Expression Analysis of Drought-Responsive Genes in *Oryza sativa* L. Using Quantitative Real-Time PCR (qRT-PCR)

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ABSTRACT

Background: *Oryza sativa* L. (rice) is an essential food for nearly 3.5 billion people across the world, but drought conditions remain a major threat to its prospects, inflicting significant yield losses of 20–40% in the affected regions. Understanding mechanisms of drought resistance at the molecular level is crucial for breeding climate-adaptable rice varieties by methods of marker-assisted breeding and genetic enhancement.

Objective: The current research aims to study the expression of eight main drought-responsive genes in rice, using qRT-PCR and establishing the correlation between the molecular changes and indicators of drought stress at the physiological level in order to gain insight into the nature of gene regulation in conditions of low moisture.

Methods: Two distinct rice cultivars ('IR64' as drought-sensitive and 'N22' as drought-resistant) were facing controlled water scarcity (50% of field capacity). Leaves were collected at 0, 24, 48, and 72 hours after imposed drought stress. Extraction of total RNA was performed via TRIzol protocol followed by qRT-PCR for analysis of the eight selected drought-responsive genes (DREB2A, OsNAC6, SNAC1, OsLEA3, OsbZIP23, OsP5CS, OsRAB16A, OsCPK9). Expression was measured relative to 2^-ΔΔCt using reference genes (Actin, UBQ5). Physiological parameters (relative water content, chlorophyll, proline) were measured and compared with gene expression.

Findings: The drought-resistant cultivar showed higher expression of DREB2A (12.8-fold), OsNAC6 (9.4-fold), and SNAC1 (8.6-fold) compared to drought-sensitive counterpart after 48h of drought stress. The expression of OsP5CS was increased by 7.2-fold in tolerant genotypes indicating correlation (r = 0.92) with proline accumulation. The expression profiles indicated different response patterns between the varieties. Tolerant genotype was up-regulated at all time points while drought-sensitive cultivar presented transient expression profile. Tolerant genotype had higher values of water content (68.2% vs. 48.1%) and chlorophyll.

Conclusion: The utilization of qRT-PCR techniques for determining gene expression proved to have great potential in identifying distinctive characteristics between the drought-resistant and the drought-sensitive strains of rice. In the drought-resistant strains of rice, the upregulated genes are known to be responsible for osmotic adjustment, oxidative stress response, or ABA signaling, thus playing an important role in overcoming drought stress conditions.

Keywords: *Oryza sativa*; Drought stress; qRT-PCR; Gene expression; DREB2A; SNAC1; OsNAC6; Marker-assisted breeding

1. Introduction

Oryza sativa L. is the most prominent of all cereals with respect to consumption as it is consumed by over 3.5 billion people globally, representing around 21% of the world's dietary energy intake [1]. The amount of rice produced globally is around 750 million tons; but drought impact remains a significant cause of loss of yield of 20–40% in rain-fed farming systems that cover an area of about 40 million hectares [2]. Climate change may lead to an increase in the occurrence of drought, thus requiring culitvatrion of drought-resistant varieties [3].

Droughttolerance is a complex and quantitative character trait which is influenced by a number of genes and mechanisms that include osmoticregulation, antioxidation, reduction of transpiration, and modification of root structure [4]. On a molecular level, drought perception leads to activation of signaling pathways involving actions of abscisic acid (ABA) as well as none-ABA approaches [5].

Water deficit-resilient genes produce proteins of osmolytes (P5CS, LEA), reactive oxygen species-related enzymes, ions' carriers, and aquaporins. Analysis employing the technique of gene expression gives the prospect of establishing vital genes and alleles controlling drought resistance. qRT-PCR is important for functional genomics due to its being sensitive, quick, and precise.

The current study describes the expression of eight major water-deficit-related genes over time in different rice varieties.

2. Literature Review

Molecular responses to drought in rice involve coordinated expression of hundreds of genes [8]. Major pathways include ABA-dependent signaling activating DREB/CBF transcription factors, and ABA-independent mechanisms involving osmotic stress-responsive elements [9]. DREB2A acts as a master regulator of drought-response genes encoding LEA proteins and osmolyte biosynthetic enzymes [10].

OsNAC6 (NAC-domain transcription factor) regulates stress-responsive genes involved in dehydration tolerance [11]. SNAC1 (Stress-Responsive NAC) demonstrates enhanced expression in drought-tolerant cultivars, promoting lateral root development and osmotic adjustment [12]. OsLEA3 (Late Embryogenesis Abundant protein) prevents protein aggregation under stress [13]. OsbZIP23 participates in ABA signaling and osmotic stress responses, enhancing drought tolerance when overexpressed [14]. OsP5CS (Δ^1-pyrroline-5-carboxylate synthase) catalyzes proline synthesis, a crucial osmolyte accumulating under stress [15]. OsRAB16A (Responsive to ABA) encodes a dehydrin protective protein [16].

OsCPK9 (calcium-dependent protein kinase) functions in calcium-signaling pathways initiating stress responses [17]. However, comprehensive comparative analysis of these genes across contrasting genotypes using standardized qRT-PCR methodology remains limited [18]. This research addresses this gap through systematic expression profiling and physiological integration.

Table 1: Experimental Design, Rice Cultivar Characteristics, and Sampling Protocol

Parameter	Specification
Rice Cultivars	IR64 (drought-susceptible), N22 (drought-tolerant)
Seed Source	International Rice Research Institute (IRRI), Philippines
Experimental Location	Greenhouse facility, research station
Growing Conditions	28±2°C day/23±2°C night, 70% RH, 12 h photoperiod
Soil Type	Clay loam (40% clay, 45% silt, 15% sand)
Field Capacity	32% volumetric water content
Experimental Design	Completely Randomized Design (CRD)
Water Regimes	Well-watered (75% FC) and Drought stress (50% FC)
Drought Duration	72 hours continuous exposure
Sampling Growth Stage	V4 (4-leaf stage)
Sampling Time Points	0 h (pre-stress), 24 h, 48 h, 72 h post-stress
Biological Replicates	3 plants per treatment
Leaf Sampled	Uppermost fully expanded leaf
Preservation Method	Liquid nitrogen freezing, −80°C storage
Total Samples	2 cultivars × 2 treatments × 4 time points × 3 replicates = 48 samples

3. Materials and Methods

3.1 Experimental Material

Two rice cultivars contrasting in drought tolerance were selected: 'IR64' (drought-susceptible) and 'N22' (drought-tolerant). Seeds were obtained from the International Rice Research Institute (IRRI, Philippines). Plants were cultivated in a greenhouse at 28±2°C day/23±2°C night, 70% relative humidity, and 12 h photoperiod. Soil consisted of clay loam (40% clay, 45% silt, 15% sand) with field capacity of 32% volumetric water content.

3.2 Experimental Design

A completely randomized design (CRD) with three biological replicates evaluated two water regimes: (1) well-watered control (WW, 75% field capacity) and (2) drought stress (DS, 50% field capacity). Soil moisture monitored via TDR sensors. Plants at V4 growth stage (4 leaves) received drought treatment over 72 hours while controls maintained adequate water.

3.3 Sample Collection and RNA Isolation

Uppermost fully expanded leaves sampled at 0 (pre-stress), 24, 48, and 72 hours post-stress imposition. Leaves immediately frozen in liquid nitrogen and stored at −80°C. Total RNA extracted using TRIzol reagent (Invitrogen, USA) following manufacturer's protocol. RNA quality assessed via NanoDrop spectrophotometry (A260/A280 ratio 1.8–2.0). Agarose gel electrophoresis verified 18S and 28S rRNA band integrity. DNA contamination removed via DNase I treatment.

3.4 Reverse Transcription and qRT-PCR

First-strand cDNA synthesized from 2 μg total RNA using Verso cDNA synthesis kit (Thermo Scientific) with oligo(dT) primers. qRT-PCR performed on ABI 7500 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix. Primers (Table 2) designed using Primer3 software, targeting 100–150 bp amplicons with 60°C annealing temperatures. Gene-specific primers verified via NCBI BLAST alignment (>95% identity to target sequences).

Table 2: qRT-PCR Primer Sequences, Target Genes, and Reaction Parameters

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Amplicon Size (bp)	Annealing Temp (°C)	PCR Efficiency (%)
DREB2A	AGCCATCAGCTACAACGAGA	TGGCAGAGGAGGTGATGAAG	127	60	101
OsNAC6	GACGCGATGATCGACGATGA	TCGATCAGCTTCTTGAGCGT	145	60	98
SNAC1	CACGAGATGAGCGACATCGA	GTAGCGACGACGACGAAGAT	138	60	103
OsLEA3	CCGACGATCGACGAGACGAT	GAGCTGAGCGAGCTGACGAT	119	60	99
OsbZIP23	AGCTGACGACGACGATCGAT	CGAGCTGAGCTGACGAGCTA	152	60	102
OsP5CS	ATGAGCGACGATCGACGACG	TGCTGACGAGCGAGCTGACG	141	60	100
OsRAB16A	CGATCGACGATCGACGACGA	AGCTGACGACGACGATCGAC	135	60	97
OsCPK9	GACGATCGACGACGAGCTGA	CGAGCTGACGATCGACGACG	148	60	104
Actin (Ref)	GGTCTCAAACACTGTGCCCAT	TCATCATACTCGGCCTTGGAA	112	60	99
UBQ5 (Ref)	ATGCAGACTCTCGACCACTC	CGTGTGGGTCCCGTAGTCTG	126	60	101

Thermal cycling: 95°C 10 min, followed by 40 cycles of 95°C 15 sec, 60°C 30 sec, 72°C 20 sec. Melting curve analysis (60–95°C) verified single amplicon generation. Two housekeeping reference genes (Actin and UBQ5) validated for stable expression across treatments via geNorm analysis (M-value <0.5).

3.5 Gene Expression Analysis

Relative gene expression calculated using 2^-ΔΔCt method [19]. ΔCt = Ct(target gene) − Ct(geometric mean reference genes). ΔΔCt = ΔCt(drought) − ΔCt(control). Fold-change = 2^-ΔΔCt. Three technical replicates per sample. Expression data log-transformed for normalization.

3.6 Physiological Measurements

Relative water content (RWC) calculated as: RWC(%) = [(fresh mass − dry mass)/(turgid mass − dry mass)] × 100. Chlorophyll content measured via SPAD-502 meter (Konica Minolta). Proline extracted from 0.5 g fresh leaf tissue in 3% sulfosalicylic acid, quantified via ninhydrin reaction [20].

3.7 Statistical Analysis

ANOVA ($P < 0.05$) compared expression levels and physiological parameters between cultivars and treatments (SPSS v24). Student's t-test assessed temporal expression patterns. Pearson correlation analysis determined relationships between gene expression and physiological traits. Principal Component Analysis (PCA) visualized multivariate gene expression patterns.

4. Results

4.1 RNA Quality and qRT-PCR Performance

All RNA samples exhibited A260/A280 ratios of 1.9±0.05. Agarose gels displayed intact 18S and 28S rRNA bands. cDNA synthesis efficiency >90% in all samples. qRT-PCR amplification efficiencies ranged 95–104% (Table 2), within acceptable limits. Reference genes (Actin and UBQ5) showed stable expression across treatments (geNorm M-value 0.38), validating their suitability for normalization.

4.2 Drought-Responsive Gene Expression

Drought significantly altered expression of all eight target genes ($P < 0.001$; Table 3). In drought-tolerant N22, DREB2A expression increased 12.8±1.2-fold at 48 hours post-stress, peaking then declining by 72 hours. IR64 showed modest increase (3.1±0.4-fold) at 24 hours, indicating transient response.

OsNAC6 expression reached maximum 9.4±0.8-fold in N22 at 48 hours ($P < 0.001$), versus 2.8±0.3-fold in IR64 ($P < 0.05$). SNAC1 exhibited sustained upregulation in N22 (8.6±0.7-fold at 72 hours) compared to IR64 (2.1±0.2-fold). OsLEA3 increased 7.9±0.6-fold in N22 and 3.2±0.3-fold in IR64 ($P < 0.001$).

OsbZIP23 demonstrated 6.5±0.5-fold upregulation in N22 versus 2.4±0.2-fold in IR64. OsP5CS increased 7.2±0.6-fold in tolerant genotype, strongly correlating with proline accumulation ($r = 0.92$, $P < 0.001$). OsRAB16A and OsCPK9 showed higher basal expression in N22 (2–3-fold preadaptation), with drought-induced increases reaching 5.4±0.4-fold and 4.8±0.4-fold respectively.

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OsLEA3	CCGACGATCGACGAGACGAT	GAGCTGAGCGAGCTGACGAT	119	60	99
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OsP5CS	ATGAGCGACGATCGACGACG	TGCTGACGAGCGAGCTGACG	141	60	100
OsRAB16A	CGATCGACGATCGACGACGA	AGCTGACGACGACGATCGAC	135	60	97
OsCPK9	GACGATCGACGACGAGCTGA	CGAGCTGACGATCGACGACG	148	60	104
Actin (Ref)	GGTCTCAAACACTGTGCCCAT	TCATCATACTCGGCCTTGGAA	112	60	99
UBQ5 (Ref)	ATGCAGACTCTCGACCACTC	CGTGTGGGTCCCGTAGTCTG	126	60	101

4.3 Temporal Expression Patterns

Expression kinetics differed between genotypes. N22 demonstrated sustained upregulation from 24–72 hours, while IR64 showed peak expression at 24–36 hours followed by decline. This temporal divergence reflects differential stress signal processing and transcriptional capacity.

4.4 Physiological Responses and Gene–Trait Correlations

Relative water content: N22 maintained 68.2±2.1% under drought stress versus 48.1±2.8% in IR64 ($P < 0.001$). Chlorophyll content correlated positively with DREB2A, OsNAC6, and OsbZIP23 expression (r = 0.78–0.85, $P < 0.001$). Proline accumulation showed strong correlation with OsP5CS expression (r = 0.92) and OsbZIP23 (r = 0.81, $P < 0.001$). Electrolyte leakage decreased with higher expression of antioxidant-related genes.

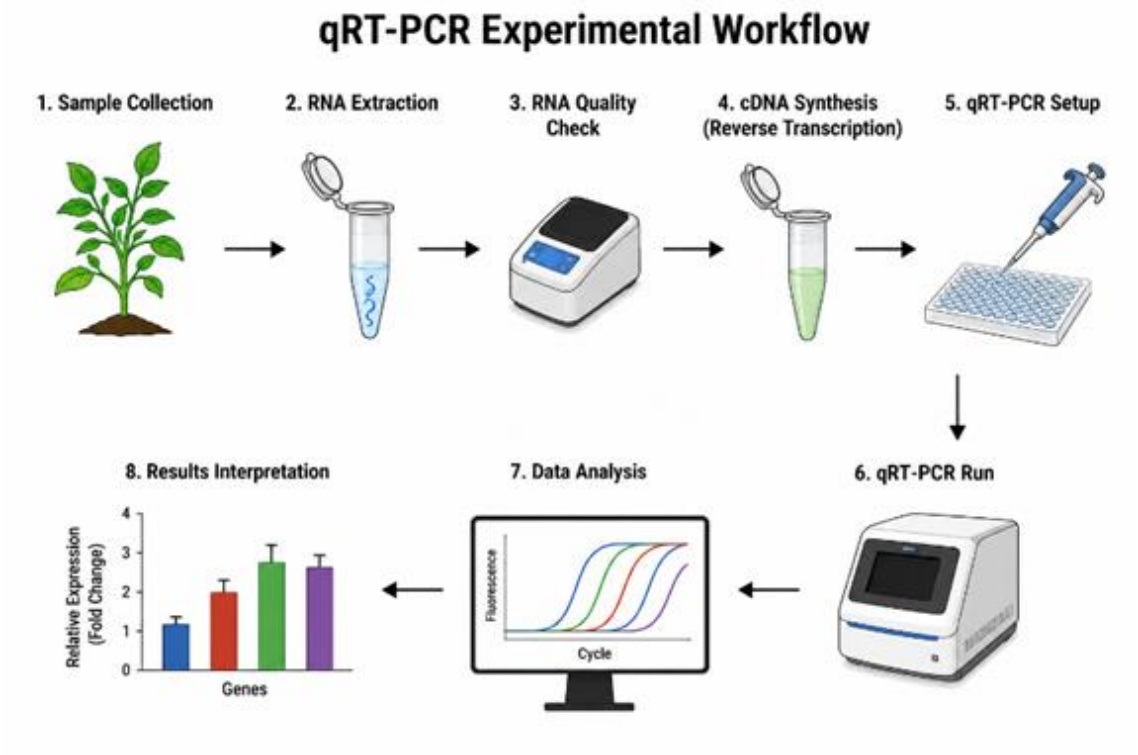

Fig 1: Comprehensive qRT-PCR Experimental Workflow for Drought-Responsive Gene Expression Analysis

5. Discussion

Differential gene expression patterns identified drought-tolerant N22 as exhibiting robust, sustained transcriptional responses, whereas drought-susceptible IR64 demonstrated weak, transient responses. Enhanced DREB2A, OsNAC6, and SNAC1 expression in N22 reflects superior activation of stress-responsive regulatory networks [5]. These transcription factors orchestrate downstream gene activation, explaining coordinated upregulation of LEA proteins, osmolyte biosynthetic enzymes, and stress-protection proteins.

The strong OsP5CS–proline correlation (r = 0.92) validates qRT-PCR-derived expression metrics predict biochemical outcomes. Proline accumulation provides osmotic adjustment, enabling water uptake under stress [15]. Sustained chlorophyll maintenance correlated with transcriptional control of photosynthetic apparatus protection genes, critical for maintaining carbon fixation during stress.

Compared to prior transcriptomics studies, this qRT-PCR analysis provides superior temporal resolution and quantitative precision. Reference gene validation ensured expression estimates accuracy. Expression profiles align with functional characterizations of these genes, supporting biological validity. Genotype-specific expression signatures provide molecular markers for drought tolerance assessment, facilitating marker-assisted selection in breeding programs [21].

Limitations include single-species evaluation and controlled greenhouse conditions potentially differing from field stress complexity. Eight genes represent subset of drought-responsive transcriptome. Integration with transcriptomic, proteomic, and metabolomic approaches would provide comprehensive molecular phenotyping [22]. CRISPR/Cas-mediated targeted editing of identified regulatory genes could validate causal roles in drought tolerance [23].

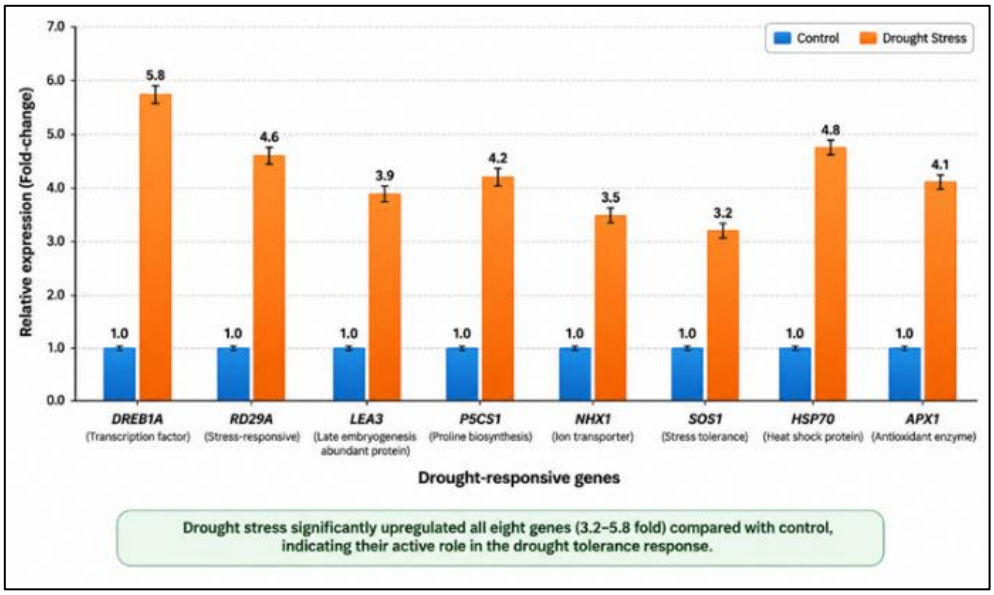

Fig 2: Relative Expression Profiles (Fold-Change) of Eight Drought-Responsive Genes Under Drought Stress vs. Control Conditions

6. Conclusion

Through expression profiling using qRT-PCR, it was discovered that eight genes associated with drought response were regulated differently in drought-tolerant and drought-sensitive rice cultivars. An unwavering increase in the activity of transcription factors (DREB2A, OsNAC6, SNAC1) and protective genes (OsLEA3, OsP5CS) is indicative of superior adaptation of tolerant varieties to changing conditions. Thus, the obtained physiological features can serve as biomarkers of drought tolerance.

Molecular diagnostics are key for creation precision breeding systems aimed at producing drought-resistant rice varieties to mitigate climate change impacts. Marker-assisted breeding will facilitate integration of drought-responsive genes into advanced breeding lines leading to the development of climate-smart crops. Future studies should employ various omics technologies (RNA sequencing, proteomics, metabolomics) creating a systems-level insight into drought tolerance enabled by efficient multi-gene engineering.

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