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De novo transcriptome analysis identifies key genes involved in dehydration stress response in kodo millet (*Paspalum scrobiculatum* L.)

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ABSTRACT

Kodo millet (*Paspalum scrobiculatum* L.) is a small millet species known for its excellent nutritional and climate-resilient traits. To understand the genes and pathways underlying dehydration stress tolerance of kodo millet, the transcriptome of cultivar 'CO3' subjected to dehydration stress (0 h, 3 h, and 6 h) was sequenced. The study generated 239.1 million clean reads that identified 9201, 9814, and 2346 differentially expressed genes (DEGs) in 0 h vs. 3 h, 0 h vs. 6 h, and 3 h vs. 6 h libraries, respectively. The DEGs were found to be associated with vital molecular pathways, including hormone metabolism and signaling, antioxidant scavenging, photosynthesis, and cellular metabolism, and were validated using qRT-PCR. Also, a higher abundance of uncharacterized genes expressed during stress warrants further studies to characterize this class of genes to understand their role in dehydration stress response. Altogether, the study provides insights into the transcriptomic response of kodo millet during dehydration stress.

1. Introduction

Small millets are known for their nutritional and climate-resilient traits, and they are cultivated in the arid and semi-arid regions of the world. Among different small millet species, kodo millet (*P. scrobiculatum* L.) is grown predominantly in the marginal regions with limited rainfall and poor soil fertility [1]. This crop is reported to be domesticated in India (~3000 years ago), and presently, it is cultivated in countries including India, Thailand, Indonesia, Philippines, Vietnam, and West Africa [2]. India is the largest contributor of kodo millet, and its state-wise production includes Gujarat (0.07 lakh tons/yr), Chhattisgarh (0.17 lakh tons/yr), Uttar Pradesh (0.07 lakh tons/yr), Madhya Pradesh (0.5 lakh tons/yr) and Tamil Nadu (0.12 lakh tons/yr) [3]. Nutritionally, kodo millet is rich in fiber (9%), carbohydrates (66 g/100 g grain), proteins (11%), and calcium (27/100 mg grain) [4–6]. The higher lecithin content promotes easy digestibility, making the crop suitable for food and feed [7]. Further, the kodo millet grains have superior seed longevity, making them suitable for long-term storage [8]. Though the crop is one of the underutilized and neglected species, it is now gaining importance due to its potential in addressing food (hunger) and nutritional (hidden hunger) securities among the population [9,10]. From a research perspective, foxtail millet, pearl millet, and finger millet have received much research attention, while other millets are yet to be studied to understand their important traits. As kodo millet is known for its climate-resilient traits and better adaptation potential to different climatic conditions [11–13], studies on this crop to dissect the genetic determinants of these traits are imperative. In particular, dehydration tolerance is an important trait that is the immediate consequence of drought, salinity, and cold stresses [14]. The present-day crops (major cereals) are highly vulnerable to dehydration stress [15].

The survivability of plants during dehydration stress depends on the extent of stress and the ability of species to withstand the stress. Plants

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often utilize dehydration avoidance and tolerance mechanism to combat the water deficit conditions during dehydration stress [16,17]. Enhancing water uptake and retention by modifying root system and stomata closure are the crucial mechanisms that plants adopt during dehydration, which is regulated by a series of signaling pathways and hormone-dependent gene expressions [18,19]. Thus, studying the genes underlying dehydration mechanisms using transcriptomics approaches will provide insights into the repertoire of genes having a role in response to stress, signaling, and downstream activation of tolerance mechanisms [20,21]. Transcriptome sequencing has been a powerful tool to understand the molecular stress response in various crop plants. For instance, leaf epidermal transcriptome analysis unraveled the hormonal crosstalk and signaling mechanisms underlying drought stress in wild barley [22]. Comparative transcriptome study of drought-resistant and sensitive wheat cultivars revealed both induced and repressed genes involved in resistance and susceptibility responses [23]. Further, stress-responsive mechanisms have also been elucidated in millets by employing a transcriptomic approach [24,25]. In foxtail millet, comparative transcriptome analysis revealed several differentially expressed genes under dehydration stress [26]. The study identified 327 differentially expressed transcripts in tolerant cultivar, which were further validated by Reverse Northern and qPCR analyses. Similarly, transcriptome analysis identified drought-responsive genes in finger millet [27]. Well-watered and low moisture stressed finger millet samples were sequenced using the Illumina platform to identify several protein families associated with drought tolerance genes [27].

In pearl millet, de novo transcriptome profiling revealed the role of purine and tryptophan metabolism under drought stress [28]. In another study, comparative transcriptome analysis at two developmental stages pinpointed several drought-responsive genes involved in stress response [29]. Transcriptome and metabolite profiling revealed the role of phenylpropanoid-related pathways in drought tolerance in foxtail millet [30]. Also, de novo transcriptome analysis in little millet unveiled drought-responsive genes and pathways [31]. Despite these reports, no study has been made to dissect the transcriptomic complexity in kodo millet during the dehydration stress. Given this, the present study describes the RNA-seq analysis of kodo millet cultivar 'CO3' to identify dehydration-responsive genes under control and stress conditions. De novo assembly led to the identification of several known as well as novel genes, indicating their potential role in stress response. GO, and KEGG analysis highlighted the intricated pathways underlying dehydration-responsive signaling in kodo millet. Thus, the present study provides a comprehensive report on genome-wide transcriptome sequencing in kodo millet subjected to dehydration stress.

2. Materials and methods

2.1. Plant material and stress treatments

Seeds of kodo millet cultivar 'CO3' were obtained from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The seeds were sown in composite soil (agropeat and vermiculite in 3:1 ratio) and grown in Phytotron chamber (Percival Scientific Inc.) at 28 °C (day) and 24 °C (night) temperature. The relative humidity was maintained at 70% with a photoperiod of 14 h using a photon flux density of 500 μmol m^{-2} s^{-1}. The 21-day old seedlings were then uprooted, and the roots were rinsed in running tap water and blot-dried to remove the soil and water molecules adhering to the roots. The seedlings were then placed on beakers containing 20% polyethylene glycol 6000 (PEG-6000). The roots were completely immersed in 20% PEG-6000, followed by the collection of whole seedlings at 0 h (control), 3 h (early), and 6 h (late) by snap-freezing them in liquid nitrogen and storing at −80 °C until RNA isolation. Stress treatment and sample collection were performed in triplicates to ensure accuracy and reproducibility.

2.2. RNA isolation, library construction, and Illumina sequencing

The total RNA was isolated from control and stress-treated seedlings using Trizol reagent (Invitrogen), followed by purification with RNase-free DNaseI (Qiagen, Germany). The quality of isolated RNA was ascertained by agarose gel electrophoresis, spectrophotometer (NanoDrop, Thermo Scientific, USA), and Agilent 2100 bioanalyzer (Agilent Technologies, USA). Further, oligo (dT) beads were used for poly(A) mRNA enrichment from high-quality total RNA, which was further used for cDNAs library construction by following the instructions of Illumina TruSeq RNA Library Prep Kit. The library preparation and Illumina sequencing were performed in triplicates.

The paired-end sequencing of cDNA libraries was accomplished on an Illumina HiSeq 4000 platform, resulting in 100-bp long paired-end reads from each sample. The raw data files have been submitted to the NCBI SRA database under the BioProject PRJNA735015 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA735015?reviewer=6c5pqnv03l8fv5besidaki9m64). The quality check of raw sequence data was performed by considering various parameters, including base quality score distribution, average base content per read, and GC distribution in the reads. The fastq files were pre-processed by adapter removal and quality trimming based on the quality cutoff $Q \geq 30$ using the AdapterRemoval tool (v2.3.1) [32]. The rRNA was removed by aligning the sequences with the SILVA database using bwa (v0.7.17) aligner [33]. Further, the cleaned reads were assembled using Trinity (v2.8.5) [34] with default settings, which generated 218,058 transcripts. The redundant sequences were removed by clustering similar sequences using CD-HIT-EST (v4.6) [35]. The clustered transcripts were then filtered using Transdecoder (v5.3.0) (http://transdecoder.github.io) [36], leading to the identification of 132,887 transcripts.

2.3. Differential gene expression analysis

The assembled transcripts were aligned against the UniProt database using BlastX (v2.6.0) [37] with an *E*-value cutoff of $1e^{-3}$ [38]. The best BlastX hits were selected based on the query coverage, identity, similarity score, and description of each transcript. The transcript quantification was carried out with Salmon (v0.14.1) [39] using the Perl script in Trinity. DESeq2 (v1.20.0) program [40] was used for differential gene expression analysis with adjusted *P*-value cutoff <0.001 and Log2 fold-change up to (+1/−1).

2.4. Functional annotation and pathway analysis

The transcription factors involved in dehydration stress were annotated using BlastX against *Setaria italica* in the plant transcription factor database (PlantTFDB) (http://planttfdb.gao-lab.org/doewnload.php#tf_idseq) with the e-value cutoff of 10^{-10} [41]. Both upregulated and downregulated gene loci during dehydration stress were identified and represented through heatmap using the Microarray Experiment Viewer (MeV v5.2) [42]. Venn diagrams illustrating common and exclusive DEGs among control, 3 h, and 6 h of treatment were plotted using InteractiVenn [43]. The GO analysis of dehydration-responsive DEGs was performed using BLAST2Go [44]. REduce & VIsualize Gene Ontology (REVIGO) (http://revigo.irb.hr/) visualization tool was used to summarize GO terms based on their semantic similarities [45]. Pathway analysis was performed by mapping the DEGs to Kyoto Encyclopedia Genes and Genomes (KEGG) pathways database [46]. The network analysis to elucidate protein-protein interaction among putative genes was performed using STRING (Search Tool for Recurring Instances of Neighbouring Genes) database (http://string-db.org/; vX11.0) [47].

2.5. Validation by quantitative real-time PCR analysis

The transcriptome data was validated by quantitative real-time PCR

(qRT-PCR) analysis. Candidate dehydration-responsive genes showing significant differential expression were chosen based on the FPKM values and gene annotation data. The gene-specific primers (Supplementary Table S1) were designed for selected dehydration responsive genes using Primer Express Version 3.0. RNA was extracted from control and PEG treated kodo millet seedlings using Trizol reagent. cDNA was synthesized from respective RNA samples using the AffinityScript QPCR cDNA synthesis kit (Agilent Technologies). The qRT-PCR was performed using the AriaMx Real-Time PCR system (Agilent Technologies). *Actin2* was used as an internal standard for normalization. The experiments were performed with three biological and three technical replicates. The expression analysis of all transcripts was performed by calculating the fold change using $2^{-\Delta\Delta CT}$ method [48].

3. Results

3.1. De novo assembly and transcriptome analysis

To study the genes expressed during dehydration stress in kodo millet, the RNA-seq analysis was performed on 21 days-old seedlings under control conditions and PEG treatment. Pearson correlation analysis, based on the TPM (Transcripts Per Million) values, revealed the high R-value (≥0.78) between biological replicates that signifies great reproducibility and increased consistency in the RNA-seq data (Fig. 1A). The paired-end sequencing generated 351.3 million reads from three samples (including control and dehydration stress conditions with three biological replicates) with minimum 16.85 million reads per sample. About 239.1 million clean reads were obtained after data filtration (quality cut off Q ≥ 30) that led to the generation of 1,32,887 assembled transcripts (Supplementary Table S2). The average contigs length distribution was observed between 250 bp and 5000 bp, with maximum transcripts having a length between 1000 and 1500 bp and the longest transcript length of 16,692 bp. The average GC content of all transcripts was approximately 50.86 (Supplementary Table S2). The assembled transcripts were searched against the UniProt database using BlastX program with an *E*-value cutoff of 10^{-3}. Overall, 72,518 of 132,887 transcripts were annotated. Of these, 51.79% contigs showed an 80–100 similarity score, followed by 32.55% and 12.98% contigs with 60–80 and 40–60 similarity scores, respectively (Supplementary Fig. S1). Further, 17,222 transcripts shared similarities with *S. italica*, followed by 14,982 with *Sorghum bicolor*, 10,357 with *Zea mays*, 9105 with *Dichanthelium oligosanthes,* 4331 with *Arundo donax,* 1951 with *Triticum aestivum,* 1748 with *Oryza sativa* subsp. *japonica,* 1236 with *Brachypodium distachyon,* and 719 with *Hordeum vulgare* (Supplementary Fig. S2).

3.2. Analysis of dehydration responsive differentially expressed genes

Comparative analysis was performed between control and treated samples to identify differentially expressed genes (DEGs) in different combinations, viz. control vs. 3 h (C vs. 3 h), control vs. 6 h (C vs. 6 h) and 6 h vs. 3 h. In C vs. 3 h, 24,251 DEGs were identified, among which the significantly upregulated and downregulated genes were 5353 and 3848, respectively. Furthermore, 25,644 DEGs were estimated in C vs. 6 h, with 5462 upregulated and 4352 downregulated genes. Similarly,

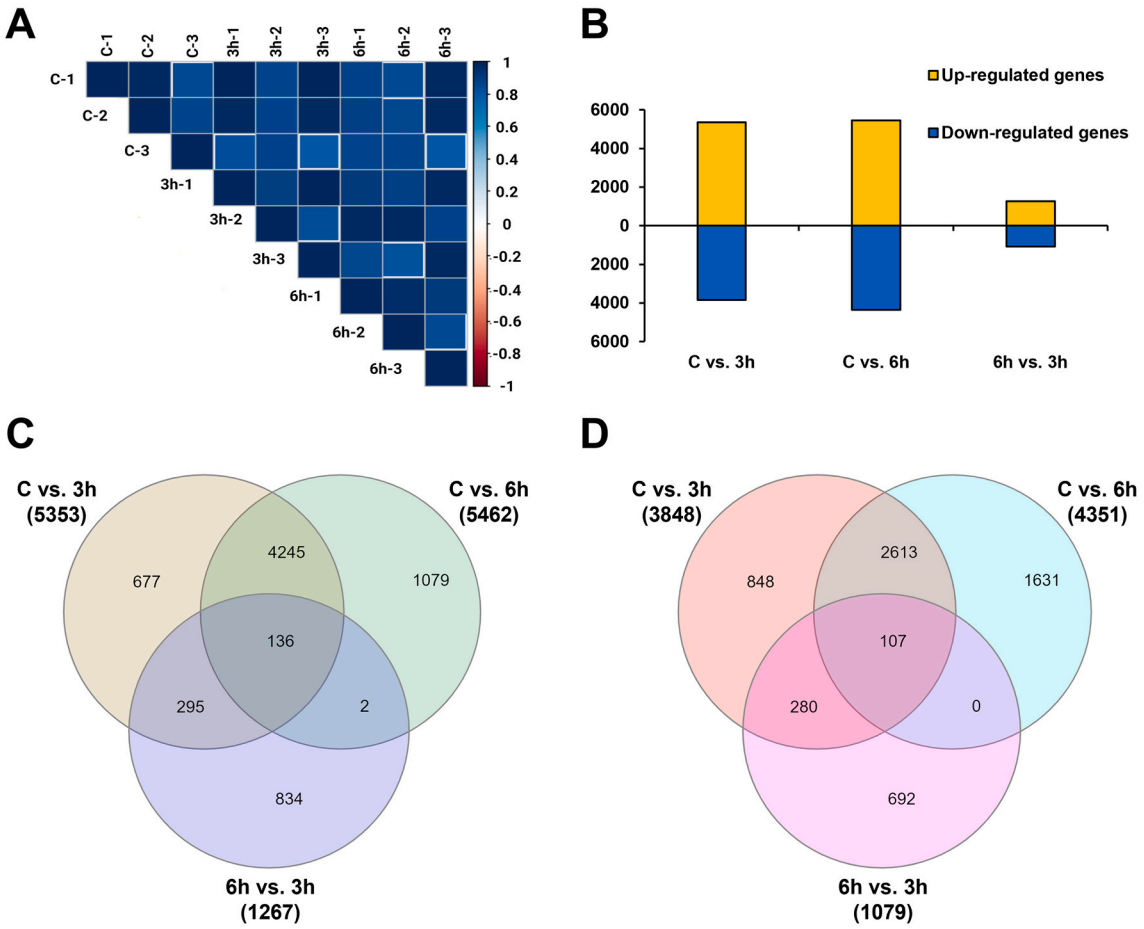

Fig. 1. Statistical analysis of transcriptome data and overview of DEGs. (A) Pearson correlation analysis of three biological replicates from control, 3 h, and 6 h post dehydration treatment. (B) The number of dehydration responsive differentially expressed genes (upregulated and downregulated) in C vs. 3 h, C vs. 6 h and 6 h vs. 3 h. Venn diagram representing the number of common and unique (C) upregulated and (D) downregulated DEGs in all the samples.

1267 upregulated and 1079 downregulated genes were observed in 6 h vs. 3 h (late versus early timepoint after stress) (Fig. 1B). The Venn diagram represented 4245 upregulated and 2613 downregulated genes common among C vs. 3 h and C vs. 6 h, respectively (Fig. 1C, D). Similarly, 295 upregulated and 280 downregulated genes were found to be common between C vs. 3 h and 6 h vs. 3 h, respectively (Fig. 1C, D). However, only two upregulated genes were common between C vs. 6 h and 6 h vs. 3 h (Fig. 1C). Additionally, 136 upregulated and 107 downregulated genes were common among all the three groups of DEGs (Fig. 1C, D). The number of DEGs identified in 6 h were higher than 3 h, suggesting the complex and active nature of dehydration stress responses at a later timepoint. Further, the volcano plots were generated to visualize the distribution of differentially expressed genes (Fig. 2A, B, C). Few of the most significant DEGs in C vs. 3 h were

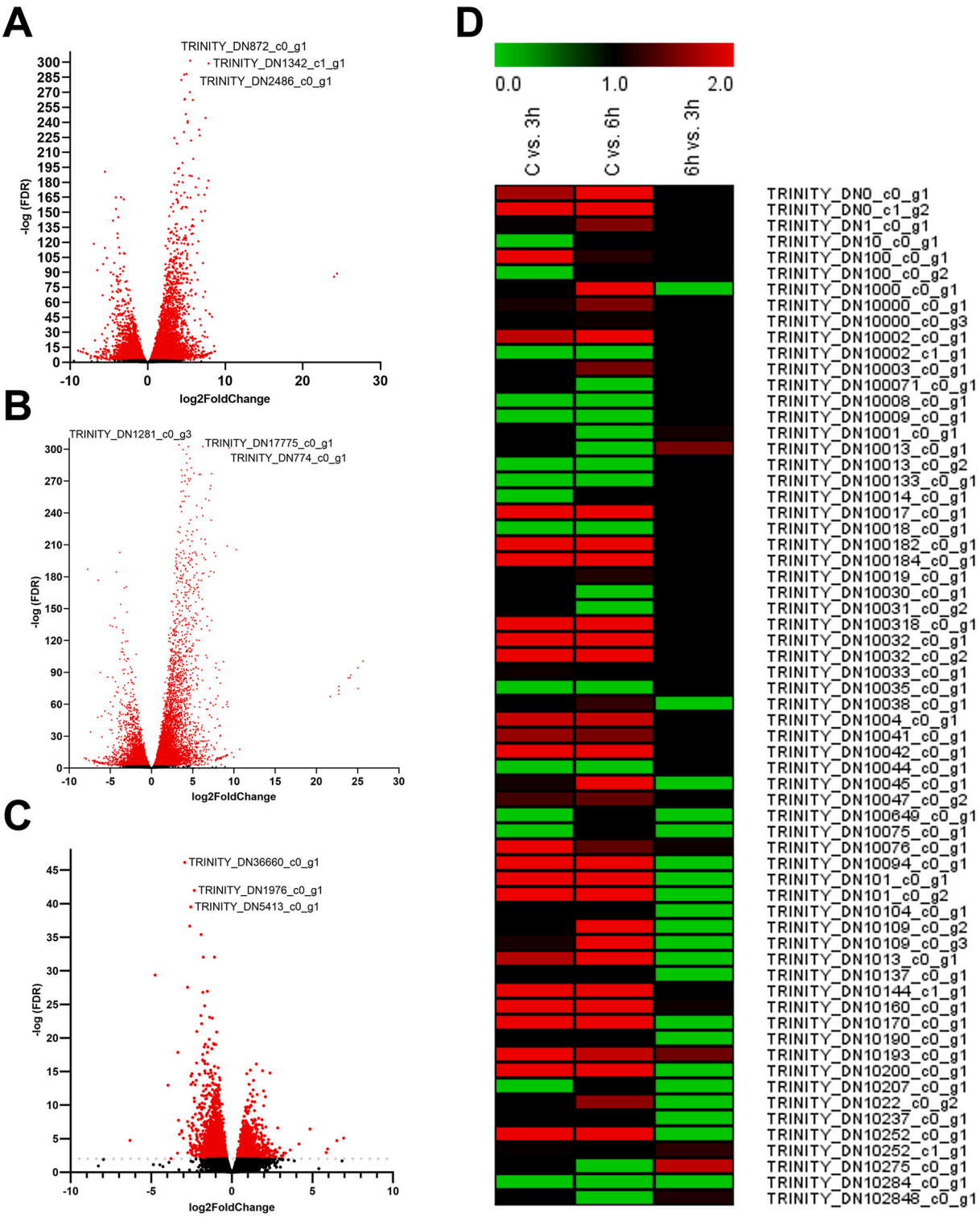

Fig. 2. Statistical significance and expression of top 30 dehydration stress-responsive DEGs. Volcano plot of statistically significant dehydration-responsive DEGs identified from the de novo RNA-seq analysis for, (A) C vs. 3 h, (B) C vs. 6 h, and (C) 6 h vs. 3 h. (D) Heat map expression of top 30 dehydration stress-responsive DEGs in C vs. 3 h, C vs. 6 h, and 6 h vs. 3 h.

TRINITY_DN872_c0_g1 (*WRK28_ORYSI*), *TRINITY_DN1342_c1_g1* (*WRK76_ORYSI*), *TRINITY_DN14325_c0_g1* (*TI11D_ORYSI*), and *TRINITY_DN4405_c0_g2* (*UGDH5_ORYSJ*) (Fig. 2A). Likewise, *TRINITY_DN17775_c0_g1* (*CRPK1_ARATH*), *TRINITY_DN1281_c0_g3* (*XTH22_ARATH*), *TRINITY_DN12801_c0_g2* (*RVE8_ARATH*), *TRINITY_DN5492_c0_g1* (*PIX13_ARATH*) and *TRINITY_DN6509_c0_g1* (*NDL1_ARATH*) were significantly differentially expressed in C vs. 6 h (Fig. 2B). Furthermore, the genes with significant differential expression identified in 6 h vs. 3 h group were *TRINITY_DN36660_c0_g1*, *TRINITY_DN1976_c0_g1* (*LHY_PETHY*), *TRINITY_DN5413_c0_g1*, and *TRINITY_DN60966_c0_g1* (*ACSS_MAIZE*) (Fig. 2C). The *WRKY28* and *WRKY76* were previously found to have a crucial role in regulating the root architecture and responses towards osmotic stress in rice [49]. Differential expression of these genes during the early phase (C vs. 3 h) suggested their role in affecting root system architecture under dehydration stress in kodo millet. Further, we analyzed the expression pattern of top 30 dehydration-responsive genes through heatmap scaled on expression values, and observed that all DEGs showed differential expression in at least one or both time points (3 h and 6 h) as compared to the control (Fig. 2D). The heatmap represented the upregulation of genes such as *PME51_ARATH* (*TRINITY_DN10033_c0_g1*), *TPRL2_ERYCB* (*TRINITY_DN10144_c1_g1*) and *B561P_ARATH* (*TRINITY_DN10193_c0_g1*) under C vs. 3 h or C vs. 6 h treatment, supporting the fact of their involvement in suppression of drought tolerance and root development in kodo millet [49,50]. However, many genes such as Zinc finger protein *DTX54_ARATH* (*TRINITY_DN10207_c0_g1*), *CTR1_ARATH* (*TRINITY_DN10018_c0_g1*), *RITF1_ARATH* (*TRINITY_DN10013_c0_g2*), *FTIP3_ARATH* (*TRINITY_DN10030_c0_g1*), and *EXPA4_ORYSJ* (*TRINITY_DN1001_c0_g1*) were exclusively downregulated under treatments, advocating their role in stress responses, suppression of cell elongation and shoot growth inhibition during dehydration stress in kodo millet [51,52].

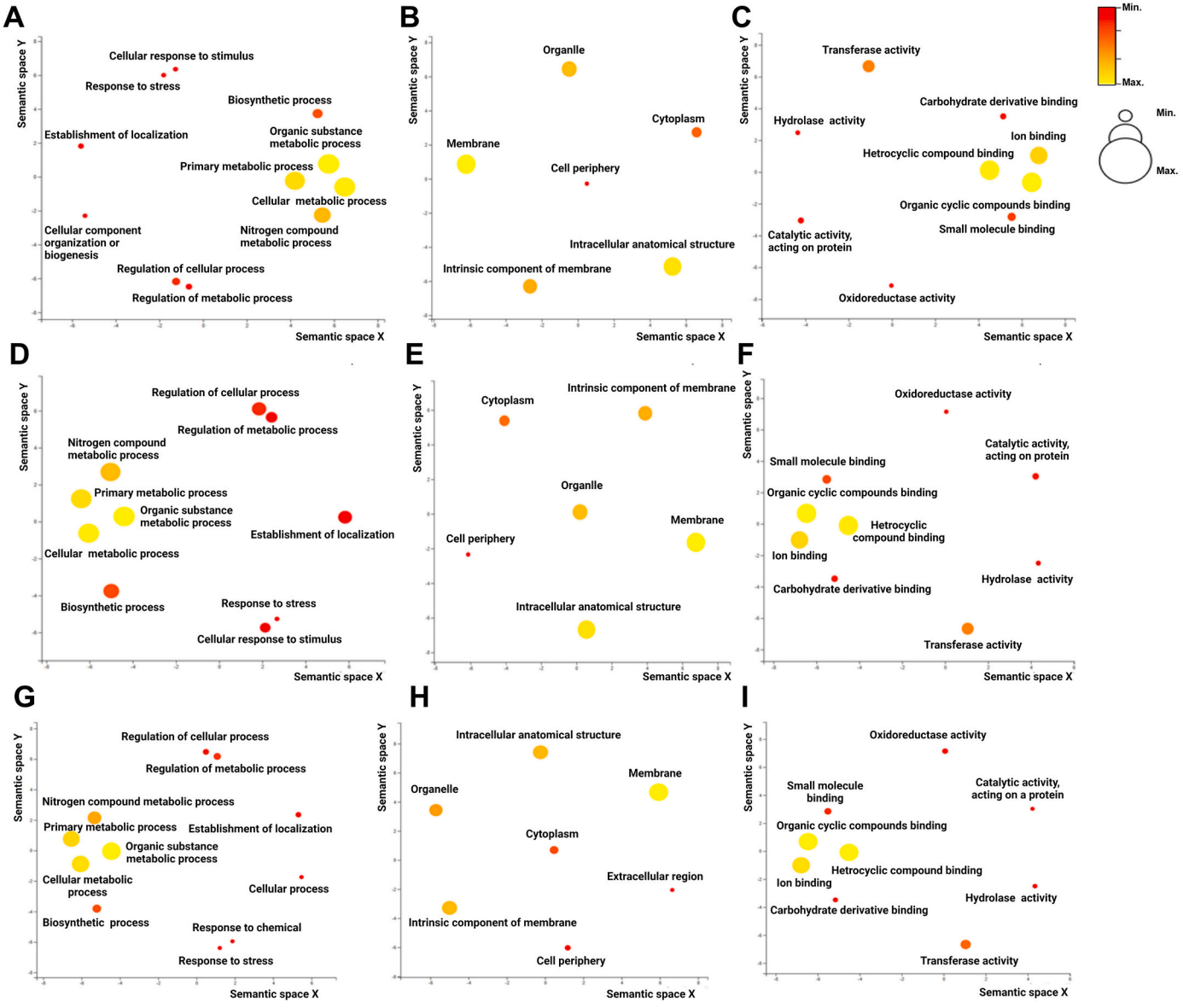

Fig. 3. Gene Ontology (GO) analysis of dehydration-responsive DEGs. REVIGO scatterplots showing GO analysis of dehydration-responsive DEGs. The scatterplots were derived by multidimensional scaling (MDS) of GO terms pairwise semantic similarities. (A, B, C) C vs. 3 h, (D, E, F) C vs. 6 h and (G, H, I) 6 h vs. 3 h. The yellow colour and size of node increase with the number of DEGs. Abbreviation: Biological process (BP), molecular function (MF) and cellular component (CC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Functional annotation and GO analysis of dehydration responsive DEGs

GO analysis of dehydration responsive DEGs indicated that a significant fraction of DEGs from all the treatments was found to be involved in metabolic and cellular processes, biosynthetic processes, response to stress, transferase activity, heterocyclic compound binding, membrane, intracellular anatomical structures and cytoplasm (Fig. 3). The GO terms were visualized by REVIGO scatterplots, derived by multidimensional scaling (MDS) of GO terms pairwise semantic similarities. Notably, the biological processes highly represented in all the samples were organic substance metabolic process, cellular metabolic process, primary metabolic process, biosynthetic processes, regulation of cellular and metabolic processes, response to stimulus, and stress (Fig. 3A, D, G). Moreover, the most represented GO terms in molecular function were compound and ion binding, transferase activity, small molecule and carbohydrate derivative binding, catalytic activity, and hydrolase activity (Fig. 3C, F, I). In addition, the significantly enriched GO terms in the cellular components category were membrane, intracellular anatomical structure, organelle, membrane compounds, and cytoplasm (Fig. 3B, E, H).

3.4. DEGs mediating dehydration stress response in kodo millet

Several DEGs were identified in our study with significant expression change and might have a potential role in mediating the dehydration stress responses (Supplementary Table S3), including *Beta-amylase 1*, Abscisic acid 8′-hydroxylase 3 (*ABA 8′-hydroxylase 3*), *Abscisic stress-ripening protein 1*, *BTB/POZ* and *MATH domain-containing protein 4*, *Calmodulin-binding protein 25*, Dehydrin *DHN1*, Protein *EARLY-RESPONSIVE TO DEHYDRATION 7*, *Late embryogenesis abundant protein 2*, *Probable protein phosphatase 2C 27*, and various transcription factors (TFs). These DEGs were involved in regulating abscisic acid activated signaling pathway, protein ubiquitination, cell wall organization, redox homeostasis, ethylene activated signaling, ion homeostasis, and developmental processes such as seed maturation flowering, stomatal complex development, meristem, and root development. Plants recognize the dehydration stress conditions in their roots and translocate the signals to other distant organs. Dehydrated plants accumulate ABA by activating ABA biosynthetic pathways. In kodo millet, ABA biosynthetic pathway gene such as Probable lysophospholipase *BODYGUARD 1* (*BDG1*) (*TRINITY_DN15179_c0_g2*) was found to be upregulated at an early stage. We observed the downregulation of cysteine biosynthetic gene, *methionine gamma-lyase* (*MGL*) (*TRINITY_DN6995_c0_g1*), which might be linked with reduced ABA accumulation during dehydration. Also, the genes involved in ABA catabolism, *Abscisic acid 8′-hydroxylase 3* (*TRINITY_DN744_c0_g1*) were 2-fold upregulated. Leucine-rich repeat receptor-like serine/threonine-protein kinase *BAM1* (*TRINITY_DN4443_c0_g1*), which perceives CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED (CLE) peptides produced in response to dehydration stress in roots for inducing ABA synthesis in the aerial tissue, was 2-fold downregulated in kodo millet. Aldehyde dehydrogenase family 3 member *I1* (*TRINITY_DN2703_c2_g1*), involved in detoxifying lipid peroxidation products, was only 1-fold upregulated during 3 h of dehydration stress; however, no expression was observed in the later stages. DEGs regulating phytohormone signaling and their metabolism such as, ABA pathway genes (*TRINITY_DN744_c0_g1, TRINITY_DN9015_c1_g1, TRINITY_DN4365_c1_g1, TRINITY_DN138_c0_g2*), ethylene-responsive genes (*TRINITY_DN1078_c1_g1, TRINITY_DN5570_c0_g1, TRINITY_DN1799_c0_g1*) were differentially expressed. *Abscisic acid 8′-hydroxylase 3* (*TRINITY_DN744_c0_g1*), involved in the oxidative degradation of ABA, was upregulated during dehydration stress, indicating dehydration-induced regulation of ABA level in kodo millet. Abscisic acid receptor *PYL9* (*TRINITY_DN9015_c1_g1*), involved in ABA-mediated stomatal closure, was 3-fold downregulated. Another ABA-responsive gene, *Late embryogenesis abundant protein 14* (*TRINITY_DN27896_c0_g1*) was also found to be downregulated under dehydration stress. Calcium transporter (*TRINITY_DN9889_c0_g2*) and Ca^{2+} binding proteins (*TRINITY_DN3891_c0_g2*) were differentially expressed in contrast to the *Calcium-transporting ATPase 5*, which did not show altered expression, which could be the reason for enhanced accumulation of Ca^{2+} in the cytosol. Ca^{2+} accumulation might induce calmodulin protein, followed by the activation of the calmodulin-binding protein, which is a negative regulator of stress tolerance. In consistence, we observed the upregulation of *calmodulin-binding protein 25* by 3-fold in our dataset. Several ubiquitin pathway genes (*TRINITY_DN10833_c0_g1, TRINITY_DN7065_c0_g1, TRINITY_DN10351_c0_g1*) were many-fold upregulated in our data under dehydration stress.

3.5. Analysis of metabolic pathways triggered under dehydration

Dehydration responsive DEGs were aligned against the KEGG (Kyoto and Encyclopedia of Genes and Genomes) database to identify potential pathways underlying dehydration stress response. KEGG analysis showed that 27, 36, and 12 pathways were significantly enriched in C vs. 3 h, C vs. 6 h, and 6 h vs. 3 h, respectively. Notably, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, glycolysis/gluconeogenesis, purine metabolism, carbon fixation, and sugar metabolism pathways were majorly abundant in C vs. 3 h and C vs. 6 h (Fig. 4A, B). Further, in 6 h vs. 3 h, the significantly enriched pathways were starch and sucrose metabolism, pyruvate metabolism, phenylpropanoid biosynthesis and glycolysis/gluconeogenesis (Fig. 4C). Abiotic stresses induce remobilization of starch reserves to release various sugars and metabolites to mitigate stress. Genes involved in starch and sucrose metabolism such as endo-1,4-beta-D-glucanase (EC:3.2.1.4) and trehalose 6-phosphatase (EC:3.1.3.12) were downregulated under dehydration stress. Genes involved in ascorbate metabolism, a potential oxidant scavenger, were highly enriched during the early and late stages of dehydration (Fig. 4A, B). (EC:1.3.2.3) L-galactono-1,4-lactone dehydrogenase 2 was downregulated in kodo millet under dehydration stress, which obstructs the ascorbic acid-mediated modulation of physiological and biochemical processes. Enzymes essential for sugar metabolism (EC:2.7.1.90–1-phosphotransferase, EC:5.3.1.5 - isomerase), fatty acid metabolism (EC:6.2.1.3 - ligase, EC:2.3.1.85 - synthase system, EC:1.2.1.3 - dehydrogenase (NAD+)) and secondary metabolite biosynthesis (EC:2.5.1.84 - diphosphate synthase [geranyl-diphosphate specific], EC:1.3.1.77 - reductase [(2*R*,3*R*)-flavan-3-ol-forming]) also exhibited altered expression in our data. In addition, glutamine synthetase (EC:6.3.1.2 - synthetase), involved in nitrogen assimilation, was not upregulated significantly, leading to reduced amino acid accumulation followed by a decrease in carbon reservoir during dehydration. Further, genes involved in inositol phosphate metabolism, such as L-myo-inositol phosphate synthase (MIPS; EC:5.5.1.4), did not show altered expression, whereas DEGs involved in phytohormonal metabolic pathways such as JA pathway (EC:5.3.99.6 - cyclase) exhibited differential expression under dehydration stress.

3.6. STRING-based network analysis of dehydration responsive DEGs

Among dehydration responsive genes, protein-protein interaction was studied using STRING network analysis database with a confidence score of >0.5 (Supplementary Fig. S3; Supplementary Table S4). Our study revealed that ABI5 (Abscisic Acid INSENSITIVE 5), an important member of ABA-dependent stress response, showed interaction with SnRK2.2/3 (SNF1-related protein kinase 2), and SIZ1. This SUMO E3 ligase is a major regulator of developmental processes during water deficit conditions. ABI5 also showed interaction with KEG (ubiquitin-protein ligases), a negative regulator of ABA signaling and responsible for ABI5 degradation during the stress response. Likewise, bHLH148, involved in regulating JA-induced gene expression, interacted with other JA signaling components having a similar role in regulating stress-

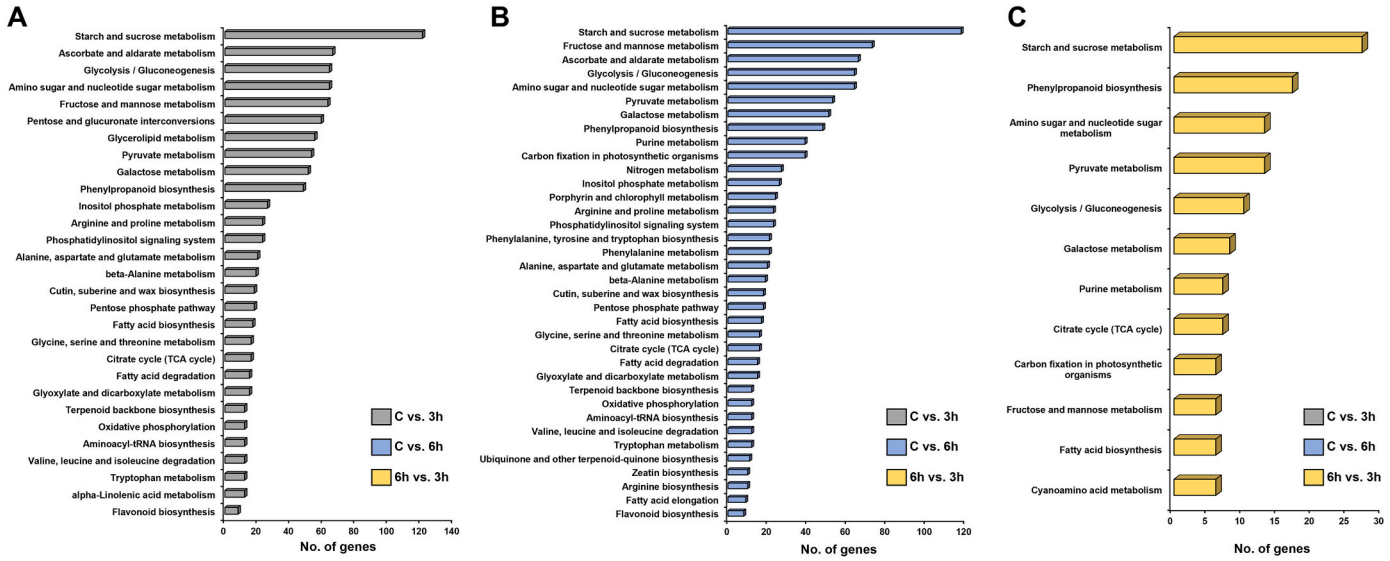

Fig. 4. KEGG pathway analysis. KEGG-enriched metabolic and hormonal pathways in (A) C vs. 3 h, (B) C vs. 6 h, and (C) 6 h vs. 3 h dehydration stress.

mediated JA pathway (Supplementary Fig. S3). Notably, dehydrin (DHN1) exhibited interaction with several proteins known to be involved in dehydration stress response (Supplementary Fig. S3). Further, TFs such as ABF3 (bZIP46), ERF113, and WRKY70 showed interactions with dehydration responsive, DREB2A, SARD1 (Calmodulin binding protein-like), SAPK2 (Stress/ABA activated protein kinase 2) and SUT (Sucrose Transporters) (Supplementary Fig. S3). Another important calmodulin (CAM)-binding protein, CAMBP25, induced by dehydration stress, showed interaction with stress-responsive proteins, WRKY33, MKS1 (MAP kinase substrate 1), WRKY25, Sigma factor binding protein 1 (SIB1) and DIC2 (Mitochondrial uncoupling protein 4) (Supplementary Fig. S3).

3.7. Identification of dehydration responsive transcription factors

A total of 1305 transcription factors (TFs) representing 48 TF families were identified (Supplementary Table S5). TF families with five or more genes are shown in Fig. 5A, which revealed that the most abundant TFs categories were MYB-related family proteins (319), WRKY (124), NAC (120), bHLH (99), MYB (93), ERF (85), C2H2 (56) and bZIP (48). Altogether, 507 TFs were upregulated, and 467 were downregulated at C vs. 3 h, whereas 487 were upregulated and 602 were downregulated in C vs. 6 h (Fig. 5B). MYBs play a crucial role in the regulation of development and stress responses in plants [53]. Overexpression of MYB confers dehydration tolerance in plants by reducing water loss and malondialdehyde level [53]. Similarly, WRKY TFs are major regulators of various abiotic stresses, including dehydration [54]. Constitutive expression of WRKY enhanced the tolerance against dehydration [54]. WRKYs identified in our data showed differential expression during dehydration stress at different time points, suggesting their significant role in regulating dehydration stress responses in kodo millet. Further, 120 NAC TFs were identified, of which 67 were upregulated, whereas 53 showed downregulation at both time points, indicating their role in the dehydration responsive signaling pathways (Fig. 5C; Supplementary Table S5). Several TFs, like *bZIP* (TRINITY_DN1840_c0_g1, TRINITY_DN14052_c0_g1), *ERF* (TRINITY_DN1078_c1_g1, TRINITY_DN1553_c1_g2), *NAC* (TRINITY_DN23685_c1_g1, TRINITY_DN2799_c2_g3), *WRKY* (TRINITY_DN383_c0_g1), *bHLH* (TRINITY_DN6018_c0_g2, TRINITY_DN23435_c0_g1), *MYB* (TRINITY_DN4708_c1_g1, TRINITY_DN13022_c0_g1, TRINITY_DN16749_c0_g1) are known to regulate dehydration responses, and were showing significant differential expression in kodo millet under dehydration stress. Differential expression of TFs such as AP2/ERF2 C2H2, bZIP, GRAS, HD-ZIP, ARF, HSF, and Trihelix were consistent with the expression of orthologous genes, suggesting their crucial role in mediating dehydration stress response in kodo millet. The TFs families with five or less genes are shown in Fig. 5C. Among these, CAMTA, EIL, M-type MADS, RAV, and NF-YB transcription factor families were identified. These TFs were previously known to regulate various developmental and stress responses during dehydration [55–58].

3.8. Validation of dehydration-responsive differentially expressed genes

The expression data based on the RNA-Seq experiment were validated using qRT-PCR analysis. Eight DEGs were chosen from the expression and annotation data for the validation, and the data showed a higher correlation of qRT-PCR data with the FPKM values predicted for each gene using RNA-seq (Fig. 6; Supplementary Fig. S4). Five genes, viz., *TRINITY_DN12801_c0_g2, TRINITY_DN1342_c1_g1, TRINITY_DN21125_c0_g1,* and *TRINITY_DN4676_c0_g1* showed upregulated expression during dehydration stress compared to control. Notably, the expression of *TRINITY_DN1342_c1_g1, TRINITY_DN21125_c0_g1,* and *TRINITY_DN12801_c0_g2* was found to be significantly upregulated at C vs. 3 h sample. Two genes, viz., *TRINITY_DN30318_c0_g1* and *TRINITY_DN8821_c0_g3,* showed significantly downregulated expression during dehydration stress conditions compared to control (Fig. 6).

4. Discussion

The transcriptome of kodo millet cultivar ‘CO3’ was studied to unravel the intricate signaling pathways and identify the key players underlying dehydration stress response. This study identified dehydration-responsive DEGs/transcripts in kodo millet, and their expression profile in the seedling showed their involvement in regulating the stress response. DEGs involved in several biological, cellular and molecular processes and metabolic pathways under dehydration stress were identified (Fig. 7). Previously, it has been shown that dehydration stress responses are mediated by complex signaling and metabolic pathways which are regulated by various hormones, particularly the stress hormones ABA [19]. The onset of dehydration stress induces ABA-mediated stomatal closure to minimize the water loss, followed by a reduction in the photosynthetic efficiency [17]. These dehydration stress responses may be attributed to ROS production, which leads to the degradation of proteins, lipids, and DNA [59]. In the present study, various transporters have been identified, which are known to maintain the spatial

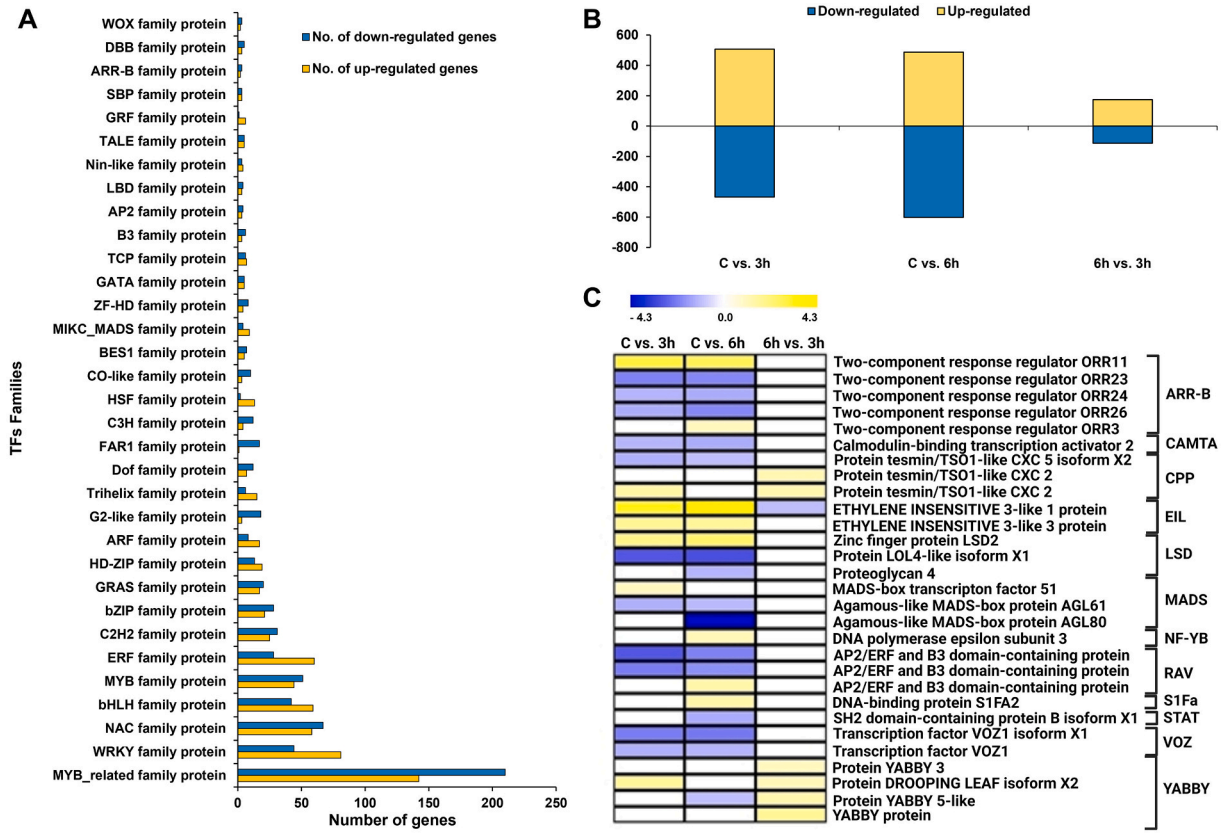

Fig. 5. Overview of differentially expressed transcription factors (TFs). (A) Transcription factor families are highly represented in kodo millet under dehydration stress. (B) The number of upregulated and downregulated transcription factors in stress, C vs. 3 h, C vs. 6 h, and 6 h vs. 3 h. (C) Expression profiling of candidate transcription factors in C vs. 3 h, C vs. 6 h, and 6 h vs. 3 h treatments under dehydration stress.

concentration of the hormone across various tissues [57,60]. The transporter family, DETOXIFICATION EFFLUX CARRIER 50 (DTX50), has been identified, which is majorly expressed in vascular tissue. DTX50 upregulation during dehydration stress reduces the ABA concentration in guard cells, leading to a stomatal opening that enhances sensitivity towards dehydration conditions [61]. We observed a similar expression of DTX50 in kodo millet under dehydration stress, which could be linked to reduced stomatal closure. Further, ABA-mediated responses are regulated by various receptors, which upon ABA binding activates several TFs that stimulate ABA-responsive genes, leading to growth and developmental changes under stress conditions [62]. For instance, downregulation of *bZIP12* and *bZIP46* was observed in kodo millet. This observation was similar to the results obtained in sesame and rice [63,64], therefore suggesting their involvement in negative regulation of dehydration stress response in kodo millet. Further, leaf senescence is also a crucial ABA-responsive developmental change in plants under dehydration conditions, which enhances the translocation of nutrients to developing and storage tissues of plants to maintain growth and productivity [65]. We observed the reduced expression of ABA-receptor PYL9, which is a prime regulator of leaf senescence. These results suggest delayed senescence and subsequent obstruction of nutrient translocation to developing tissue, thereby reducing survival under dehydration conditions. In addition, differential expression of different classes of ABA-responsive genes was observed, such as catabolic enzyme: ABA 8′-hydroxylase, transcription factor: ABA Insensitive 5 (*ABI5*), ABA signaling component: Aspartic Protease in Guard Cell 1 (*ASPG1*), ABA-regulated RNA binding protein (*ARP1*), Glycine-rich RNA-binding protein 4 (*RBG4*), Abscisic acid Stress Ripening (*ASR*), *EARLY RESPONSIVE TO DEHYDRATION 15 (ERD15),* Dehydrins (*DHNs*) and late embryogenesis abundant (LEA) proteins. These results were comparable to other crops, which advocate the ABA-mediated regulation of growth retardation, activation of stress signaling, stomatal closure, and altered germination in kodo millet [66–72].

The calcium signaling components showed the differential expression in kodo millet. The enhanced expression of Ca^{2+}ATPases triggers stress-responsive signaling and maintains growth and development during dehydration [73]. Although, the expression of Ca^{2+}ATPases was not significantly altered under dehydration in kodo millet. Further, the transient changes in Ca^{+2} level are recognized by various sensors such as calmodulin (CaM) and calmodulin-like protein (CMLs) to mediate downstream signaling [74]. A gene encoding calmodulin-binding protein, *CaMBP25,* was more than 3-fold upregulated at 6 h of dehydration stress in kodo millet. This observation is comparable with the results observed in Arabidopsis, where *AtCaMBP25* overexpressing lines showed increased sensitivity against osmotic stress [75].

The downregulation of RING-H2 finger protein-encoding gene, *ATL61,* enhances the stress susceptibility in kodo millet, and this observation is in comparison with the results obtained in tomato, where constitutive expression of *ShATL78L* showed enhanced abiotic stress tolerance [76]. RING E3 ligase *RGLG1* and U-box E3 ubiquitin ligase *PUB22* were upregulated in kodo millet. These results suggest the regulation of dehydration response via ubiquitination [76,77]. However, negative regulation of drought stress in Arabidopsis by *AtPUB22* and *AtPUB23* is well studied, supporting the fact that more than 4-fold expression of these genes in kodo millet reduces stress tolerance by inducing the ABA receptor PYL9 degradation and subsequently hampering ABA signaling [78]. Further, plant cuticle is an important component to protect the plant from excessive water loss during dehydration stress [79]. Lysophospholipase BODYGUARD 1, is very crucial for cuticle structure. However, the allelic form of *bdg, cool breath (cb)*, which is responsible for defective cuticle formation, was found to be upregulated in kodo millet, resulting in enhanced transpiration under

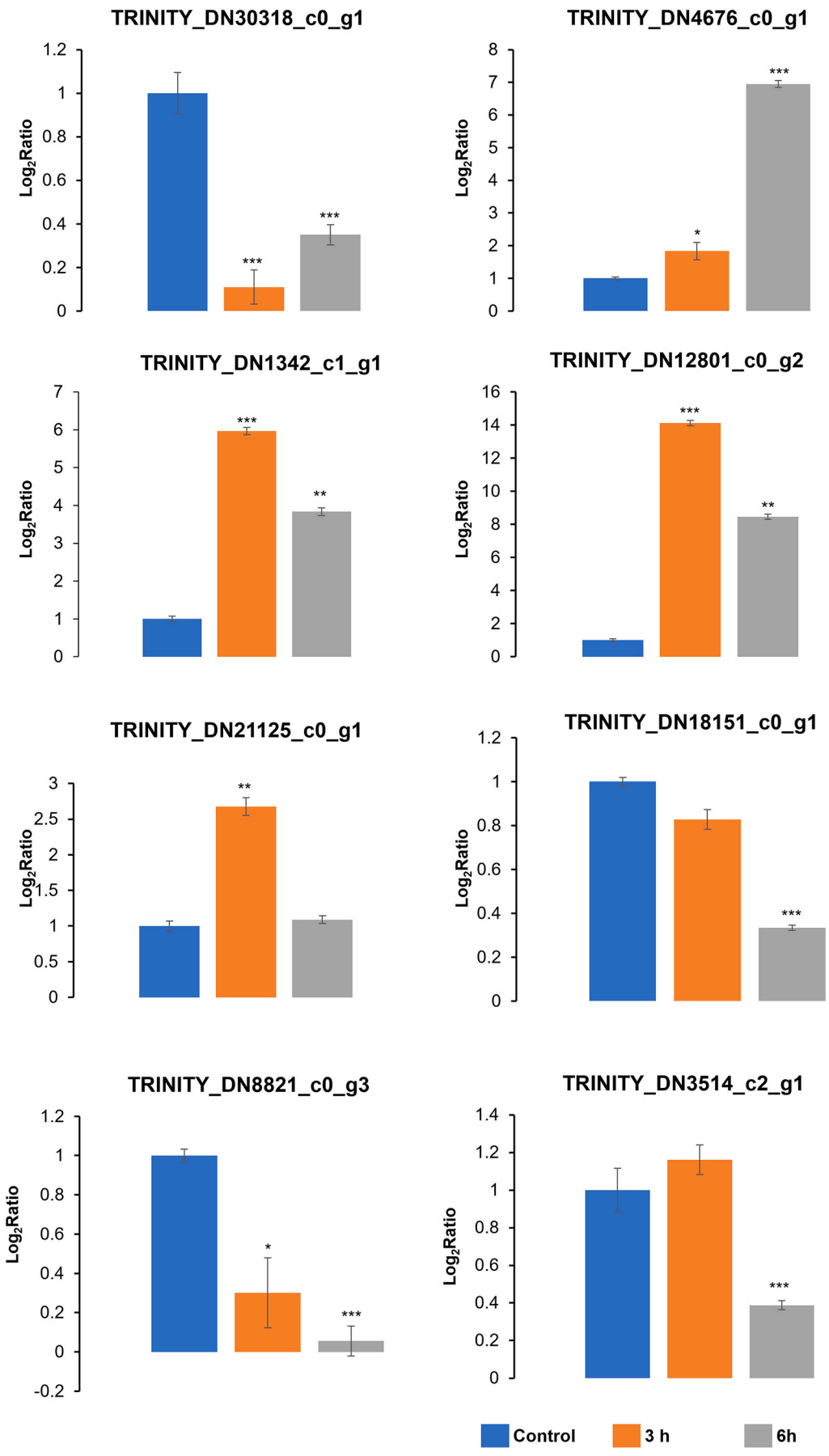

Fig. 6. Expression of candidate genes deduced using qRT-PCR. Validation of RNA-seq data of eight DEGs, namely, TRINITY_DN12801_c0_g2, TRINITY_DN1342_c1_g1, TRINITY_DN4676_c0_g1, TRINITY_DN3514_c2_g1, TRINITY_DN18151_c0_g1, TRINITY_DN8821_c0_g3, TRINITY_DN30318_c0_g1, and TRINITY_DN21125_c0_g1, representing differential expression under dehydration stress in kodo millet. The bars represent mean fold-change calculated from biological and technical replicates of samples along with their corresponding standard deviation. The asterisks indicate significant difference calculated by Student *t*-test with *P*-value. **P*-value <0.05; ***P*-value <0.001; ****P*-value <0.0001 by t-test.

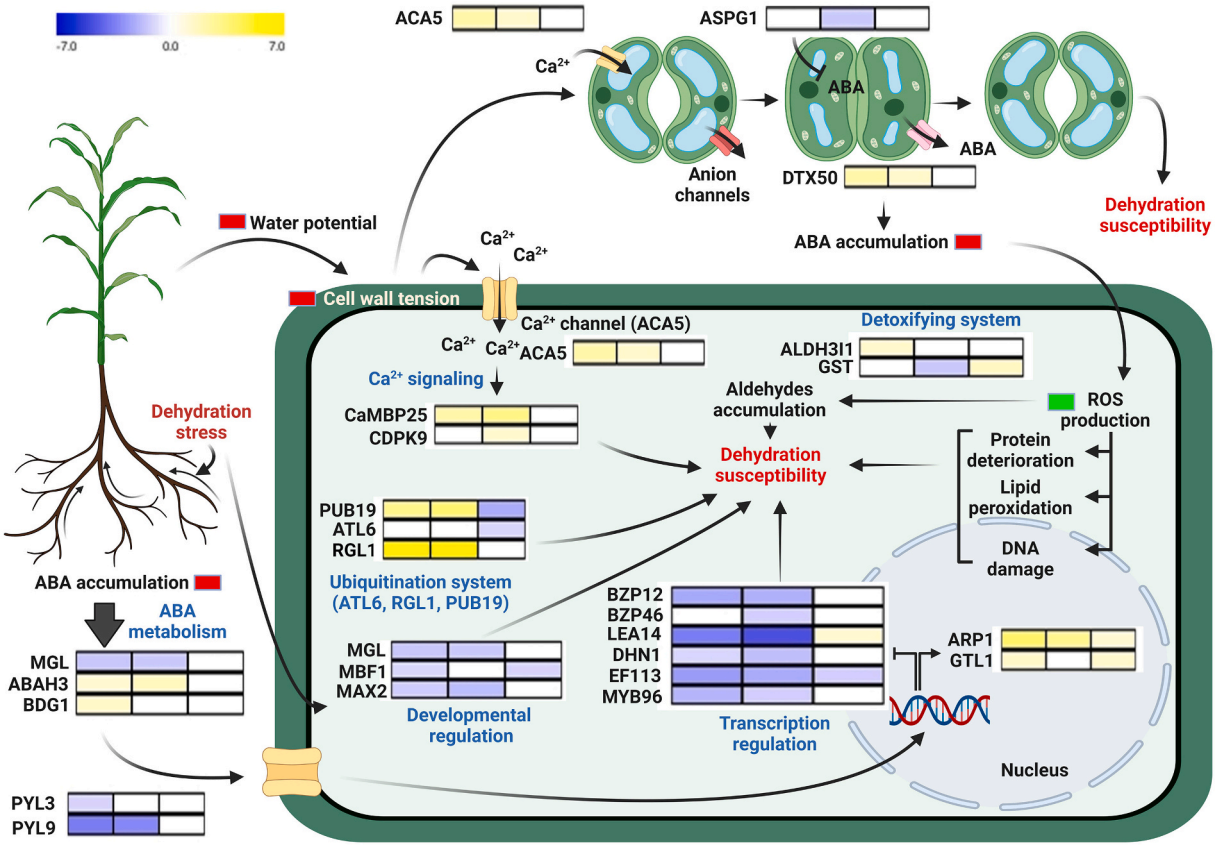

Fig. 7. Schematic representation of molecular mechanisms and the underlying differentially expressed genes involved in dehydration stress response in kodo millet. Red and green box indicates repression and induction of the biological process, respectively, during dehydration stress. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dehydration [80].

Transcription factors play a crucial role in regulating signaling pathways. We identified transcripts encoding ERF113, MYB96, MYB3R-2, SRM1 (MYB), bZIP12, bZIP46, ABI5 (bZIP) and NAC48, which showed differential expression during dehydration stress in kodo millet. These TFs are known for regulating stomatal closure, antioxidant enzyme activity, cuticular wax biosynthesis, ABA biosynthesis and signaling, pollen maturation, seed germination, membrane modification, phosphoadenosine phosphosulfate accumulation and root growth under dehydration stress various crops [81–87]. Upregulation of transcripts encoding WRKY70, bHLH112, and bHLH148 suggested their regulatory role in suppressing brassinosteroid, jasmonic acid, ethylene mediated stress responses, preventing stomatal closure, and enhanced osmotic stress [88–90]. Similar results were shown in Arabidopsis, which demonstrated negative regulation of senescence and defense signaling pathways by WRKY70 [89]. Trihelix transcription factor *GTL1* (GTL-1) is known to enhance stomatal development and density; hence, dehydration-responsive upregulation in kodo millet increases water loss due to increased stomatal density [91]. Further, upregulation of an ethylene-responsive TF, *RAP2–4*, causes defects in various developmental processes and suppresses dehydration tolerance in kodo millet [92]. It is known that developmental genes such as *More Axillary Growth2 (MAX2)* and *Multiprotein bridging factor 1 (MBF1)* regulate shoot and lateral root growth by mediating ethylene-response signal transduction [93–96]. Dehydration responsive downregulation of these genes makes kodo millet sensitive. The metabolic profiles also change during dehydration stress in plants; thus, the metabolic enzymes showed stress-responsive differential expression. Downregulation of Met γ-lyase (*MGL*), which is involved in Met (Methionine) homeostasis and Ile (Isoleucine) synthesis, would confer susceptibility response in kodo millet under dehydration in the similar way as *AtMGL* in Arabidopsis [97]. Two genes, viz., TRINITY_DN13040_c0_g1, and TRINITY_DN23293_c0_g2, showed 24-fold upregulation under C vs. 3 h and C vs. 6 h, respectively, of dehydration stress, respectively, suggesting their role in modulating stress response in kodo millet. Also, TRINITY_DN18151_c0_g1 and TRINITY_DN12488_c0_g1 showed 8-fold downregulation under C vs. 3 h and C vs. 6 h, respectively. Therefore, functional characterization of these genes to understand their role in modulating dehydration stress-responses in kodo millet might identify novel candidates to develop climate-resilient cultivars.

The de novo RNA-seq analysis of dehydration sensitive, kodo millet cv. 'CO3' has thus provided a comprehensive understanding of dehydration response mechanisms. It demonstrated several molecular and metabolic pathways regulated by various hormones and stress-responsive genes under dehydration conditions. DEGs encoding transporters, transcription factors, and metabolic enzymes associated with dehydration response were identified in kodo millet. The study also identified several uncharacterized genes with differential expression during dehydration stress, eventually paving the way for functional characterization of these genes to understand their role in dehydration stress response. Further downstream characterization of candidate genes identified in the present study will provide additional insights into their precise functioning in stress tolerance.

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Declarations of competing interest

The authors declare no conflict of interest.

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