings in either the control or stress conditions. This suggests that these genes are likely not expressed in the tissue or sampling time used in this study, or are below the detection limit of the sequencing technology used. Overall, these findings support the role of the *Os01t0165000* gene as a strong candidate in the molecular response of rice plants to drought stress, and strengthen our understanding of the role of *DREB* transcription factors in abiotic stress tolerance. *DREB2* (Dehydration-Responsive Element-Binding Protein 2) is a member of the AP2/ERF family of transcription factors that plays a key role in regulating plant responses to drought stress. Activation of *DREB2* expression triggers a stress protection pathway by binding to the DRE/C-repeat element in the promoter region of target genes, which subsequently induces the expression of various stress response genes such as LEA (Late Embryogenesis Abundant) genes, dehydrins, starch-degrading proteins, and HSP (Heat Shock Proteins). These proteins function to protect cell structure and maintain plant physiological stability under water deficit conditions (Kumar et al., 2024).

Increased expression of *OsDREB2A*, thought to be represented by the *Os01t0165000* gene in this study, may increase the accumulation of protective proteins and osmolytes, which play a role in maintaining turgor and cellular stability during stress. Previous research has shown that transgenic rice plants overexpressing *OsDREB2A* have a higher survival rate under drought stress than control plants, confirming the important role of this gene in adaptation to abiotic stress (Cui et al., 2011; Berchembrock et al., 2022). The very high $\log_2FC$ value indicates dramatically

increased expression under drought stress conditions. This strengthens the hypothesis that increased *DREB2* expression, particularly *OsDREB2A*, is positively correlated with increased rice plant drought tolerance.

Based on the literature, the *DREB* (Dehydration-Responsive Element-Binding) gene family is divided into two main subgroups, *DREB1/CBF* and *DREB2*, which are regulated by distinct signalling pathways. The *DREB1/CBF* genes are predominantly induced by low temperatures (cold stress), while *DREB2* is activated by drought, high salinity, and high temperatures (heat stress) (Ma et al., 2016). Thus, *DREB1* is generally associated with the response to cold stress, while *DREB2* plays a key role in drought adaptation mechanisms. This finding aligns with the analysis in this study, where 8 hours of drought stress significantly increased *DREB2* gene expression, particularly *OsDREB2A* (*Os01t0165000*), while the *DREB1/CBF* gene showed no significant expression changes. This reinforces the interpretation that the observed regulation of *DREB* expression is directly related to drought tolerance mechanisms, and not a consequence of cold exposure. The literature also reports that overexpression of *DREB1* or *DREB2* genes can enhance plant resistance to various abiotic stresses. However, this increase is often accompanied by phenotypic side effects, such as stunted plant growth (Ma et al., 2016).

The RNA-seq analysis identified *OsDREB2A* (*Os01t0165000*) as a differentially expressed *DREB*-family transcription factor. Because *DREB* transcription factors are important regulators of plant responses to abiotic stress, *DREB4* (*OsDREB4-2*) was subsequently selected for independent qRT-PCR analysis. Among the *DREB*-family genes identified in the transcriptomic dataset, *OsDREB2A* (*Os01t0165000*) was detected as a differentially expressed transcript under drought stress. In contrast, *DREB4/OsDREB4-2* (*Os04g46400*) was not identified as a significant DEG under the predefined RNA-seq criteria. Therefore, *DREB4* was subsequently evaluated independently using qRT-PCR in the Inpago cultivars rather than being described as a transcriptomic DEG.

3.3. *DREB4* Gene Expression under Mild PEG-Induced Osmotic Stress

The qRT-PCR analysis showed that *OsDREB4-2* expression differed between the evaluated rice cultivars under mild PEG-induced osmotic stress. To analyze gene expression, isolates verified to have the target gene were subjected to qRT-PCR using a pair of primers specific for the *Actin* gene and the target gene *DREB4*. The qRT-PCR results are depicted as an amplification plot. For the housekeeping *Actin* gene, the curves in all three test samples (Inpago 6, Inpago 9, and IR64) reached the detection threshold (threshold cycle or Ct) at around 20–25 cycles, indicating relatively high and consistent expression. This indicates that *Actin* gene expression is stable and unaffected by drought stress.

Meanwhile, the amplification results for the target gene (*DREB4*) reflected varying levels of expression (Fig. 3). In some samples, the amplification curves crossed the detection threshold early (Ct around 28–32), indicating induction of gene expression. Conversely, several other curves did not reach the detection threshold, indicating very low or undetectable expression (control condition). The *DREB4* gene showed low expression in the control and was strongly induced by drought treatment, which can be quantified using the $\Delta\Delta C_t$ method.

Quantitative calculation of qRT-PCR results using the $\Delta\Delta C_t$ method, with the reference gene *ACTIN*, is shown in a bar chart (Fig. 4). In the Inpago 6 cultivar under normal, non-stress conditions, *DREB4* gene expression was relatively low. A high Ct indicates minimal transcriptional activity of the *DREB4* gene under control conditions. An increase then occurred after drought treatment, with *DREB4* gene expression increasing approximately 2.5-fold (in the Inpago 6) compared to the control (Fig. 4). This indicates that *DREB4* is induced by drought stress, signifying the activation of the molecular response pathway to dehydration (Lata & Prasad, 2011).

In Inpago 9, *DREB4* gene expression was very low under control conditions. Expression then increased 250-fold change after drought treatment compared to the control. This is the highest expression among the three cultivars, indicating that Inpago 9 has a highly responsive molecular mechanism to drought through the *DREB4* regulatory pathway. On the other hand, the IR64 cultivar also showed very low *DREB4* expression under normal conditions. The high Ct in the amplification plot supports that this gene is almost inactive in the absence of environmental stress. After drought stress, *DREB4* expression in IR64 increased drastically to 7.5-fold compared to the control. This increase was the highest of all cultivars, indicating a strong genetic induction ability of *DREB4* when facing drought (Susanto et al., 2022).

4. DISCUSSION

Drought stress induces complex molecular responses in rice involving changes in water status, osmotic adjustment, oxidative stress regulation, and transcriptional control (Khan et al., 2021; Bhandari et al., 2023). Among the transcription factors associated with abiotic stress responses, the *DREB/AP2-ERF* family has an important regulatory role because *DREB* proteins recognize dehydration-responsive elements in the promoters of stress-responsive genes. However, members of the *DREB* family are not functionally interchangeable, and individual genes may exhibit distinct expression patterns depending on genotype, stress intensity, developmental stage, and duration

of treatment. Therefore, the identification of a *DREB*-related transcript in the public RNA-seq dataset and the subsequent analysis of another *DREB* family member by qRT-PCR should be interpreted as complementary observations rather than evidence that both analyses represent the same molecular event.

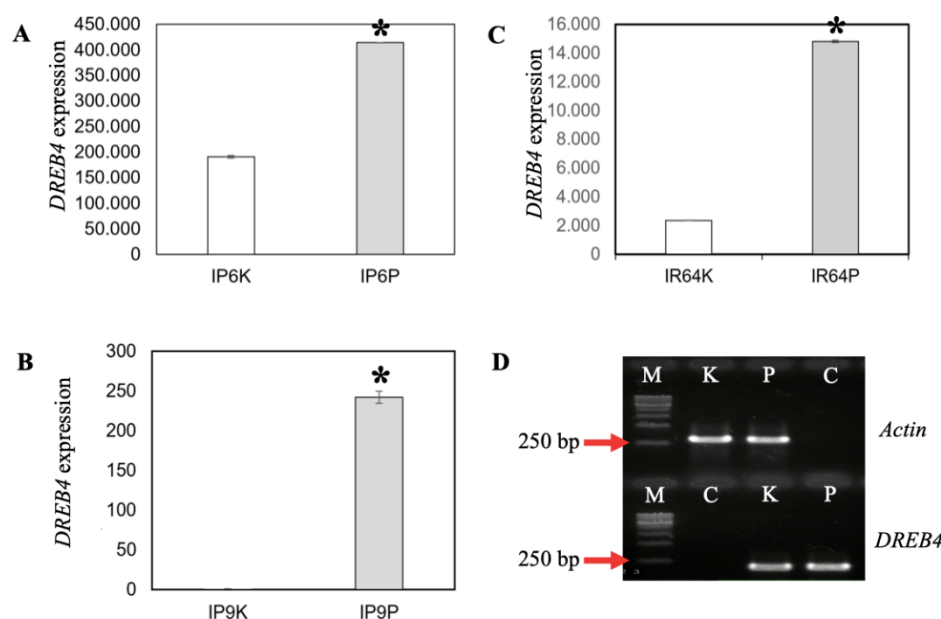


Fig. 4: Gene expression of *DREB4* in rice cv. Inpago 6 (A), Inpago 9 (B) and IR64 (C) between control and drought stress treatment. Electrophoregram of RT-PCR of *DREB4* gene of rice in control and drought stress treatment (D). Housekeeping gene: *Actin* as reference gene. M= marker 1kb, K= control and P= drought treatment, C= ddH₂O or non-template cDNA. Asterisk (*) represents statistically significant difference based on Welch-T test (p-value < 0.05) (Supplementary I for raw data).

In the present study, re-analysis of the public RNA-seq dataset from rice cv. Nipponbare subjected to short-term drought stress identified *OsDREB2A* (*Os01t0165000*) as a differentially expressed transcript. The strong transcriptional response of *OsDREB2A* is biologically consistent with its established involvement in abiotic stress signaling. *DREB2*-type transcription factors are generally associated with responses to dehydration and other osmotic stresses and can regulate downstream genes involved in cellular protection and water-stress responses. Previous studies have also demonstrated that enhanced expression of *OsDREB2A* can improve the ability of rice plants to withstand water limitation, supporting its importance as a candidate regulator of drought-responsive pathways (Yuliza et al., 2024; Chrisnawati et al., 2022). Nevertheless, the high expression of *OsDREB2A* observed in the present RNA-seq dataset should be interpreted as evidence of transcriptional responsiveness rather than direct evidence of increased drought tolerance, because transcript abundance alone cannot establish physiological or agronomic tolerance.

Dehydration-Responsive Element Binding (DREB) genes were initially identified in *Arabidopsis thaliana*. The *AtDREB1* and *AtDREB2* genes have been demonstrated to enhance plant tolerance to drought and low temperatures (Sakuma et al., 2002). *DREBs* are part of the AP2/ERF superfamily and contain highly conserved AP2/ERF domains, which facilitate their interaction with the DRE/CRT cis-elements in target gene promoters (Huang et al., 2020). In numerous plant species, including rice, *DREBs* are instrumental in regulating the expression of genes involved in abiotic stress defence mechanisms (Sakuma et al., 2002). *DREB* genes are pivotal in enabling plants to manage stress independently of ABA (Lata & Prasad, 2011). These genes contribute to the expression of various stress-responsive genes. The initial cDNAs isolated for DRE-binding proteins, specifically CBF1, *DREB1A*, and *DREB2A*, were discovered through a yeast one-hybrid screening method from *Arabidopsis*. Subsequently, numerous other *DREB* genes have been isolated from various plant species. These proteins possess the specific capability to bind DRE sequences, thereby triggering the expression of associated genes (Lata & Prasad, 2011).

Transcriptomics is a scientific discipline focused on the study of transcriptomes, which are the complete set of RNA molecules transcribed from the genome of a specific organism or cell, along with their expression levels. Research in transcriptomics entails the analysis of gene expression patterns through the detection and quantification of RNA transcripts (Shahid, 2024). RNA sequencing (RNA-Seq) is a widely employed technique in this field, involving the sequencing of RNA molecules to achieve a comprehensive and precise representation of the transcriptome. This technology facilitates the measurement of gene expression levels and aids in identifying variations in gene expression across different samples or conditions. The analysis of transcriptomics data necessitates the application of various computational and statistical methodologies. This analytical process encompasses read sequence alignment, transcript assembly, quantification of gene expression levels, differential expression analysis, and functional annotation. Bioinformatics tools and databases are indispensable for managing and analyzing the

substantial volumes of data generated from transcriptomics experiments (Shahid, 2024).

Transcriptomics holds significant potential in the realms of biological and medical research. Within the agricultural sector, transcriptomics is employed to elucidate the mechanisms underlying plant tolerance to abiotic stresses, such as drought and salinity. By examining alterations in gene expression under stress conditions, researchers can pinpoint key genes implicated in plant adaptive responses (Xia et al. 2020; Zargar et al. 2022). Additionally, transcriptomics facilitates the discovery of potential disease biomarkers, the identification of novel metabolic pathways, and the comprehension of complex gene-environment interactions (Huang et al., 2020; Xia et al. 2020; Zargar et al. 2022).

DREB genes are categorized into several subgroups based on their structural and functional attributes, encompassing six primary groups (A1–A6) or four clades (I–IV) (Chai et al., 2020). A prominent example is *OsDREB4*, a member of the rice *DREB* gene family. This gene, comprising two homologs, *OsDREB4-1* and *OsDREB4-2*, modulates responses to drought and salinity stress and functions as a transactivating factor in stress signalling pathways (Tian et al., 2005). Both genes encode proteins with a conserved AP2 domain, which binds to DRE elements in DNA. They also possess an N-terminal region that potentially serves as a nuclear localization signal (NLS) and a C-terminal region that may act as a transcriptional activation domain. The *OsDREB4* gene plays a crucial role in regulating plant responses to abiotic stress, particularly stress induced by drought and salinity (Tian et al., 2005).

Interestingly, *OsDREB2A* was identified from the transcriptomic analysis, whereas *OsDREB4-2* was evaluated independently by qRT-PCR. The differential response of these two *DREB*-family members may indicate that drought-responsive regulation in rice involves multiple *DREB* transcription factors with potentially distinct expression patterns or regulatory functions. However, because *OsDREB4-2* was not identified as a significant DEG in the analyzed RNA-seq dataset, its qRT-PCR expression pattern should not be interpreted as transcriptomic validation of *OsDREB2A*. Instead, the result provides complementary evidence that members of the *DREB* family may respond differently to mild osmotic stress.

Most plants are inherently immobile and thus incapable of evading adverse environmental changes. Consequently, they have evolved physiological mechanisms to recognize and adapt to such conditions (Batool et al., 2025). A notable form of this adaptation is the response to drought stress, which can be simulated in vitro using polyethylene glycol (PEG). PEG serves as an osmotic agent that reduces the water potential in the root environment without being absorbed by plant tissues, thereby effectively replicating drought-like conditions in a laboratory setting (Sobahan et al., 2022). The application of PEG at low concentrations, such as 1% (w/v), can induce mild osmotic stress, sufficient to trigger initial stress signals in plants (Sobahan et al., 2022). This response involves the activation of specific genes, such as *DREB*, which are crucial transcription factors in regulating the expression of protective genes against water stress. In this early activation phase, the plant prepares its internal defense mechanisms to withstand hostile environmental conditions (Sobahan et al., 2022; Zaffar et al., 2025).

The qRT-PCR results demonstrated differences in *OsDREB4-2* expression among Inpago 6, Inpago 9, and IR64 following 1% PEG-6000 treatment. The magnitude of induction differed among the cultivars, indicating that the transcriptional response to mild osmotic stress was genotype-dependent. Inpago 9 showed a stronger relative induction than Inpago 6, whereas IR64 exhibited a different response pattern. Such differences may reflect variation in upstream stress perception, signal transduction, transcription-factor regulation, or basal expression levels among rice genotypes. In particular, very low transcript abundance under control conditions can result in a large relative fold change after treatment; therefore, a high fold-change value should not automatically be interpreted as evidence of superior drought tolerance. The biological meaning of the response is better evaluated by considering the absolute or normalized expression levels, replicate variability, statistical significance, and corresponding physiological phenotypes.

The differences among the three cultivars are particularly relevant because Inpago 6 and Inpago 9 were developed for cultivation under environments with water limitations, whereas IR64 is commonly used as a high-yielding rice cultivar but is generally considered more sensitive to drought than drought-adapted cultivars. The relatively strong *OsDREB4-2* induction observed in Inpago 9 may indicate a greater transcriptional responsiveness to mild osmotic stress. However, transcriptional responsiveness and drought tolerance are not equivalent characteristics (Melandri et al., 2021). A genotype may strongly induce a stress-responsive gene without necessarily maintaining growth, water status, or yield under prolonged drought (Xia et al., 2020). Conversely, a tolerant genotype may maintain relatively stable gene expression because its constitutive physiological and molecular mechanisms already provide protection. Thus, the present results support differences in molecular response, but they do not by themselves establish that Inpago 9 has greater whole-plant drought tolerance than Inpago 6 or IR64.

The growth observations provide additional context for interpreting the molecular data. Although differences in leaf and root length were observed visually among treatments and cultivars, the statistical analysis did not detect

significant effects of drought treatment or cultivar on these traits. Consequently, the observed morphological tendencies should not be interpreted as statistically demonstrated differences in drought tolerance. The combination of relatively limited morphological responses and differential *DREB4* expression suggests that the molecular response may occur before substantial changes in growth become statistically detectable. Mild osmotic stress can activate early signaling pathways without necessarily producing large phenotypic effects during a short experimental period (Susilawati et al., 2022; Chakraborty et al., 2022). This interpretation is consistent with the concept that transcriptional regulation is one of the early components of plant stress responses. However, longer stress exposure and additional physiological measurements would be required to determine whether the observed transcriptional responses contribute to sustained drought adaptation.

The use of 1% PEG-6000 in the present experiment is also important when interpreting the expression data. PEG-6000 is commonly used experimentally to reduce the water potential of the growth medium and generate controlled osmotic stress without the physical complications associated with soil drying. At a relatively low concentration, PEG treatment can impose a mild osmotic challenge and activate early stress-responsive signalling (Susilawati et al., 2022; Chakraborty et al., 2022). Therefore, the response observed in this study is more appropriately described as a response to mild PEG-induced osmotic stress rather than as a direct simulation of field drought. This distinction is important because drought in soil involves several interacting processes, including progressive soil-water depletion, changes in root-soil interactions, altered nutrient availability, and changes in atmospheric demand. Accordingly, the molecular responses observed under PEG treatment provide a controlled model of osmotic stress but cannot fully reproduce the complexity of field drought (Susilawati et al., 2022; Chakraborty et al., 2022).

An important strength of the present study is the integration of public transcriptomic data with cultivar-level qRT-PCR analysis. The RNA-seq analysis provided an unbiased overview of transcriptional changes under short-term drought stress and identified *OsDREB2A* as a responsive *DREB*-family candidate. The qRT-PCR experiment subsequently examined *OsDREB4-2* in three rice cultivars with different genetic backgrounds. This combination provides complementary information on *DREB*-family regulation (Zha et al., 2023) and highlights the possibility that different *DREB* genes may contribute differently to osmotic-stress responses. However, these datasets cannot be regarded as a direct within-genotype validation because the public RNA-seq dataset was generated from Nipponbare, whereas the qRT-PCR experiment used Inpago 6, Inpago 9, and IR64. Differences in genotype, experimental conditions, stress duration, and molecular background may contribute to the differences between the two datasets. Therefore, the RNA-seq and qRT-PCR findings should be considered supportive evidence at the level of the *DREB* stress-response network rather than direct validation of one another.

Several limitations should be considered when interpreting these findings. First, the RNA-seq dataset was derived from a public experiment using Nipponbare and therefore does not directly represent the transcriptome of the three cultivars evaluated by qRT-PCR. Second, the qRT-PCR analysis focused on *OsDREB4-2*, which was not identified as a significant DEG in the RNA-seq analysis; consequently, its expression pattern represents an independent candidate-gene analysis rather than transcriptomic validation of *OsDREB2A*. Third, the use of mild PEG-induced osmotic stress for a relatively short period provides information about an early molecular response but does not establish long-term drought tolerance (Melandri et al., 2021). Finally, gene expression alone does not demonstrate downstream protein activity or whole-plant performance. Future studies should therefore integrate cultivar-specific RNA-seq, physiological measurements, protein-level analyses, and longer-duration drought experiments to determine whether differential *DREB* expression is functionally associated with drought adaptation (Xia et al., 2020; Zargar et al., 2022).

RNA-seq was performed to identify differentially expressed genes associated with the response to PEG-induced osmotic stress. *OsDREB2A* (*Os01t0165000*) was identified among the drought-responsive transcripts. Because *DREB* transcription factors constitute an important regulatory family involved in abiotic stress responses, *OsDREB4-2* was subsequently selected as an additional candidate for independent qRT-PCR analysis. The stronger induction of *DREB4* in Inpago 9 indicates a greater transcriptional responsiveness to the imposed mild osmotic stress, but this response alone does not establish superior whole-plant drought tolerance.

5. CONCLUSION

In conclusion, re-analysis of public RNA-Seq data identified several *DREB* family genes responsive to mild PEG-6000-induced osmotic stress, with *OsDREB2A* (*Os01t0165000*) showing the strongest transcriptomic response ($\log_2FC=21.03$). Independent qRT-PCR analysis further showed that *OsDREB4-2* expression increased under mild osmotic stress in Inpago 6, Inpago 9, and IR64, with approximately 2.5-fold, 250-fold, and 7.5-fold increases, respectively, relative to their corresponding controls. These findings indicate that *OsDREB4-2* is responsive to mild osmotic stress and may represent a candidate gene for further investigation of rice stress-response mechanisms; however, the present data do not establish a causal role of *OsDREB4-2* in whole-plant drought tolerance. The

differential expression observed among cultivars suggests potential variation in molecular responsiveness, but further validation using physiological traits, additional genotypes, and functional analyses is required before *OsDREB4-2* can be considered a reliable candidate marker for drought-resilient rice breeding.

Declarations

Funding: This research was funded by the Indonesian Endowment Fund for Education (LPDP/Lembaga Pengelola Dana Pendidikan) and the National Research and Innovation Agency (BRIN/ Badan Riset dan Inovasi Nasional), through number 152/IV/KS/11/2023, for their financial support of the Riset dan Inovasi untuk Indonesia Maju Gelombang 4 (RIIM 4) research, particularly for the Fiscal Year (FY) 2023-2026 grant PI Miftahul Huda Fendiyanto (number: 37/II.7/HK/2023 and 92/II.7/HK/2025). In addition, this research was supported by the Ongoing Research Funding Program (ORF-2026-751), King Saud University, Riyadh, Saudi Arabia.

Acknowledgement: This research was fully supported by the Indonesian Endowment Fund for Education (LPDP/Lembaga Pengelola Dana Pendidikan) and the National Research and Innovation Agency (BRIN/ Badan Riset dan Inovasi Nasional), through number 152/IV/KS/11/2023, for their financial support of the Riset dan Inovasi untuk Indonesia Maju Gelombang 4 (RIIM 4) research, particularly for the Fiscal Year (FY) 2023-2026 grant PI Miftahul Huda Fendiyanto (contract number: 37/II.7/HK/2023 and 92/II.7/HK/2025). The authors also acknowledge the Ongoing Research Funding Program (ORF-2026-751), King Saud University, Riyadh, Saudi Arabia.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study

Data Availability: The datasets analyzed during the current study are available at <https://doi.org/10.5281/zenodo.19642846>, including RNA-seq data processing and gene expression data, including p-values, gel electrophoresis, and amplification plots from qRT-PCR. The RNA-Seq data used in this study are RNA data with accession number PRJNA922103, available on NCBI.

Ethics Statement: Not Applicable.

Author's Contributions: MHF: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Software, Visualization, Writing – original draft, Writing – review & editing, MPP: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing, EGC: Methodology, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing, IRK: Methodology, Formal analysis, Writing – original draft, Writing – review & editing, ES: Writing – review & editing, FSF: Writing – review & editing, AKB: Writing – review & editing, MFA: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Generative AI Statements: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

Publisher's Note: All claims stated in this article are exclusively those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated/assessed in this article or claimed by its manufacturer is not guaranteed or endorsed by the publisher/editors.

REFERENCES

- Arnon, D. I., & Hoagland, D. R. (1944). The investigation of plant nutrition by artificial culture methods. *Biological Reviews*, 19(2), 55–67. <https://doi.org/10.1111/j.1469-185X.1944.tb00302.x>
- Aslam, M. M., Rashid, M. A. R., Siddiqui, M. A., Khan, M. T., Farhat, F., Yasmeen, S., Khan, I. A., Raja, S., Rasool, F., Sial, M. A., & Yan, Z. (2022). Recent insights into signaling responses to cope with drought stress in rice. *Rice Science*, 29(2), 105–117. <https://doi.org/10.1016/j.rsci.2021.08.001>
- Azzahroh, E. Q., Vardhana, D. P., & Fendiyanto, M. H. (2024). Carotenoid content test of drought-tolerant Inpago paddy cultivars to support national food security. *MUNISI: Military Mathematics and Natural Sciences*, 2(2), 54–57.
- Batool, I., Ayyaz, A., Qin, T., Wu, X., Chen, W., Hannan, F., Zafar, Z. U., Naeem, M. S., Farooq, M. A., & Zhou, W. (2025). Morphological, physiological, and molecular responses to heat stress in Brassicaceae. *Plants*, 14(2), 152. <https://doi.org/10.3390/plants14020152>

Citation: Fendiyanto, M.H., Pratami, M.P., Cahyani, E.G., Kurniyanto, I.R., Setiawan, E., Fastanti, F.S., Syaiful, S.A., Baharuddin, A.K., & Anshori, M.F. (2026). Mild PEG-induced osmotic stress affects the regulation of the dehydration-responsive element-binding (DREB) family genes in rice cv. Inpago 6 and Inpago 9 based on transcriptome and gene expression analysis. *Agrobiological Records*, 26, 1–18. <https://doi.org/10.47278/journal.abr/2026.079>

- Berchembrock, Y. V., Pathak, B., Maurya, C., Botelho, F. B. S., & Srivastava, V. (2022). Phenotypic and transcriptomic analysis reveals early stress responses in transgenic rice expressing Arabidopsis DREB1A. *Plant Direct*, 6(10), e456. <https://doi.org/10.1002/pld3.456>
- Bhandari, U., Gajurel, A., & Khadka, B. (2023). Morpho-physiological and biochemical response of rice (*Oryza sativa* L.) to drought stress: A review. *Heliyon*, 9(3), e13744. <https://doi.org/10.1016/j.heliyon.2023.e13744>
- Cahyani, E. G., Azzahroh, E. Q., Fendiyanto, M. H., & Pratami, M. P. (2024). Enhancing food security: Chlorophyll B content in several rice genotypes as an indicator of drought stress tolerance. *MUNISI: Military Mathematics and Natural Sciences*, 2(2), 49–53.
- Chai, M., Cheng, H., Yan, M., Priyadarshani, S., Huang, X., Lai, L., Chen, H., & Cai, H. (2020). Identification and expression analysis of the DREB transcription factor family in pineapple (*Ananas comosus* (L.) Merr.). *PeerJ*, 8, e9006. <https://doi.org/10.7717/peerj.9006>
- Chakraborty, K., Jena, P., Mondal, S., Dash, G. K., Ray, S., Baig, M. J., & Swain, P. (2022). Relative contribution of different members of OsDREB gene family to osmotic stress tolerance in indica and japonica ecotypes of rice. *Plant Biology*, 24(2), 356–366. <https://doi.org/10.1111/plb.13379>
- Chrisnawati, L., Ernawati, E., Yulianti, & Hariri, M. R. (2022). Polymorphism analysis of drought tolerance gene OsDREB2A in Indonesian local rice from Lampung, Indonesia. *Biodiversitas*, 23(12), 6352–6357. <https://doi.org/10.13057/biodiv/d231232>
- Conesa, A., Madrigal, P., Tarazona, S., Gomez-Cabrero, D., Cervera, A., McPherson, A., Szczesniak, M. W., Gaffney, D. J., Elo, L. L., Zhang, X., & Mortazavi, A. (2016). A survey of best practices for RNA-seq data analysis. *Genome Biology*, 17(1), 13. <https://doi.org/10.1186/s13059-016-0881-8>
- Cui, M., Zhang, W., Zhang, Q., Xu, Z., Zhu, Z., Duan, F., & Wu, R. (2011). Induced over-expression of the transcription factor OsDREB2A improves drought tolerance in rice. *Plant Physiology and Biochemistry*, 49(12), 1384–1391. <https://doi.org/10.1016/j.plaphy.2011.09.012>
- Ereful, N. C., Laurena, A., Liu, L. Y., Kao, S. M., Tsai, E., Greenland, A., Powell, W., Mackay, I., & Leung, H. (2021). Unraveling regulatory divergence, heterotic malleability, and allelic imbalance switching in rice due to drought stress. *Scientific Reports*, 11(1), 13489. <https://doi.org/10.1038/s41598-021-92938-x>
- Fendiyanto, M. H., Satrio, R. D., Pratami, M. P., Nikmah, I. A., Sari, N. I. P., Widana, I. D. K. K., & Darmadi, D. (2021). Analysis of superoxide dismutase (Ossod) gene expression using qRT-PCR, its morphophysiological characters and path analysis in rice variety IR64 under aluminum stress. *Journal of Tropical Life Science*, 11(3), 275–286. <https://doi.org/10.11594/jtls.11.03.04>
- Fendiyanto, M. H., Anshori, M. F., Pratami, M. P., Wasonga, D. O., & Seleiman, M. F. (2024). Metabolite comparative variation related to lipid metabolisms among fruit, leaf, and stem of *Jatropha curcas*. *Heliyon*, 10(15), e35861. <https://doi.org/10.1016/j.heliyon.2024.e35861>
- Fendiyanto, M. H., Kurniyanto, I. R., Setiawan, E., Pratami, M. P., Fastanti, F. S., Wosonowati, A., Azzahroh, E. Q., & Vardhana, D. P. (2025a). Engineering of sgRNA targeting DREB4 isolated from *Oryza sativa* using pRGE32 in CRISPR/Cas9 gene editing. *SABRAO Journal of Breeding and Genetics*, 57, 989–998. <https://doi.org/10.54910/sabao2025.57.3.11>
- Fendiyanto, M. H., Setiawan, E., Pratami, M. P., Kurniyanto, I. R., & Fastanti, F. S. (2025b). Construction of a CRISPR/Cas9-mediated genome editing system in manipulating OsART1 from *Oryza sativa* cv. Inpago 5. *Biodiversitas*, 5, 1–14. <https://doi.org/10.13057/biodiv/d260241>
- Giri, J., Parida, S. K., Raghuvanshi, S., & Tyagi, A. K. (2021). Emerging molecular strategies for improving rice drought tolerance. *Current Genomics*, 22(1), 16–25. <https://doi.org/10.2174/1389202921999201231205024>
- Guo, Z., Cai, L., Liu, C., Chen, Z., Guan, S., Ma, W., & Pan, G. (2022). Low-temperature stress affects reactive oxygen species, osmotic adjustment substances, and antioxidants in rice (*Oryza sativa* L.) at the reproductive stage. *Scientific Reports*, 12(1), 6224. <https://doi.org/10.1038/s41598-022-10420-8>
- Henry, A., Wehler, R., Grondin, A., Franke, R., & Quintana, M. (2016). Environmental and physiological effects on grouping of drought-tolerant and susceptible rice varieties related to rice (*Oryza sativa*) root hydraulics under drought. *Annals of Botany*, 118, 711–724. <https://doi.org/10.1093/aob/mcw068>
- Hu, Y., Chen, X., & Shen, X. (2022). Regulatory network established by transcription factors transmits drought stress signals in plant. *Stress Biology*, 2(1), 26. <https://doi.org/10.1007/s44154-022-00048-z>
- Huang, X., Song, X., Chen, R., Zhang, B., Li, C., Liang, Y., Qiu, L., Fan, Y., Zhou, Z., Zhou, H., Lakshmanan, P., Li, Y., & Wu, J. (2020). Genome-wide analysis of the DREB subfamily in *Saccharum spontaneum* reveals their functional divergence during cold and drought stresses. *Frontiers in Genetics*, 10, 1326. <https://doi.org/10.3389/fgene.2019.01326>
- Khan, M. I. R., Palakolanu, S. R., Chopra, P., Rajurkar, A. B., Laxmi, A., & Kumar, A. (2021). Improving drought tolerance in rice: Ensuring food security through multi-dimensional approaches. *Physiologia Plantarum*, 172(2), 645–668. <https://doi.org/10.1111/ppl.13223>
- Kumar, N., Chhokar, R. S., Meena, R. P., Tripathi, S. C., Gill, S. C., Sharma, R. K., Jasrotia, P., & Singh, G. P. (2021). Challenges and opportunities in productivity and sustainability of rice cultivation system: A critical review in Indian perspective. *Cereal Research Communications*, 50(4), 573–601. <https://doi.org/10.1007/s42976-021-00214-5>
- Kumar, P., Paul, D., Jhahhriya, S., Kumar, R., Dutta, S., Siwach, P., & Das, S. (2024). Understanding heat-shock proteins' abundance and pivotal function under multiple abiotic stresses. *Journal of Plant Biochemistry and Biotechnology*, 33(4), 492–513. <https://doi.org/10.1007/s13562-024-00932-x>
- Lata, C., & Prasad, M. (2011). Role of DREBs in regulation of abiotic stress responses in plants. *Journal of Experimental Botany*, 62(14), 4731–4748. <https://doi.org/10.1093/jxb/err210>

Citation: Fendiyanto, M.H., Pratami, M.P., Cahyani, E.G., Kurniyanto, I.R., Setiawan, E., Fastanti, F.S., Syaiful, S.A., Baharuddin, A.K., & Anshori, M.F. (2026). Mild PEG-induced osmotic stress affects the regulation of the dehydration-responsive element-binding (DREB) family genes in rice cv. Inpago 6 and Inpago 9 based on transcriptome and gene expression analysis. *Agrobiological Records*, 26, 1–18. <https://doi.org/10.47278/journal.abr/2026.079>

- Lathif, Y., & Listyorini, D. (2018). Varietas padi lokal Jawa Timur tahan cekaman kekeringan berdasarkan gen DREB2A. *Journal of Tropical Biology*, 6(3), 89–95. <https://doi.org/10.21776/ub.biotropika.2018.006.03.03>
- Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12), 550. <https://doi.org/10.1186/s13059-014-0550-8>
- Ma, X., Xia, H., Liu, Y., Wei, H., Zheng, X., Song, C., Chen, L., Liu, H., & Luo, L. (2016). Transcriptomic and metabolomic studies disclose key metabolism pathways contributing to well-maintained photosynthesis under drought and the consequent drought tolerance in rice. *Frontiers in Plant Science*, 7, 1886. <https://doi.org/10.3389/fpls.2016.01886>
- Ma, Z., Hu, L., & Jiang, W. (2024). Understanding AP2/ERF transcription factor responses and tolerance to various abiotic stresses in plants: A comprehensive review. *International Journal of Molecular Sciences*, 25(13), 6845. <https://doi.org/10.3390/ijms25136845>
- Ma, Z., Jin, Y. M., Wu, T., Hu, L., Zhang, Y., Jiang, W., & Du, X. (2022). OsDREB2B, an AP2/ERF transcription factor, negatively regulates plant height by conferring GA metabolism in rice. *Frontiers in Plant Science*, 13, 1007811. <https://doi.org/10.3389/fpls.2022.1007811>
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, 17(1), 10–12. <https://doi.org/10.14806/ej.17.1.200>
- Melandri, G., AbdElgawad, H., Floková, K., Jamar, I., Asard, H., Beemster, G. T. S., Ruyter-Spira, C., & Bouwmeester, H. J. (2021). Drought tolerance in selected aerobic and upland rice varieties is driven by different metabolic and antioxidative responses. *Planta*, 254(1), 13. <https://doi.org/10.1007/s00425-021-03659-4>
- Michel, B. E., & Kaufmann, M. R. (1973). The osmotic potential of polyethylene glycol 6000. *Plant Physiology*, 51(5), 914–916. <https://doi.org/10.1104/pp.51.5.914>
- Miftahudin, M., Fendiyanto, M. H., Satrio, R. D., Turhadi, & Chikmawati, T. (2021). Genomic improvement of rice for drought, aluminum, and iron toxicity stress tolerance. In *Genomic designing for abiotic stress resistant cereal crops* (pp. 1–69). Springer. https://doi.org/10.1007/978-3-030-75875-2_1
- Nowicka, M., & Robinson, M. D. (2016). DRIMSeq: A Dirichlet-multinomial framework for multivariate count outcomes in genomics. *Fl000Research*, 5, 1356. <https://doi.org/10.12688/fl000research.8900.2>
- O'Connell, T. M. (2025). Pathway volcano: An interactive tool for pathway guided visualization of differential expression data. *Bioinformatics*, 41(7). <https://doi.org/10.1093/bioinformatics/btaf367>
- Pereira, M. B., Wallroth, M., Jonsson, V., & Kristiansson, E. (2018). Comparison of normalization methods for the analysis of metagenomic gene abundance data. *BMC Genomics*, 19(1), 274. <https://doi.org/10.1186/s12864-018-4637-6>
- Petrany, A., Chen, R., Zhang, S., & Chen, Y. (2024). Theoretical framework for the difference of two negative binomial distributions and its application in comparative analysis of sequencing data. *Genome Research*, 34(10), 1636–1650. <https://doi.org/10.1101/gr.278843.123>
- Ramadhan, D. P., Fendiyanto, M. H., Sihombing, O. D. E., & Vardhana, D. P. (2024). *Oryza sativa* cv. Inpago 10 shows significant drought tolerance differences based on relative water content (RWC) for national food sovereignty. *MUNISI: Military Mathematics and Natural Sciences*, 2(2), 44–48.
- Rio, D. C., Ares, M., Hannon, G. J., & Nilsen, T. W. (2010). Nondenaturing agarose gel electrophoresis of RNA. *Cold Spring Harbor Protocols*, 2010(6), pdb-prot5445. <https://doi.org/10.1101/pdb.prot5445>
- Roy, N., Debnath, P., & Gaur, H. S. (2026). Adoption of multi-omics approaches to address drought stress tolerance in rice and mitigation strategies for sustainable production. *Molecular Biotechnology*, 68(2), 422–434. <https://doi.org/10.1007/s12033-025-01400-0>
- Sakuma, Y., Liu, Q., Dubouzet, J. G., Abe, H., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2002). DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochemical and Biophysical Research Communications*, 290(3), 998–1009. <https://doi.org/10.1006/bbrc.2001.6299>
- Sari, A., Fendiyanto, M. H., & Miftahudin, M. (2026). Interrelation among MOC1, MOC3, and TN1 gene expressions with the architecture of five rice genotypes. *HAYATI Journal of Biosciences*, 33(4), 904–914. <https://doi.org/10.4308/hjb.33.4.904-914>
- Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, 3(6), 1101–1108. <https://doi.org/10.1038/nprot.2008.73>
- Shahid, M. (2024). *Molecular dynamics of plant stress and its management*. Springer. <https://doi.org/10.1007/978-981-97-1699-9>
- Sindiya, V., Fendiyanto, M. H., Hamim, H., & Miftahudin, M. (2024). Expression of TAD1 (Tillering & Dwarf1) gene in Hawara Bunar and IR64 rice cultivars. *Journal of Tropical Life Science*, 14(3), 553–564. <https://doi.org/10.11594/jtls.14.03.11>
- Singh, P., Sundaram, K. T., Vinukonda, V. P., Venkateshwarlu, C., Paul, P. J., Pahi, B., Gurjar, A., Singh, U. M., Kalia, S., Kumar, A., Singh, V. K., & Sinha, P. (2024). Superior haplotypes of key drought-responsive genes reveal opportunities for the development of climate-resilient rice varieties. *Communications Biology*, 7(1), 89. <https://doi.org/10.1038/s42003-024-05769-7>
- Sobahan, M. A., Akter, N., & Rana, M. M. (2022). Polyethylene glycol mediated drought stress impacts on germination, growth and accumulation of proline in rice (*Oryza sativa* L.). *SAARC Journal of Agriculture*, 20(1), 107–119. <https://doi.org/10.3329/sja.v20i1.60544>
- Susanto, U., Rohaeni, W. R., & Arifin, S. (2022). Drought tolerance screening of selected rice varieties by limited semi controlled field system. *IOP Conference Series: Earth and Environmental Science*, 978(1), 012020. <https://doi.org/10.1088/1755-1315/978/1/012020>

Citation: Fendiyanto, M.H., Pratami, M.P., Cahyani, E.G., Kurniyanto, I.R., Setiawan, E., Fastanti, F.S., Syaiful, S.A., Baharuddin, A.K., & Anshori, M.F. (2026). Mild PEG-induced osmotic stress affects the regulation of the dehydration-responsive element-binding (DREB) family genes in rice cv. Inpago 6 and Inpago 9 based on transcriptome and gene expression analysis. *Agrobiological Records*, 26, 1–18. <https://doi.org/10.47278/journal.abr/2026.079>

- Susilawati, P. N., Tajima, R., Giamerti, Y., Suhartini, S., Nugraha, Y., Rumanti, I. A., & Homma, K. (2022). Application of consecutive polyethylene glycol treatments for modeling the seminal root growth of rice under water stress. *Scientific Reports*, 12(1), 2096. <https://doi.org/10.1038/s41598-022-06053-6>
- Tian, X. H., Li, X. P., Zhou, H. L., Zhang, J. S., Gong, Z. Z., & Chen, S. Y. (2005). OsDREB4 genes in rice encode AP2-containing proteins that bind specifically to the dehydration-responsive element. *Journal of Integrative Plant Biology*, 47(4), 467–476. <https://doi.org/10.1111/j.1744-7909.2005.00028.x>
- Wang, C., Hu, C., Yang, X., & Zhao, Y. (2025). ScJAZ15-I attenuates drought tolerance and promotes flowering through interaction with JA and auxin pathways in sugarcane. *Journal of Agricultural and Food Chemistry*, 73(46), 29459–29471. <https://doi.org/10.1021/acs.jafc.5c09279>
- Wang, K., Guo, H., & Yin, Y. (2024). AP2/ERF transcription factors and their functions in Arabidopsis responses to abiotic stresses. *Environmental and Experimental Botany*, 222, 105763. <https://doi.org/10.1016/j.envexpbot.2024.105763>
- Wang, L., Zhang, X., & She, Y. (2022). Physiological adaptation mechanisms to drought and rewatering in water-saving and drought-resistant rice. *International Journal of Molecular Sciences*, 23(22), 14043. <https://doi.org/10.3390/ijms232214043>
- Wei, H., Wang, X., Wang, K., & Tang, X. (2024). Transcription factors as molecular switches regulating plant responses to drought stress. *Physiologia Plantarum*, 176(3), e14366. <https://doi.org/10.1111/ppl.14366>
- Xia, H., Luo, Z., Xiong, J., Ma, X., Lou, Q., Wei, H., Zhou, J., Chen, L., & Liu, G. (2020). Temporal transcriptomic differences between tolerant and susceptible genotypes contribute to rice drought tolerance. *BMC Genomics*, 21(1), 776. <https://doi.org/10.1186/s12864-020-07193-7>
- Yavuz, C. (2025). A meta-analysis of the overexpression of AP2/ERF genes in response to drought stress in plants. *Eurasian Journal of Science Engineering and Technology*, 6(2), 61–68. <https://doi.org/10.55696/ejset.1582037>
- Yuliza, Y., Salamah, A., & Puspitaningrum, H. (2024). Exploration of *Oryza sativa* drought-responsive element binding protein 2A (OsDREB2A) gene in several local Indonesian rice varieties. *Biodiversitas*, 25(2), 574–582. <https://doi.org/10.13057/biodiv/d250216>
- Zaffar, A., Abhinav, Dutta, S., Mhetre, V. B., Prusty, R., Das, R., Sharma, S., Chitranshi, A., Kumar, A., Irfan, M., & Bharati, A. (2025). Transcriptomics approaches for understanding the dynamics of climate-resilient traits. In *From gene discovery to climate-resilient crops: Omics in crop improvement* (pp. 44–95). CABI. <https://doi.org/10.1079/9781800628717.0003>
- Zargar, S. M., Mir, R. A., Ebinezer, L. B., Masi, A., Agrawal, G. K., Rakwal, R., et al. (2022). Physiological and multi-omics approaches for explaining drought stress tolerance and supporting sustainable production of rice. *Frontiers in Plant Science*, 12, 803603. <https://doi.org/10.3389/fpls.2021.803603>
- Zha, Q., Yin, X., Xi, X., & Jiang, A. (2023). Heterologous VvDREB2c expression improves heat tolerance in Arabidopsis by inducing photoprotective responses. *International Journal of Molecular Sciences*, 24(6), 5989. <https://doi.org/10.3390/ijms24065989>
- Zhang, Y., & Xia, P. (2023). The DREB transcription factor, a biomacromolecule, responds to abiotic stress by regulating the expression of stress-related genes. *International Journal of Biological Macromolecules*, 243, 125231. <https://doi.org/10.1016/j.ijbiomac.2023.125231>