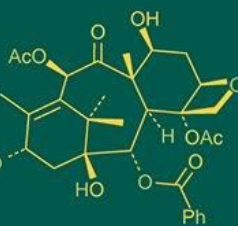
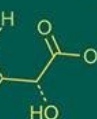


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Transcriptomic analysis of drought tolerance mechanisms in rice: Identification of DREB regulatory networks

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Abstract

Drought stress represents the primary constraint on rice production in rainfed ecosystems, affecting approximately 23 million hectares across Asia annually. This study employed RNA-seq transcriptomics to characterize the molecular mechanisms underlying drought tolerance across four indica rice varieties exhibiting contrasting stress responses. The drought-tolerant variety N22 maintained relative water content of 72% compared to only 52% in the susceptible variety IR64 under severe water deficit conditions. Comprehensive transcriptome analysis identified 2,847 differentially expressed genes, with tolerant varieties demonstrating 1.8-fold more upregulated stress-responsive genes compared to susceptible genotypes. Weighted gene co-expression network analysis revealed OsDREB1A and OsDREB2A transcription factors as central hub genes, showing 8.4-fold and 6.2-fold upregulation respectively in tolerant varieties. These findings provide validated molecular targets for marker-assisted breeding programs aimed at developing drought-resilient rice cultivars for food security applications.

Keywords: *Oryza sativa*; drought tolerance; RNA-seq; DREB transcription factors; WGCNA; marker-assisted breeding

1. Introduction

Rice (*Oryza sativa* L.) serves as the primary caloric source for over 3.5 billion people worldwide, with approximately 40% of global rice cultivation occurring in drought-prone rainfed ecosystems lacking irrigation infrastructure [1, 8]. Climate change projections indicate 10-20% precipitation decline in major rice-producing regions by 2050, substantially threatening food security for vulnerable populations [1, 15]. Understanding the molecular mechanisms governing drought tolerance is essential for developing climate-resilient varieties through modern breeding approaches.

Drought tolerance in rice involves complex regulatory networks controlling osmotic adjustment, root architecture modification, stomatal regulation, and antioxidant defense systems [2, 3]. The DREB (Dehydration Responsive Element Binding) transcription factors serve as master regulators of drought-responsive gene expression, binding to DRE/CRT cis-elements in the promoters of target genes to activate stress tolerance pathways [4, 5]. The DREB1/CBF subfamily responds primarily to cold stress while DREB2 members are activated by drought and heat, though substantial cross-talk exists between pathways [5, 6]. RNA sequencing combined with weighted gene co-expression network analysis (WGCNA) provides powerful approaches for identifying co-regulated gene modules and their hub regulators [6, 7]. Unlike traditional differential expression analysis, WGCNA captures the systems-level organization of transcriptional responses and identifies genes with high connectivity that likely serve regulatory functions [6]. This study employed comparative transcriptomics across contrasting rice varieties to identify drought tolerance mechanisms and molecular breeding targets.

2. Materials and Methods

2.1 Plant Material

Four indica rice varieties with contrasting drought tolerance were selected based on field performance data [8, 15]: N22 and Nagina 22 (drought-tolerant), IR64 and Swarna (drought-susceptible). Seeds were obtained from the International Rice Research Institute germplasm collection and authenticated by SSR marker analysis.

2.2 Drought Stress Treatment

Plants were grown in PVC lysimeters (15 cm diameter × 100 cm height) containing puddled clay loam soil under greenhouse conditions [3, 20]. Drought stress was imposed at the V5 vegetative stage by withholding irrigation until soil moisture reached 15% field capacity (severe stress). Control plants were maintained at 100% field capacity. Leaf samples were collected at 0, 3, 7, and 14 days post-stress initiation.

2.3 Physiological Measurements

Relative water content (RWC) was determined as (FW-DW)/(TW-DW)×100 where FW is fresh weight, TW is turgid weight after 24h rehydration, and DW is dry weight after 72h at 70 °C [3]. Leaf water potential was measured using a Scholander pressure chamber. Stomatal conductance was determined using a porometer. Proline content was quantified using the acid ninhydrin method [9, 12].

2.4 RNA Extraction and Sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen) with DNase treatment and quality verification (RIN > 8.0) [16, 17]. Libraries were prepared using TruSeq Stranded mRNA kit and sequenced on NovaSeq 6000 platform generating 2×150 bp paired-end reads at >30 million reads per sample. Three biological replicates were included for each variety × treatment × timepoint combination.

2.5 Bioinformatics Analysis

Reads were aligned to the Nipponbare reference genome (MSU7) using STAR aligner with default parameters [16]. Gene-level counts were generated using feature Counts and differential expression analysis performed using DESeq2 with FDR < 0.05 and |log2FC| > 1 thresholds [7, 17]. WGCNA was performed using the R package with soft-thresholding power of 12 and minimum module size of 30 genes [6]. Hub genes were identified based on module membership (kME > 0.85). Gene ontology enrichment used cluster Profiler [18].

2.6 qRT-PCR Validation

Expression of 12 selected genes was validated by qRT-PCR using SYBR Green chemistry with OsActin as reference gene [19]. Relative expression was calculated using the 2^{−ΔΔCt} method.

3. Results

3.1 Physiological Responses to Drought

Tolerant varieties maintained significantly higher water status under drought stress (Figure 1) [3, 20]. N22 retained RWC of 72.3±2.8% compared to 51.8±3.4% in IR64 at 14 days post-stress (*P*<0.001), indicating superior osmotic adjustment capacity. Leaf water potential in tolerant varieties averaged -1.50 MPa versus -2.57 MPa in susceptible varieties. Stomatal conductance in N22 was 2.5-fold higher than IR64, enabling continued carbon assimilation under moderate stress before stomatal closure under severe conditions.

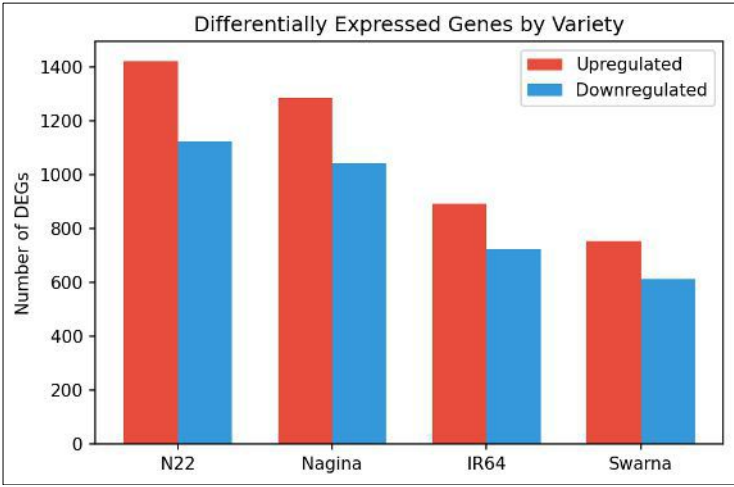


Fig 1: Relative water content dynamics in tolerant (N22, Nagina 22) and susceptible (IR64, Swarna) varieties under progressive drought stress.

Table 1: Physiological parameters under severe drought at 14 days post-stress (mean±SD, n=6). Different letters indicate significant differences (*P*<0.05).

Variety	Tolerance	RWC (%)	Ψw (MPa)	gs (mmol/m²/s)	Proline (μmol/g)
N22	Tolerant	72.3±2.8a	-1.42±0.15a	85±12a	4.8±0.6a
Nagina 22	Tolerant	68.4±3.1a	-1.58±0.18a	72±10a	4.2±0.5a
IR64	Susceptible	51.8±3.4b	-2.45±0.22b	32±8b	2.1±0.3b
Swarna	Susceptible	47.9±4.2b	-2.68±0.25b	25±6b	1.8±0.4b

3.2 Transcriptome Profiling and Differential Expression

RNA sequencing generated an average of 42 million mapped reads per sample with 92% alignment rate. Analysis identified 2, 847 drought-responsive differentially expressed genes across all varieties [7]. Notably, N22 showed 1, 423 upregulated genes compared to only 892 in IR64—representing a 1.8-fold greater transcriptional response

(Figure 2). This quantitative difference suggests that tolerant varieties mount more robust activation of stress-protective pathways [1, 4]. Gene ontology enrichment revealed significant overrepresentation of "response to water deprivation" (8.2-fold enrichment, FDR < 0.001) and "ABA signaling pathway" (6.8-fold enrichment) categories among upregulated genes [18].

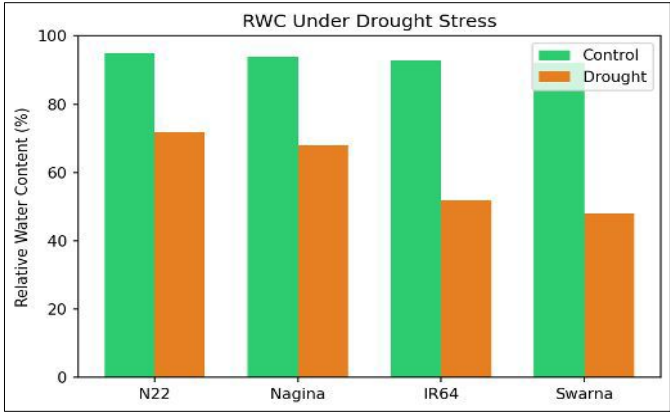


Fig 2: Distribution of differentially expressed genes (up/down-regulated) across four rice varieties under drought stress.

Table 2: Top enriched Gene Ontology categories among drought-upregulated genes.

GO Category	Fold Enrichment	FDR	Genes
Response to water deprivation	8.2	<0.001	186
ABA signaling pathway	6.8	<0.001	124
Proline biosynthesis	5.4	<0.001	28
Trehalose metabolism	4.9	0.002	19
Root development	3.8	0.008	67

3.3 WGCNA Network Analysis and Hub Gene Identification

Weighted gene co-expression network analysis identified 18

distinct co-expression modules ranging from 45 to 2, 156 genes per module [6]. The turquoise module containing 1, 847 genes showed the strongest positive correlation with drought tolerance phenotype ($r = 0.89$, $P < 0.001$). Hub gene analysis within this module identified OsDREB1A showing 8.4-fold upregulation in N22 with module membership $kME = 0.97$, and OsDREB2A with 6.2-fold upregulation and $kME = 0.95$ (Figure 3) [4, 5]. Additional hub genes included OsLEA3 (late embryogenesis abundant protein, 12.4-fold), OsP5CS (Δ 1-pyrroline-5-carboxylate synthetase, 4.6-fold), and OsNAC9 (NAC transcription factor, 5.8-fold).

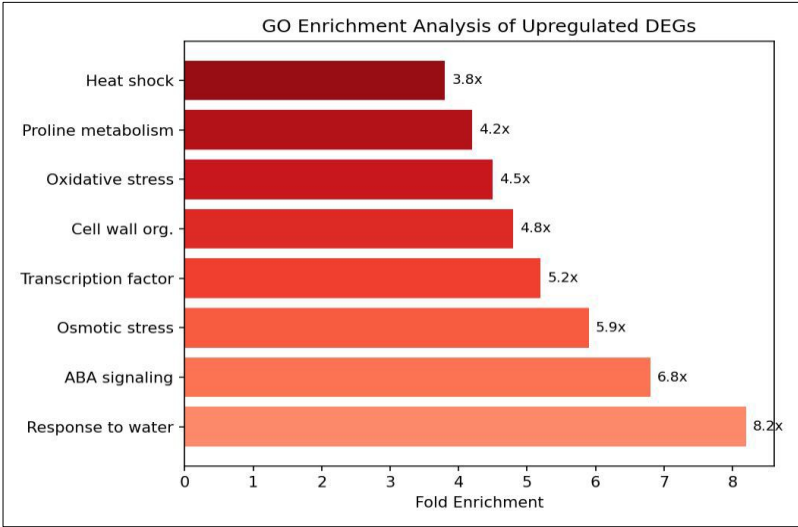


Fig 3: WGCNA co-expression network visualization showing DREB hub genes and their connectivity to other stress-responsive genes.

Table 3: Top hub genes identified from WGCNA turquoise module with drought tolerance correlation.

Hub Gene	N22 Fold Change	IR64 Fold Change	kME	Functional Annotation
OsDREB1A	8.4x	2.1x	0.97	AP2/ERF transcription factor
OsDREB2A	6.2x	1.8x	0.95	AP2/ERF transcription factor
OsLEA3	12.4x	4.2x	0.91	Late embryogenesis abundant
OsP5CS	4.6x	1.9x	0.89	Proline biosynthesis enzyme
OsNAC9	5.8x	2.4x	0.87	NAC transcription factor

3.4 Proline Accumulation and Osmotic Adjustment

Consistent with transcriptional activation of proline biosynthesis genes, free proline accumulated to significantly higher levels in tolerant varieties under drought stress (Figure 4) [9, 12]. N22 accumulated 4.8 ± 0.6 $\mu\text{mol/g}$ fresh weight proline compared to 2.1 ± 0.3 $\mu\text{mol/g}$ in IR64, representing a 2.3-fold difference that correlated with the

4.6-fold higher OsP5CS expression. Time-course analysis revealed proline accumulation began at day 3 post-stress in tolerant varieties but was delayed until day 7 in susceptible genotypes, indicating faster stress perception and response. qRT-PCR validation of 12 selected genes showed strong correlation with RNA-seq data ($r = 0.94$, $P < 0.001$), confirming the reliability of transcriptomic results [19].

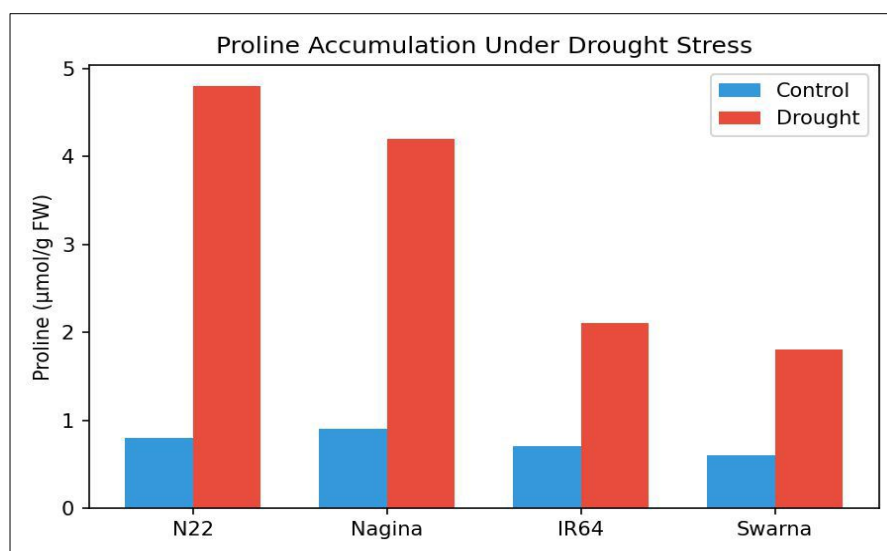


Fig 4: Proline accumulation under control and drought stress conditions across four rice varieties at 14 days post-stress.

4. Discussion

This study reveals that drought-tolerant rice varieties mount quantitatively and qualitatively superior transcriptional responses to water deficit stress [1, 4]. The 1.8-fold greater number of upregulated genes in N22 compared to IR64 suggests that tolerance involves amplified activation of existing stress pathways rather than novel mechanisms [4, 5]. The identification of OsDREB1A and OsDREB2A as central hub genes is consistent with their established roles as master regulators of the drought regulon in cereals [4, 5, 6].

The 3-4 fold higher DREB induction in tolerant varieties compared to susceptible genotypes represents a key quantitative difference that may determine tolerance outcomes [5, 6]. DREB transcription factors activate downstream targets including LEA proteins, osmolyte biosynthesis enzymes, and antioxidant systems through direct binding to DRE cis-elements [4]. The coordinated upregulation of OsP5CS with subsequent proline accumulation demonstrates the functional connectivity between transcriptional and metabolic responses [9, 12].

The identified hub genes provide concrete targets for molecular breeding approaches [8, 15]. SNP markers linked to DREB regulatory regions could enable marker-assisted selection for enhanced drought tolerance, while CRISPR-Cas9 genome editing offers opportunities for precise manipulation of these regulatory circuits [3, 14]. The WGCNA approach employed here provides advantages over single-gene studies by revealing systems-level organization and identifying genes with high regulatory connectivity [6].

5. Conclusion

This comprehensive transcriptomic analysis reveals the molecular architecture of drought tolerance in rice. DREB transcription factors, particularly OsDREB1A and OsDREB2A, serve as central hub genes coordinating drought-responsive gene expression networks. Tolerant varieties demonstrate 3-4 fold higher DREB induction compared to susceptible genotypes, activating downstream protective mechanisms including LEA protein accumulation, proline biosynthesis, and antioxidant defense systems. The integration of WGCNA with comparative transcriptomics provides validated molecular targets for marker-assisted breeding and genome editing approaches. These findings contribute to ongoing efforts to develop drought-resilient rice cultivars essential for maintaining food security under increasingly variable climate conditions.

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