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Transcription factor-mediated gene regulatory networks contributes to reproductive stage drought tolerance in rice (*Oryza sativa*)

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ABSTRACT

Present study was carried out during rainy (*kharif)* seasons of 2020–22 at ICAR-Indian Agricultural Research Institute, New Delhi, to examine transcription factors (TFs) in drought tolerance using two rice (*Oryza sativa* L.) cultivars, drought-tolerant Nagina-22 and drought-sensitive IR-64. Immature panicles were used to prepare transcriptome libraries, sequenced with Paired-End 150 bp chemistry for both control and drought-treated samples. Bioinformatic analysis of the data using TFs extracted from PlantTF database revealed that majority (335) of the TFs were up-regulated in Nagina-22, while 92 were down-regulated under stress. Conversely, a significant number of TFs (359) were down-regulated in IR-64 under stress. Gene ontology and gene regulatory network analyses of the differentially expressed TFs indicated that several crucial biological processes and molecular functions were enriched with higher number of up-regulated TFs in Nagina-22 under stress. Significantly up-regulated expression of TFs, particularly WRKY, ERF, MYB, NAC, and bHLH, in panicle of N-22 must be responsible for terminal drought tolerance. Our findings confirm the master-regulator role of TFs in gene-regulatory networks essential for tolerance to complex trait like drought. The information might be valuable in selecting potential candidate parental lines for drought tolerance breeding and genetic manipulation of rice cultivars towards the development of climate-resilient cultivars.

Keywords: Climate-resilient variety, Drought tolerance, Gene regulation, Rice, Terminal drought, Transcription factor

Rice (*Oryza sativa* L.) a vital staple food for more than half of the global population, plays a crucial role in food security but faces challenges from environmental and biological stressors in ensuring a stable supply (Kumar *et al.* 2022a, Kaur *et al.* 2023a, b). Occurrence of drought at reproductive stage of plant growth, a crucial phase in the life cycle of an organism, can have severe consequences for crop yield, grain quality, and overall food production (Liang *et al.* 2021). Drought tolerance is a complex and multigenic trait, controlled by a network of genes and regulated by various mechanisms. Transcription factors (TFs) play crucial roles in modulating stress-responsive genes, regulating water uptake/loss, and fine-tuning stomatal behaviour. Several TFs have been reported to enhance drought tolerance in rice. For instance, *OsNAC10* and *OsMYB2* are essential components of the molecular machinery enabling rice plants to respond to drought stress by influencing root development and optimizing resource acquisition responses (Welner *et al.* 2016), and by activating stress-responsive genes (Li *et al.* 2019, Yao *et al.* 2022). *OsbZIP23* is involved in abscisic acid (ABA)-mediated drought responses and *OsbHLH148* influences drought tolerance through ABA-signalling pathways while *OsERF922* regulates drought tolerance by influencing ethylene-response pathways (Li *et al.* 2020, Yoon *et al.* 2020). Thus, TFs play a central role in regulating gene expression, orchestrating the plant's responses to drought, and coordinating various adaptive processes.

Hence, we aimed at unravelling the role of TFs in conferring drought tolerance in rice. We focused on identifying the differentially expressed TFs in a pair of contrasting rice cultivars [IR-64 (drought-sensitive) and N-22 (drought-tolerant)] grown under control and reproductive stage drought conditions. A set of major TFs exhibiting differential expression and playing roles in the intricate regulatory network for drought tolerance were identified. Gene ontology analysis indicated that these TFs are involved in various biological processes and molecular functions that confer drought tolerance in rice. Utilizing insights from these comparative analyses, researchers can develop rice cultivars better equipped to withstand drought

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stress, thereby ensuring a stable food supply and contributing to the long-term sustainability of agriculture.

MATERIALS AND METHODS

Plant materials and drought treatment: The study was carried out during rainy (*kharif)* seasons of 2020–22 at ICAR-Indian Agricultural Research Institute, New Delhi. Mature seeds of Nagina-22 (drought-tolerant) and IR-64 (drought-susceptible) were grown at ICAR-Indian Agricultural Research Institute, New Delhi, during the *kharif* season (2020–22). Twenty-five-days-old seedlings were transplanted into puddled soil and either well-watered or subjected to drought stress by withholding water for 4–5 days before panicle emergence. Drought stress was assessed by measuring soil moisture and leaf relative water content (Kumar *et al*. 2022b). Immature panicles were collected, frozen in liquid nitrogen, and stored at -80°C for further analysis.

RNA extraction, cDNA library preparation, and sequencing: Total RNA was extracted from immature panicle tissues using TRIzol reagent and treated with DNase I to remove residual DNA. RNA quality was assessed via agarose gel electrophoresis and Qubit-4. High-quality RNA samples (OD260/280 ≈2.0, OD260/230 ≥2.0, RIN ≥6.0, 28S:18S ratio ≥1.0) were used to prepare 12 whole transcriptome libraries from the panicles of two rice cultivars under two conditions, with three technical replicates each. The libraries were sequenced on the Illumina HiSeq 2500 platform (Macrogen, South Korea) using PE-150 bp chemistry. Raw sequence data were submitted to the NCBI database (BioProject Submission ID SUB12273896) and used for bioinformatic analyses.

Identification of differentially expressed TFs: The raw data quality was assessed using FastQC, and TrimGalore was used to trim adapter sequences, poly-N stretches, and low-quality reads. Data processing was done with pySeqRNA (Duhan and Kaundal 2020), and feature counts were determined using featureCounts (Liao *et al*. 2014). Raw read counts were normalized to reads per kilobase of exon model per million mapped reads using pySeqRNA. Clean reads were aligned to the *Oryza sativa* Japonica IRGSP-1.0 genome using STAR (Dobin *et al*. 2013). Transcription factors (TFs) were retrieved from the plantTF database and compared with the experimental dataset. Differential expression analysis of TFs was conducted using DESeq2 (Love *et al*. 2014), with P-values adjusted using the Benjamini and Hochberg method (Srinivasan *et al*. 2013). TF genes with a fold change ≥2 (|log2Ratio| >1) and FDR <0.05 were identified as differentially expressed. A methodological outline for identifying differentially expressed TFs is shown in Fig. 1.

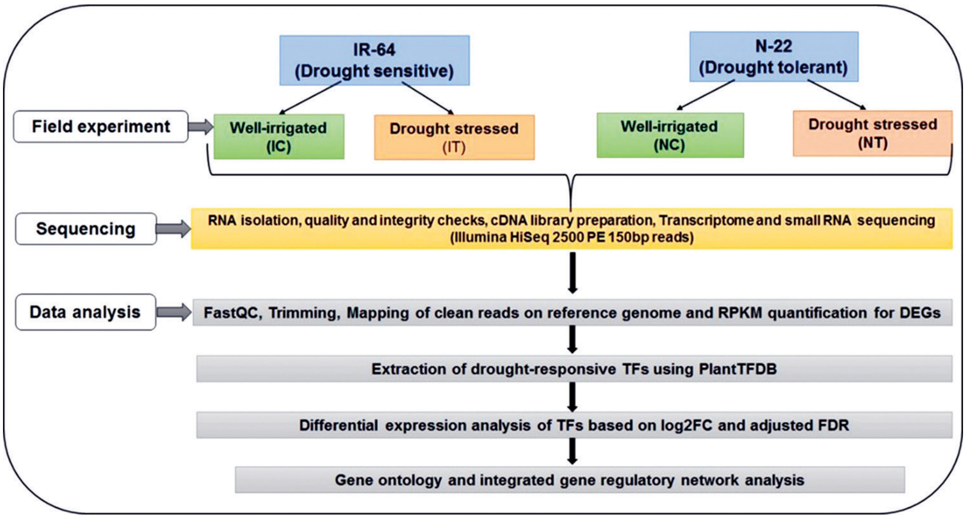

Fig. 1 Diagrammatic presentation of the methodology used to identify and annotate differentially expressed TFs in contrasting rice cultivars (IR-64 and N-22) under reproductive stage drought stress.

Functional enrichment analysis of differentially expressed TFs: Gene Ontology (GO) enrichment analysis of drought-related differentially expressed transcription factors (TFs) was performed using ShinyGO v0.76.2 with an FDR threshold of <0.05, using annotations from the Rice Genome Annotation Project (RGAP). Additionally, gene regulatory network analysis and hierarchical clustering were conducted to evaluate the interactions and dependencies among the affected pathways.

RESULTS AND DISCUSSION

Whole transcriptome analysis: Comparative whole transcriptome analysis offers a comprehensive view of genetic responses to drought stress in rice, illuminating the gene regulatory networks underlying drought tolerance (Liang *et al*. 2021). Twelve transcriptome libraries, from panicles of contrasting rice cultivars under control and drought conditions, generated over 54 million raw reads. The sequencing data were of high quality, with over 92% cleaned reads and more than 90% mapping efficiency. A previous detailed analysis of 37,849 differentially expressed genes in panicles of these rice cultivars, along with investigations into morpho-physio-biochemical changes (Kaur *et al*. 2023a), revealed numerous differentially expressed TFs under terminal drought stress. This study focuses on deciphering the role and significance of TFs in conferring reproductive stage drought tolerance in Nagina-22.

Differentially expressed TFs: To investigate the role of TFs (involved in the complex regulatory network) in providing drought tolerance, comparative analyses for the expression of TFs in the panicle of IR-64 and N-22 under control and drought stress was performed. Further analysis of the differentially expressed TFs (>2-fold change, FDR ≤ 0.05) categorized into up- and downregulated TFs comparison groups in IR-64 and N-22 (Supplementary Fig. 1). A total of 597 TFs exhibited differential expression (comprising of 238 up-regulated and 359 down-regulated) under terminal drought stress in the panicle of IR-64 (reproductive stage drought sensitive cultivar). In contrast, a total of 427 TFs were differentially expressed with a notably higher number (335) of up-regulated TFs and a comparatively lower (92) number of down-regulated TFs in the panicle of N-22 (drought tolerant cultivar) under reproductive stage drought stress (Supplementary Fig. 1). TFs function as key regulators, orchestrating a wide array of responses to environmental perturbations to mitigate the effects of stress. In rice, examples include DREB, which activates stress-associated genes to enhance drought resilience and improve water-use efficiency. TFs are central players in the plant's response to drought stress and regulate the expression of stress-responsive genes, exhibit tissue-specific functions, and interact with other signalling pathways during drought stress (Welner *et al.* 2016). MYB contributes significantly to the regulation of drought-responsive genes, particularly those involved in processes like stomatal closure and stress tolerance (Yao *et al.* 2022). Similarly, bZIP governs the expression of genes associated with osmotic stress response and ABA signalling pathways (Guo *et al.* 2021).

In N-22 panicles under drought stress, 171 transcription factors (TFs) were up-regulated, while 149 TFs up-regulated in N-22 were down-regulated in IR-64. Additionally, 199 TFs were down-regulated in IR-64, contributing to its drought sensitivity stress (Supplementary Table 1, Fig. 2).

Among the differentially expressed TF families in N-22, ERF had the highest number of members (71), followed by bHLH (66), NAC (65), WRKY (59), MYB (50), and bZIP (41). These findings align with previous studies highlighting the significant roles of TFs in abiotic stress responses (Welner *et al*. 2016, Li *et al*. 2020, Tolosa *et al*. 2020, Guo *et al*. 2021, Yao *et al*. 2022). Notably, *Os09g0457900* (AP2/ERF, which regulates internode elongation) showed over 27-fold up-regulation in N-22 panicles under drought stress, highlighting ERF's role in drought tolerance. Jin *et al*. (2018) found that *OsERF101*, primarily expressed in reproductive tissues, enhances seed setting under drought. Additionally, overexpression of bHLH from tartary buckwheat (FtbHLH3) in *Arabidopsis* increased drought and oxidative stress resilience via ABA-dependent mechanisms (Yao *et al*. 2017). The bHLH TFs play critical roles in drought response by modulating root development, stomatal closure, ABA signaling, ROS scavenging, osmotic adjustment, and LEA protein induction (Qian *et al*. 2021). Therefore, genetic manipulation of bHLH could be a promising strategy to enhance plant drought tolerance. Our findings on 29 bHLH family TFs up-regulated (more than 1−11 fold) in N-22 but most of them either not expressed or down-regulated in the panicle of IR-64 (Supplementary Table 1) are consistent with the earlier reports. NAC (NAM, ATAF1/2, and CUC2) family TFs play vital roles in regulating drought tolerance in rice by controlling the expression of stress-responsive genes, osmotic stress responses, and ABA signalling. These TFs also influence root development and stomatal behaviour, which are crucial for water uptake and conservation. Additionally, NAC family TFs contribute to oxidative stress management, cell wall integrity, and delayed leaf senescence under drought conditions (Welner *et al.* 2016). One of the NAC family TFs (*Os02g0214500*, No apical meristem protein) showed ~9-fold up-regulated expression in NIL-23, while ~19-fold down-regulated expression in the panicle of IR-64 under terminal drought stress. A total of 26 NAC family TFs showed up-regulated expression in N-22 while change in their expression under drought stress was either not significant or they were down-regulated in IR-64 on terminal drought stress. Recently, Jung *et al.* (2022) reported that *OsNAC17*, a member of NAC family TFs in rice, plays vital roles in drought tolerance by positively regulating lignin biosynthesis resulting in enhanced drought tolerance on overexpression in transgenic plants, but its knock-out increases susceptibility in plants.

WRKY family TFs modulate the expression of drought-responsive genes, enhance osmotic adjustment, control stomatal closure, and regulate ROS scavenging that collectively contribute in enhancing drought tolerance in plants. Their roles in signal transduction as well as in crosstalk with other regulatory molecules/pathways make them essential components for adaptation of plant to water-deficient conditions. We observed more than 9 and 8-fold up-regulated expression of WRKY61 and WRKY42, respectively, in the panicle of N-22 (compared to no significant change in IR-64) under terminal drought stress. Li *et al.* (2020) proposed that WRKY plays crucial roles in ABA signalling, heat/drought tolerance, and cold/

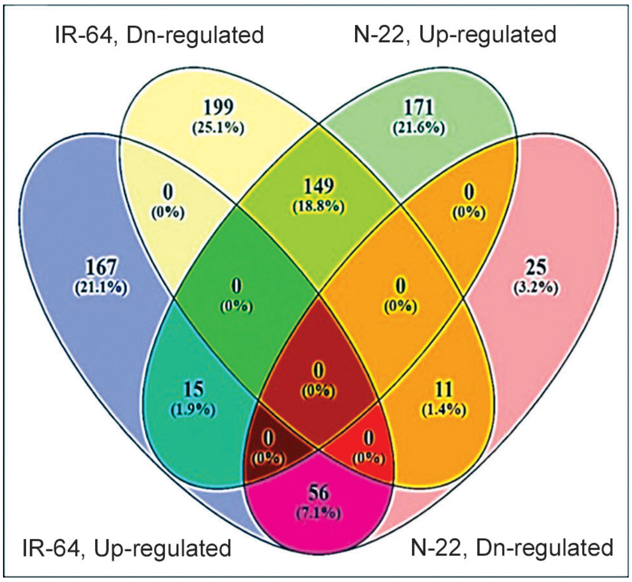

Fig. 2 Venn diagram depicting commonly and exclusively differentially expressed up- and down-regulated TFs in the panicle of contrasting rice (IR-64 and Nagina-22) cultivars under reproductive stage drought stress.

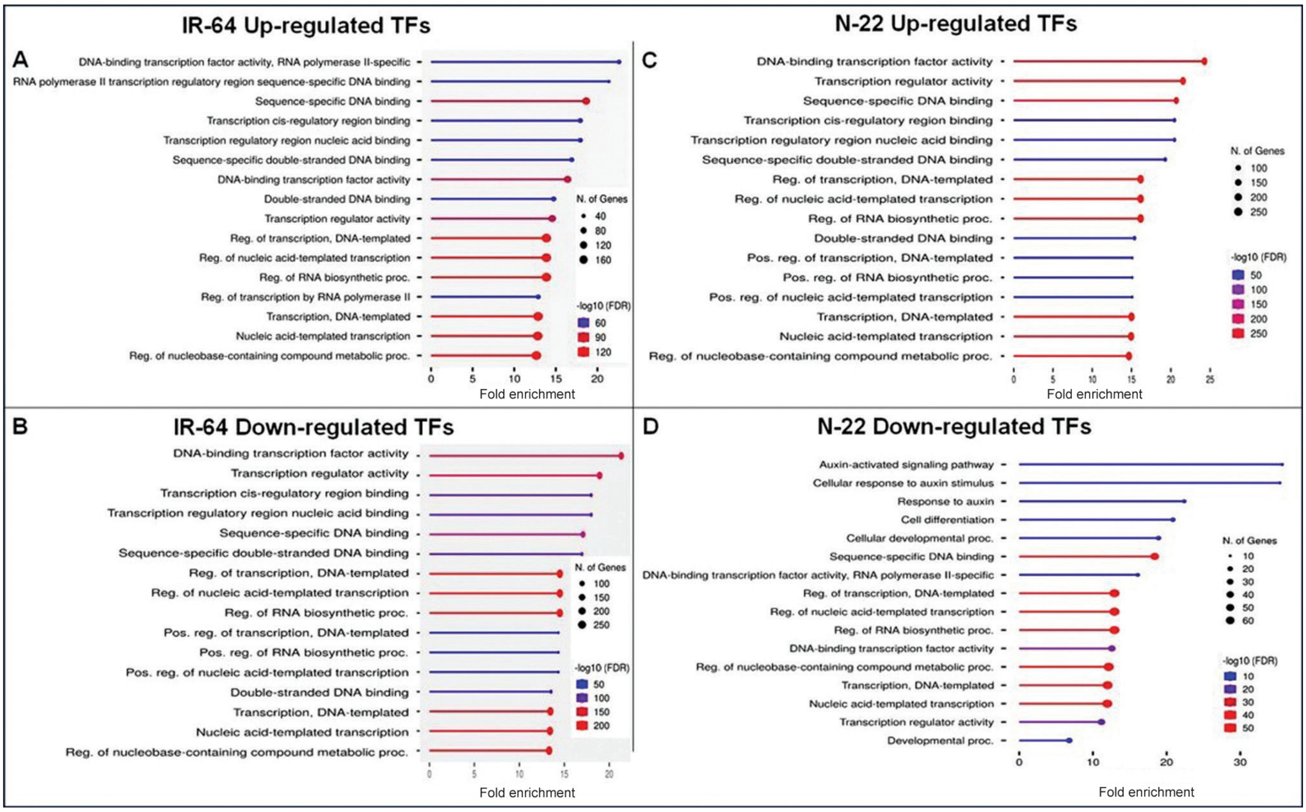

Fig. 3 Gene ontology analysis of differentially expressed TFs/genes in the panicles of contrasting rice cultivars (IR-64 and Nagina-22) under reproductive stage drought stress.

Note: The top 16 significantly enriched GO terms are shown for (A) up-regulated TFs/genes in IR-64, (B) down-regulated TFs/genes in IR-64, (C) up-regulated TFs/genes in N-22, and (D) down-regulated TFs/genes in N-22.

salt tolerance. Huang *et al.* (2021) also reported that *OsWRKY55* acts as a central regulator of drought stress responses in rice. Similarly, our observation on ~10-fold up-regulated expression of *Os03g0142600* (a MYB domain-containing protein) in the panicle of N-22, while ~5-fold down-regulated expression in IR-64, under terminal drought stress corroborates with the reports of Li *et al*. (2019) and Yao *et al.* (2022).

Gene ontology of differentially expressed TFs: Gene ontology (GO) analysis provides annotation of genes and their products with defined terms like biological process/molecular function/cellular component creating a hierarchical structure. To gain insights into the role of differentially expressed TFs in terminal drought tolerance in rice, GO functional enrichment analysis and gene regulatory network analysis were performed. We observed 177 enriched GO terms with up-regulated TFs and 102 enriched GO terms with down-regulated TFs with FDR ≤0.05. The GO terms associated with important biological processes and molecular functions in plants including 'regulation of transcription' (GO:0006355, GO:1903506), and 'regulation of RNA biosynthetic process' (GO:2001141) were enriched with up-regulated ~260 TFs/genes in the panicle of N-22 under terminal drought stress. The GO term 'regulation of transcription' has been reported to be crucial in drought tolerance in rice. Under drought stress, plants adjust transcriptional activities to adapt to the stressful conditions. These include differential expression of numerous genes related to different facets of drought stress response (e.g. water uptake and conservation), osmotic adjustment, and antioxidant defence (Kaur *et al.* 2023a). Some of the members of key TF families, such as WRKY, ERF, bHLH, MYB, C2H2, and MADS, exhibited significant upregulated expression (>10-fold) in the panicle of N-22 under drought stress. These TFs bind to specific *cis*-regulatory elements in the promoter of target genes to activate/repress their transcription in response to drought stress.

TFs play important roles in regulation of mRNA biosynthesis for the genes encoding stress-responsive proteins. Although some of the GO terms, such as 'regulation of transcription' (GO:0006355, GO:1903506), and 'regulation of RNA biosynthetic process' (GO:2001141) were enriched in the panicle of IR-64 under terminal drought stress, and they were associated with lesser number (~160) of genes, compared to (~260) genes in the case of N-22. Our comparative analysis indicated that both the cultivars exhibited enrichment of similar GO terms in response to terminal drought stress; however, N-22 exhibited a remarkably higher number of up-regulated TFs/genes associated with the GO terms (Fig. 3). Similarly, IR-64 had a larger number (254) of down-regulated transcription factors (TFs)/genes compared to N-22, which had a smaller number (60) of down-regulated TFs/genes associated with enriched GO terms in the panicle. The GO terms enriched with down-regulated TFs/genes in IR-64 under drought stress were more (254) in number than those (60) in the panicle of N-22 under stress.

Gene regulatory network analysis helps elucidating the interactions/relationships among the enriched gene sets, thereby sheds light on their functional significance. Our integrated gene regulatory network analysis for the TFs showed significant differences in terms of the enriched/interacting gene sets between N-22 and IR-64. In case of N-22, the GO term 'transcription regulator activity' showed notably higher enrichment, as depicted by the darker/bigger nodes. In the case of IR-64, GO terms such as 'DNA binding transcription factor activity' and 'transcription, DNA-templated' were more enriched with the down-regulated TF/gene sets. Such comparative analysis of the differentially expressed TFs/genes in the panicle of N-22 and IR-64 highlights the crucial role of TFs in gene regulatory networks elucidating the distinct responses of contrasting rice cultivars when imposed with reproductive stage drought stress.

The present study highlights the critical role of TFs in mitigating drought stress during the reproductive stage of rice. Nagina-22, the drought-tolerant cultivar, exhibited upregulation of numerous TFs involved in activating drought-responsive genes, significantly contributing to its drought resilience. Conversely, the drought-sensitive cultivar IR-64 showed downregulation of several essential TFs, rendering it vulnerable to reproductive stage drought stress. Our findings highlight the pivotal role of TFs in regulating gene networks to manage drought stress in rice. These insights provide valuable direction for further studies on TFs, aiding the development of rice cultivars better equipped to withstand climate challenges. This advancement is crucial for enhancing drought resistance in rice, a vital step toward ensuring global food security amid climate change.

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