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Unravelling the molecular mechanism underlying drought stress tolerance in Dinanath (*Pennisetum pedicellatum Trin.*) grass *via* integrated transcriptomic and metabolomic analyses

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Abstract

Dinanath grass (*Pennisetum pedicellatum Trin.*) is an extensively grown forage grass known for its significant drought resilience. In order to comprehensively grasp the adaptive mechanism of Dinanath grass in response to water deficient conditions, transcriptomic and metabolomics were applied in the leaves of Dinanath grass exposed to two distinct drought intensities (48-hour and 96-hour). Transcriptomic analysis of Dinanath grass leaves revealed that a total of 218 and 704 genes were differentially expressed under 48- and 96-hour drought conditions, respectively. The genes that were expressed differently (DEGs) and the metabolites that accumulated in response to 48-hour drought stress mainly showed enrichment in the biosynthesis of secondary metabolites, particularly phenolics and flavonoids. Conversely, under 96-hour drought conditions, the enriched pathways predominantly involved lipid metabolism, specifically sterol lipids. In particular, phenylpropanoid pathway and brassinosteroid signaling played a crucial role in drought response to 48- and 96-hour water deficit conditions, respectively. This variation in drought response indicates that the adaptation mechanism in Dinanath grass is highly dependent on the intensity of drought stress. In addition, different genes associated with phenylpropanoid and fatty acid biosynthesis, as well as signal transduction pathways namely phenylalanine ammonia-lyase, putrescine hydroxycinnamoyl transferase, abscisic acid 8'-hydroxylase 2, syntaxin-61, lipoxygenase 5, calcium-dependent protein kinase and phospholipase D alpha one, positively regulated with drought tolerance. Combined transcriptomic and metabolomic analyses highlights the outstanding involvement of regulatory pathways related to secondary cell wall thickening and lignin biosynthesis in imparting drought tolerance to Dinanath grass leaves.

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These findings collectively contribute to an enhanced understanding of candidate genes and key metabolites relevant to drought response in Dinanath grass. Furthermore, they establish a groundwork for the creation of a transcriptome database aimed at developing abiotic stress-tolerant grasses and major crop varieties through both transgenic and genome editing approaches.

Keywords Dinanath grass (*Pennisetum pedicellatum Trin*), Drought tolerance, Transcriptome sequencing, Metabolomics

Introduction

Grasses are C4 plants that thrive well in harsh environments like drought, salinity, and high temperature and, act as a desirable source of secondary gene pools for producing crop varieties with higher yield and resistance attributes [1, 2]. Though many grasses are under study, Dinanath grass (*Pennisetum pedicellatum Trin.*) known for its high drought tolerance and gaining popularity for its high forage yield and quality. It is one of the majorly cultivated and demanded fodder grasses belonging to Poaceae family and is widely distributed in West Africa, Southeast Asia, and northern Australia [3, 4]. Dinanath grass is known to be a quick-growing forage grass with potential drought tolerance and water loss maintenance, that enables it to thrive and perform well even on poor, eroded soils in areas receiving 500–1500 mm annual rainfall [5]. It is a multipurpose crop but due to its high nutritional value and high fodder quality Dinanath grass is prominently used as green fodder for animal feed [6].

The high demand for food productivity is increasing dramatically with the projected world population, which is predicted to be 9.1 billion by 2050 [7]. The relentless and ongoing climate change, coupled with the resulting unfavourable abiotic and biotic stresses, poses a significant threat to global crop production and quality. This escalating challenge complicates the already demanding task of ensuring food production is sufficient to meet the needs of a growing population [8]. Drought is one of the most detrimental environmental stress factors that limits crop yield and production [9]. In recent years, unpredictable weather patterns and escalating water pollution have intensified the frequency of global water shortage and drought severity, which in turn is affecting crop yield [10]. Crop breeding programs strive to enhance crop productivity by incorporating drought-tolerant genes through genetic advancements. The integration of state-of-the-art genomic tools and a deeper comprehension of physiological processes has the potential to revolutionize agricultural production, significantly amplifying the genetic gain associated with genes that provide tolerance to drought stress [11]. Meanwhile, the rapid evolution of crop species through domestication, selection, and plant breeding activities has led to a limited genetic diversity. The high selection pressure on genomic regions contributing to current ideotypes in crops such as rice, wheat, maize, bajra, and pulses has resulted in the loss of many desirable wild genes [12]. Dinanath grass, possessing wild desirable genes for drought tolerance, warrants more attention from researchers. As an apomictic crop, gene flow is restricted through cross-pollination and traditional plant breeding techniques. Consequently, it is crucial to identify genes associated with drought tolerance, which can be used to develop drought-resistant cultivars in food crops through transgenic or gene editing approaches.

De novo sequencing of transcriptomes from Dinanath grass can lead to the discovery of a set of novel genes different from those present in current cultivable crops. Meanwhile, studies have yet to be done to discover genes and molecular pathways in Dinanath grass. Transcriptome analysis was successful in crops like tea, tomatoes, rice, and wheat in deciphering major pathways regulating drought tolerance [12]. Therefore, this approach is suitable for prospecting genes underlying drought tolerance in Dinanath grass. With this concept, the present study investigated the alteration in gene expression and metabolomic changes in response to drought stress in Dinanath grass through transcriptomic and metabolomic analysis. The primary objective of this research is to elucidate the physiological mechanism of drought tolerance operating in Dinanath grass and to discover genes and molecular pathways playing a role in its ability to tolerate drought stress.

The study utilized transcriptomic analysis to investigate the variation in gene expression and the regulatory pathways associated with the drought response in Dinanath grass. RNA Sequencing (RNA-Seq) using next-generation sequencing (NGS) was utilized to analyze expression across the transcriptome of Dinanath grass leaves. Transcriptomic methods involving RNA-Seq have a dynamic range of expression levels and, thus, provide a better approach to detecting differentially expressed genes (DEGs) in drought studies [13]. On the other hand, the relationship between plants and the environment can be deeply understood from the metabolic level as metabolites are the final products of the cell regulation process [14]. Here we explore the metabolomic changes in response to drought stress at two different periods in Dinanath grass using the liquid chromatography—high resolution mass spectrometry (LC-HRMS) metabolite profiling method. With the advancement of analytical technology, the LC-MS metabolomic screening approach

has been widely used for tracking metabolomics changes. LC-MS is a rapid metabolite profiling method that is repeatable and appropriate for screening large numbers of samples. The method captures a wide range of compounds, including primary and secondary metabolites, enabling a better understanding of the regulation mechanism of plants under stress [15, 16]. The results of this research will position Dinanath grass as a model plant for investigating orphan fodder crops such as grasses and other millet crops. The generated data will facilitate the creation of genomic resources that help discover markers like SSRs and SNPs used in improving crops for drought stress tolerance.

Materials & methods

Plant materials and growth conditions

Bundel Dinanath 2 cultivar seeds were obtained from an extensive collection of Dinanath grass accessions preserved in ICAR-Indian Grassland and Fodder Research Institute, Jhansi, India. Seeds were sown and grown in pots (25 cm diameter and 30 cm depth) containing fine loamy textured soil and maintained under standard conditions in the net house of the institute. After germination, the plants were divided into three groups, namely well-watered (LC), 48-hour drought exposed (LT1), and 96-hour drought exposed (LT2), and were grown further in the same manner (Fig. 1). All plants grew under the same water conditions until 45 days; watering was withheld for 96-hours in the LT2 group and 48-hours in the LT1 group to stimulate drought. The well-watered plants were treated as a control group, which received normal water throughout the experiment. The leaf samples were collected at three biological replicates, and stored at -80℃ for further transcriptomic and metabolic profiling.

Determination of physiological indices

The collected Dinanath grass leaves under control and drought stress treatment were examined for different physiological indices. The relative water content (RWC), malondialdehyde (MDA) content, proline content, Peroxidase activity (POD) and Superoxide dismutase (SOD) were estimated according to methods described in Patanè et al. (2022) [17]. The soil-plant analysis development (SPAD) chlorophyll meter readings (SCMRs) measured using SPAD-502Plus chlorophyll meter and canopy temperature (CT) measured using Handheld Digital lnfrared thermometer. The data were processed using R software version 4.3.1. Significant differences between the control and treatments were determined by student's t- tests at a significance level of $P<0.05$.

RNA-Seq and data analysis

The transcriptome of the leaf under well-watered (LC) and 48-hour and 96-hour drought (LT1 and LT2, respectively) conditions was sequenced by Illumina NovaSeq 6000. There were three treatments, with three independent replicates per treatment, for 9 samples in total. Quality control and adapter trimming of the raw reads was done using fastp (v 0.20.0). A quality Phred score cutoff of 30 was used, and only high-quality reads were retained and used for further downstream analysis. The adaptor sequences and low-quality sequence reads were removed from the raw data using Trinity assembler. Cdhit-est was used to cluster similar nucleotide sequences within the assembly based on the sequence and length similarity. Transcripts that were 80% identical in length and sequence similarity were grouped, and only a representative transcript was retained. Quantification of transcripts was done using RSEM. It used a bowtie to align and estimate the abundance of each of the transcripts. Expression was normalized based on the Transcripts per million (TPM). All unigenes were annotated by running a BLAST (blastx) search of the transcript nucleotide sequences against the protein database. Protein sequences from the NCBI Ref Seq protein database were used as a reference for the BLAST search (NCBI Blast+2.8.1).

Quantification of gene expression and differential expression analysis

Differential expression analysis was performed using the DESeq2 R package, and principal component analysis (PCA) was performed on each sample according to the expression. Principle component 1 (PC1) accounts for the most variation across samples, PC2 the second most, and so on. These PC1 vs. PC2 plots are coloured by sample annotation to demonstrate how samples cluster together (or not) in reduced dimensional space. PCA emphasizes variation and brings out strong patterns in a dataset. It's used to make data more straightforward to explore and visualize. The package DESeq provides methods to test for differential expression using the negative binomial distribution. Read counts taken from alignment bam files are taken as input. Differential analysis compared sample A concerning sample M. Differential expression analysis was performed as "Treated vs. Control." The transcripts with log2foldChange>=two and p-value<=0.05 were considered significantly up-regulated, and the transcripts with log2foldChange <= -2 were and p-value<=0.05 considered significantly down-regulated. GLM with negative binomial distribution method was the statistical method used in DESEQ2 for differential expression analysis. Analysis of Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways based on the differentially expressed genes was done using ClueGO plugin in Cytoscape [18]. A Benjamini-Hochberg corrected p-value of <0.05 based on two-sided hypergeometric tests was selected as the threshold for

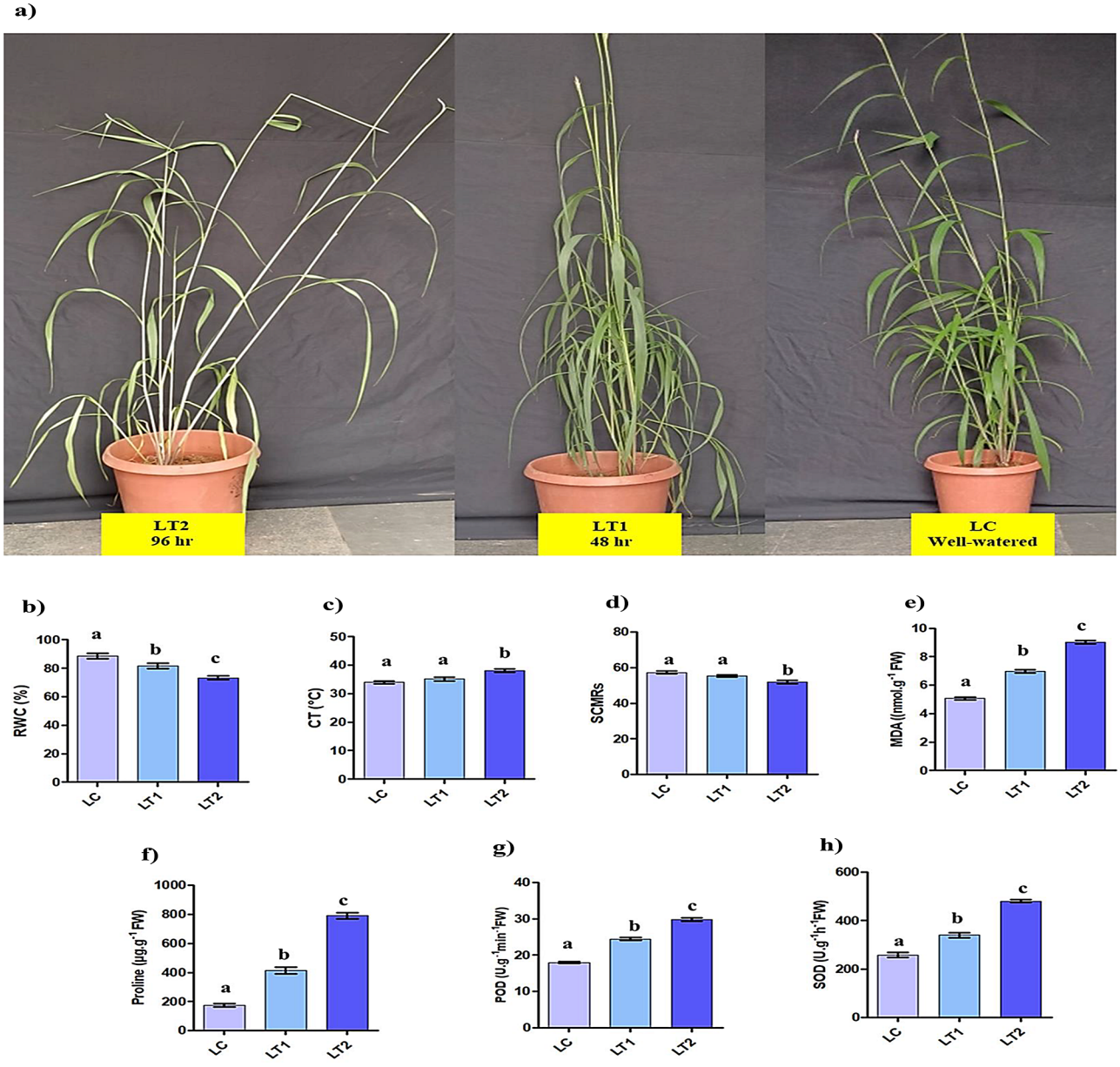

Fig. 1 (**a**) Phenotypes of Dinanath grass under drought stress; (**b-h**) Physiological property of Dinanath grass under drought stress. (**b**) RWC content; (**c**) CT; (**d**) SCMRs (**e**) MDA content; (**f**) Proline content; (**g**) POD activity and (**h**) SOD activity. Each parameter represents ± standard errors of three replicates. The different letters above the columns indicate significant difference at $P < 0.05$ level between treatments

significant KEGG pathway or GO enrichment of the gene sets.

Metabolite extraction, and LC-high-resolution MS (LC-HRMS) analysis

100 mg leaf sample was homogenized in liquid nitrogen and transferred into a 2 mL eppendorf tube. 800 µL ice-cold 80% methanol+0.1% formic acid was added and vortexed for 1 min and incubated on an orbital shaker (100 rpm) for 15 min, followed by sonication for 15 min at 40 kHz in a water bath. Samples were centrifuged (12000 rpm, 10 min, 4 °C), supernatant was transferred to a new tube and the solvent was evaporated using Speed-Vac concentrator (Concentrator-plus-eppendorf). The dried pellets were re-suspended in 200 µL 80% methanol+0.1% formic acid and filtered through Hydrophilic PVDF 0.22 µm filter (Millipore Millex-GV) into new tube50 µL filtered extract was loaded into autosampler vial and placed in the UHPLC autosampler conditioned at 10 °C. For untargeted plant metabolomics, the protocol described by De Vos et al. (2007) was followed [19]. The samples were run in positive ionization mode (Electro spray ionisation (ESI)) using ultra-high performance liquid chromatography-quadrupole time-of-flight mass

spectrometry (UHPLC-Q-TOF-MS/MS) (Xevo-G2-XS, Waters, Milford, MA, USA). For the positive ionization mode, the capillary and cone voltages were set to 2.0 kV and 40 V, desolvation temperature 400 °C; source temperature 110 °C; desolvation gas flow 800 L/h; cone gas flow 50 L/h; scanning time 1s; and scan mass range 50–2000 Da. The chromatographic separation was carried out using an Acquity UPLC BEH-C18 (100 mm×2.1 mm, 1.7 µm particle diameter) (Waters, Milford, MA, USA) maintained at 30 °C. The flow of 0.3 mL.min-1 of ultrapure water containing 0.1% formic acid water (mobile phase A) and Methanol+0.1% formic acid (mobile phase B) was used. The elution gradient was as follows: 0–2 min, 1% B; 2–6 min, 20% B; 6–10 min, 60% B; 10–15 min, 90% B; 25–31 min, 5% B; 31–35 min 1% B. Data was recorded using a multiplexed MS/MS acquisition with alternating low and high energy acquisition (MSE), operating on centroid mode and Mass Lynx 4.1 (Waters) software was used to acquire the data.

LC-HRMS output processing

The LC-HRMS output waters RAW files were converted into mzXML format. Data was processed using the MZmine-2 with the high sensitivity peak detection algorithm ADAP wavelets [20]. The observed masses and their abundance (relative intensity) were imported to MS Excel; peaks that were not consistent among replicates and those annotated as isotopes and adducts were excluded from further analyses.

Identification of resistance-related (RR) metabolites

The data on intensity of peaks of monoisotopic masses (m/z=mass/charge ratio, subtracted with a proton mass because of negative ionization) was subjected to pairwise Student's t-test analysis. The peaks significant at $P<0.05$ were retained. The metabolites with significantly higher abundances in water stress (WS) than in well-watered (WW) condition, based on a t-test, were considered as drought induced metabolites. For these RR metabolites, the fold change (FC) in abundance, relative to well-watered (WS/WW), was calculated. The metabolites were putatively identified based on accurate mass match (accurate mass error (AME<10 ppm) with metabolites reported in different databases: METLIN, Plant Metabolic Network (PMN), LIPIDMAPS, and KEGG.

Metabolomic data analysis

Statistical analyses were conducted using MetaboAnalyst version 5.0, with log transformation as the sole normalization, data transformation, and data scaling method. Sum or median sample normalizations were utilized for PCA and PLS-DA analyses. Metabolomic Pathway Analysis (MetPA) generated by MetaboAnalyst was employed to analyse the metabolite datasets, pathways were represented as circles, with colour denoting p-values and size indicating pathway impact scores. Annotation highlighted the most impacted pathways with high statistical significance scores. This approach facilitated a comprehensive understanding of metabolomic alterations and pathway perturbations in the respective datasets. Differentially accumulated metabolites were selected using the criteria of $P<0.05$ and log2FC>1.0. Volcano plots were used to visualize and screen differential metabolites based on p-value and fold change value. The volcano plots were generated using SR plot online (https://www.bioinformatics.com.cn/en?keywords=volcano). Venn and heatmap diagrams were generated using Venn Diagram (https://bioinformatics.psb.ugent.be/webtools/Venn/), and MeV software (Version 4.8, USA), respectively.

Validation of RNA-Seq data by real-time RT-PCR

For quantitative reverse transcription polymerase chain reaction (qRT-PCR), total RNA was isolated from the frozen leaf samples collected at 48 and 96-hour of water stress and well-watered condition using the RNeasy Plant Mini kit (Qiagen, Tokyo, Japan), in three replications. A 2.0 µg aliquot of purified RNA was used for cDNA synthesis following the recommended protocol (Thermoscript RT-PCR system, Invitrogen, Carlsbad, CA, USA). Quantitative PCR was carried out with gene specific primers (Table S1) in a CFX96TM Real-Time System (Bio-Rad, India). The pearl millet ELF4 gene was used as housekeeping genes for normalization of cycle threshold (Ct) values. qRT-PCR reaction conditions were set in triplicates as: 2 min at 95 °C, 40 cycles of 10s at 95°C, 20s at 60°C followed by fluorescence measurement at each 0.5 °C variation from 60°C to 95°C in 20 min was included to obtain the melting curve. Relative fold expression was calculated by $2^{-\Delta\Delta Ct}$ method [21].

Results

Physiological responses of Dinanath grass under drought stress

To study the effect of drought stress on the Dinanath grass we examined the changes of physiological parameters on drought stress (Fig. 1a). The results revealed that significant changes were observed in LT1 and LT2 conditions as compared with control condition (LC). The RWC content steadily decreased from 88.67% in LC to 82.00% and 73.33% in LT1 and LT2, respectively (Fig. 1b). In terms of SCMRs and canopy temperature, there was no significant difference between LC and LT1. However, as the duration of drought increased to 96-hour (LT2), SCMRs tended to decrease (Fig. 1d), while CT values increased (Fig. 1c). The contents of MDA and proline increased significantly in LT1 and LT2 compared with the LC, the MDA in LT1 and LT2 increased by 37.47% and 78.11%, while proline increased by 137.86% and

Table 1 Assembly statistics for Dinanath leaves

	Dinanath Leaves
Transcripts	51,878
Total bases (bp)	8,66,41,222
Minimum sequence length (bp)	500
Max sequence length (bp)	30,455
Average sequence length (bp)	1,670
N50 length (bp)	2,084
(G + C)s	50.19

Filtered reads were assembled using Trinity assembler. Cdhit-est was used to cluster together similar nucleotide sequences within the assembly based on the sequence and length similarity. Transcripts which were 80% identical in length and sequences similarity were grouped together and only a representative transcript was retained

355.20% (Fig. 1e and f). Meanwhile, for the antioxidant enzyme activity in Dinanath grass leaves, the activities of POD and SOD increased compared with the control. The POD activity increased from 17.9 U/g min in LC to 24.40 U/g min and 29.80 U/g min in LT1 and LT2, respectively (Fig. 1g). Further, the SOD activity reached to 480.97 U/g hour at LT2 from 258.93 U/g hour at LC (Fig. 1h). In brief, Dinanath grass, when subjected to varying degrees of drought, displayed drought tolerance by maintaining leaf water holding capacity and mitigating the extent of cell membrane damage.

Transcriptome profiling of Dinanath grass leaves in response to drought

Total RNAs were extracted to explore the mechanism of drought response in Dinanath grass. RNA-seq was performed in theleaves of Dinanath grass subjected to two different drought intensities. Transcriptome sequencing done using Illumina NovaSeq 6000 NGS platform yielded an average of 11.21 GB, 10.44 GB and 11.53 GB raw reads from the experimental groups LC, LT1, and LT2, respectively (Table S2). After quality checks and adapter trimming using fastp (v 0.20.0), the respective average clear reads (Phred score of ≥30) obtained from LC, LT1 and LT2 samples were 7.86 GB, 10.7 GB and 11.8 GB (Table S2). The sequence reads have been submitted to NCBI-SRA database (BioProject: PRJNA1107114).

Due to the absence of Dinanath grass genome sequences, *de novo* assembly on Dinanath grass leaves transcriptome was done using Trinity assembler. Assembly of clean reads resulted in 51,878 transcripts, ranging from 500 to 30,455 bp in size, with an N50 length of 2,084 bp, total bases of 8,66,41,222 bp, and GC content of 50.19% (Table 1). Subsequently, to evaluate the variation in gene expression levels between well-watered and drought-exposed Dinanath grass leaves, we performed PCA of all three biological replicates of 9 samples. The first two principal components, PC1 and PC2, accounted for 92% and 6% variance, respectively (Figure S1). From this analysis, it is evident that drought stress significantly impacts gene expressions in Dinanath grass leaves. Additionally, among the drought-exposed groups, the leaves of Dinanath grass subjected to 96-hour of drought stress exhibited a more significant alteration in gene expression compared to the 48-hour samples. This observation suggests distinct gene expression patterns in Dinanath grass under varying durations of drought stress.

Table 2 Upregulated and downregulated genes under well-watered and drought conditions

Comparison	Upregulated	Downregulated
LC vs. LT1	830	1709
LC vs. LT2	7549	9037
LT1 vs. LT2	7383	8616

Transcripts with log2foldChange>=2 and p-value<=0.05 were considered as significantly upregulated and the transcripts with log2foldChange <= -2 were and p-value<=0.05 considered as significantly downregulated. LC, well-watered; LT1 & LT2, 48- and 96-hour drought exposed Dinanath leaves, respectively

Identification and analysis of drought-related DEGs in Dinanath grass

To identify the significantly changed genes regulated by drought stress in Dinanath grass, DEGs were analysed in Dinanath grass leaves exposed to two different drought conditions and compared with the control. Differential expression analysis was performed using the DESeq2 R package, and the DEGs were identified based on absolute log2FC>2 (p-value≤0.05) for up-regulated genes and log2FC<-2 (p-value≤0.05) for down-regulated genes. In details, 830 and 1,709 DEGs were up-regulated and down-regulated respectively in LT1 vs. LC, 7,549 and 9,037 DEGs were up-regulated and down-regulated respectively in LT2 vs. LC, and 7,383 and 8,616 DEGs were up-regulated and down-regulated respectively in LT2 vs. LT1 (Table 2 and Figure S2). It is to be noted that the number of down-regulated DEGs is higher in all 3 set of comparisons, suggesting that drought response in Dinanath grass mainly involves down-regulation of a set of stress-responsive genes.

Transcriptomic analysis disclosed a number of drought-responsive genes differentially expressed in Dinanath grass (Table S3). Comprehensive overview of these genes revealed that many DEGs showed similar expression pattern in both LT1 and LT2 leaf samples, including transcription factors (TFs), receptor genes, calcium-related genes, ROS genes, and genes encoding phytohormones. Concurrently, the expression pattern of few DEGs differed between LT1 and LT2 samples. For instance, higher number of cell wall-related DEGs were up-regulated in LT1 while mostly down-regulated in LT2 group (Table S3). This variation in gene expression pattern among LT1 and LT2 groups indicates a possible divergent drought response by Dinanath grass to different drought intensities. The top 5 significantly

Table 3 List of top 5 significantly upregulated and downregulated DEGs in well-watered and drought (48- and 96-hour) exposed Dinanath leaves

ID	log2FoldChange	pvalue	padj	Refseq	Description
Upregulated genes in LC vs. LT1					
TRINITY_DN13299_c1_g1_i2	7.87255721	2.24E-09	1.03E-07	XP_044340397.1	probable LRR receptor-like serine/threonine-protein kinase At3g47570
TRINITY_DN30951_c0_g1_i1	6.91987053	3.20E-06	6.43E-05	XP_015643426.1	Os06g0589800 protein (Putative receptor-like protein kinase 2)
TRINITY_DN35140_c0_g1_i1	5.64051254	0.0011403	0.0093828	NP_001151029.1	DNA-binding WRKY
TRINITY_DN29683_c0_g3_i1	5.52290373	0.00108151	0.0089889	XP_008681358.1	Transcription factor bHLH71
TRINITY_DN9116_c0_g1_i2	5.06916667	0.00024722	0.002673	XP_044396982.1	probable disease resistance protein At1g61300
Downregulated genes in LC vs. LT1					
TRINITY_DN44656_c0_g2_i1	-8.400087585	1.71E-15	3.02E-13	XP_044362626.1	AP2-like ethylene-responsive transcription factor CRL5 isoform X1
TRINITY_DN11925_c0_g2_i1	-7.614308134	7.67E-08	2.40E-06	XP_002458420.1	Protein kinase domain-containing protein
TRINITY_DN4485_c2_g2_i2	-7.495239351	2.52E-10	1.49E-08	XP_015078742.1	putative calcium-binding protein CML19
TRINITY_DN10709_c1_g1_i1	-6.85476982	1.67E-08	6.15E-07	XP_015638020.1	probable serine/threonine-protein kinase At1g01540
TRINITY_DN18702_c0_g2_i1	-6.764991879	6.03E-07	1.49E-05	XP_044345771.1	tyrosine decarboxylase-like
Upregulated genes in LC vs. LT2					
TRINITY_DN7268_c0_g1_i2	12.74045808	1.13E-25	4.69E-24	XP_025882971.1	disease resistance protein RGA5
TRINITY_DN24823_c0_g2_i1	12.36836672	9.89E-25	3.72E-23	XP_044390747.1	disease resistance protein RGA4-like isoform X1
TRINITY_DN5417_c0_g1_i2	11.48387192	9.22E-21	2.34E-19	XP_044408515.1	disease resistance protein RGA5-like
TRINITY_DN786_c0_g1_i2	11.34308123	6.74E-21	1.73E-19	NP_001147982.1	Tyrosine/DOPA decarboxylase 1
TRINITY_DN60687_c0_g1_i1	11.16856792	1.43E-20	3.55E-19	XP_044366556.1	disease resistance protein RGA4-like
Downregulated genes in LC vs. LT2					
TRINITY_DN4426_c0_g1_i1	-14.71654165	8.32E-35	7.01E-33	XP_044417866.1	disease resistance protein RGA5-like
TRINITY_DN726_c0_g2_i2	-13.57589467	8.54E-30	5.09E-28	XP_044380694.1	calcium-dependent mitochondrial ATP-magnesium/phosphate carrier protein 1-like
TRINITY_DN726_c0_g2_i1	-12.97160026	2.61E-27	1.25E-25	XP_044396486.1	calcium-dependent mitochondrial ATP-magnesium/phosphate carrier protein 1-like
TRINITY_DN395_c0_g2_i1	-12.8501388	3.64E-27	1.71E-25	XP_044389889.1	disease resistance protein RGA5-like isoform X2
TRINITY_DN1855_c0_g1_i1	-12.78004268	6.25E-27	2.89E-25	XP_044424991.1	disease resistance protein RPV1-like isoform X2

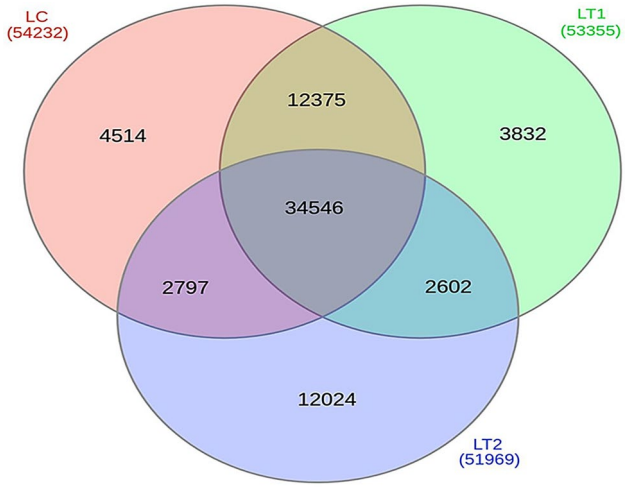

Fig. 2 Venn diagram analysis of DEGs Venn diagram representing shared and unique DEGs of Dinanath leaves transcriptome under well-watered and drought (48- and 96-hour) conditions. LC, well-watered; LT1 & LT2, 48-hour and 96-hour drought-exposed Dinanath leaves, respectively

up-regulated and down-regulated DEGs were given in Table 3. It was observed that receptor-like kinases (RLKs) and TFs related to stress response were highly altered in LT1 samples, while disease resistance genes (R genes) were majorly regulated in LT2 group suggesting that these might be the key genes involved in the drought resistance mechanism in Dinanath grass.

Venn diagram analysis was done to explore the DEGs specific to and common between well-watered (LC) and drought (LT1 and LT2) treatments in Dinanath grass (Fig. 2). A total of 72,690 DEGs were identified during drought stress against the control samples: 34,546 genes were shared between well-watered and both drought (LT1 and LT2) conditions, and 4514, 3832 and 12,024 were specific to LC, LT1 and LT2 groups, respectively. The gene number shared between LC and LT1 samples was 12,375, while 2,797 genes were shared between LC and LT2 samples. It was observed that both drought groups LT1 and LT2 shared 2,602 genes. This analysis indicates that in Dinanath grass the drought-responsive genes were specific to the intensity of drought stress,

which may account for the differences in drought tolerance concerning the length of the exposure period.

Functional enrichment analysis of DEGs by GO and KEGG

All DEGs were further subjected to GO and KEGG pathway enrichment analyses in order to explore the potential functions of the core drought-responsive genes in Dinanath grass. GO annotation was carried out to assess the biological functions of DEGs in Dinanath grass leaves exposed to drought stress for two different periods. GO analysis predominantly categorized DEGs into three major functional ontologies, namely biological process (BP), molecular function (MF), and cellular component (CC) (Fig. 3). These DEGs were further classified into 48 functional GO terms. Within the biological process group, the top three subcategories with the most gene numbers were "cellular process," "metabolic process," and "biological regulation." In the MF category, the most abundant terms were "catalytic activity," "binding," and "transporter activity." in the CC processes, genes involved in 'cell,' 'cell part,' and 'organelle' were most highly represented.

GO enrichment analysis was performed for DEGs of three treatment comparisons. A total of 13 (in 2,539 DEGs), 14 (in 16,586 DEGs), and 16 (in 15,999 DEGs) different processes were significantly enriched in DEGs from LT1 vs LC, LT2 vs LC, and LT2 vs LT1 comparisons, respectively (Table S4, S5 and S6). GO analysis of LT1 vs LC indicated that the majority of genes differentially expressed were enriched in BP associated with response to stimulus (GO:0050896), plant-type primary cell wall biogenesis (GO:0009833), cellulose biosynthetic process (GO:0030244), pyrimidine nucleobase biosynthetic process (GO:0019856) and 'de novo' CTP biosynthetic process (GO:0044210) and also, in MF including UDP-glycosyltransferase activity (GO:0008194), serine-type endopeptidase activity (GO:0004252), pyridoxal phosphate binding (GO:0030170), phospholipase activity (GO:0004620) and L-ascorbate oxidase activity (GO:0008447). Meanwhile, the extracellular region (GO:0005576) was enriched in the largest number in CC (Fig. 4a and Table S4). In LT2 vs LC comparison, GO terms involved in DEGs were mainly concentrated in MF related to ATP binding (GO:0005524), ADP

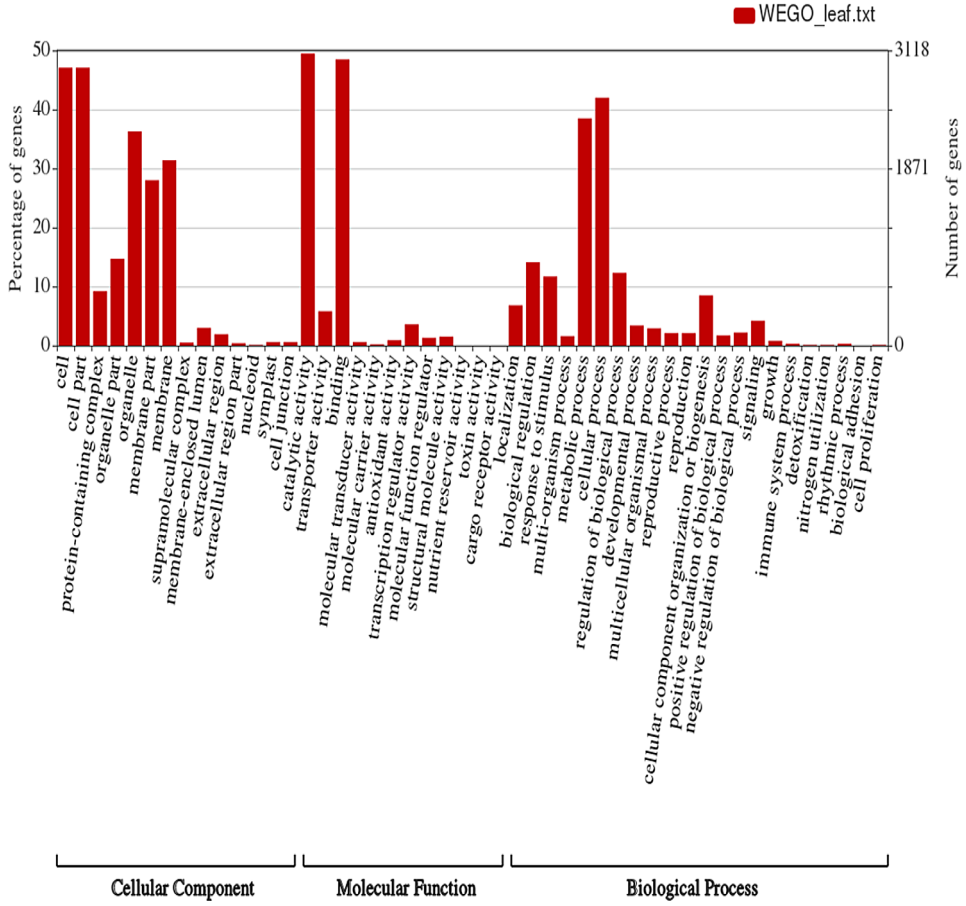

Fig. 3 Gene ontology (GO) analysis of unigenes Gene ontology (GO) functional annotation of unigenes. Abscissa indicates the functional categories; ordinate indicates the number of genes

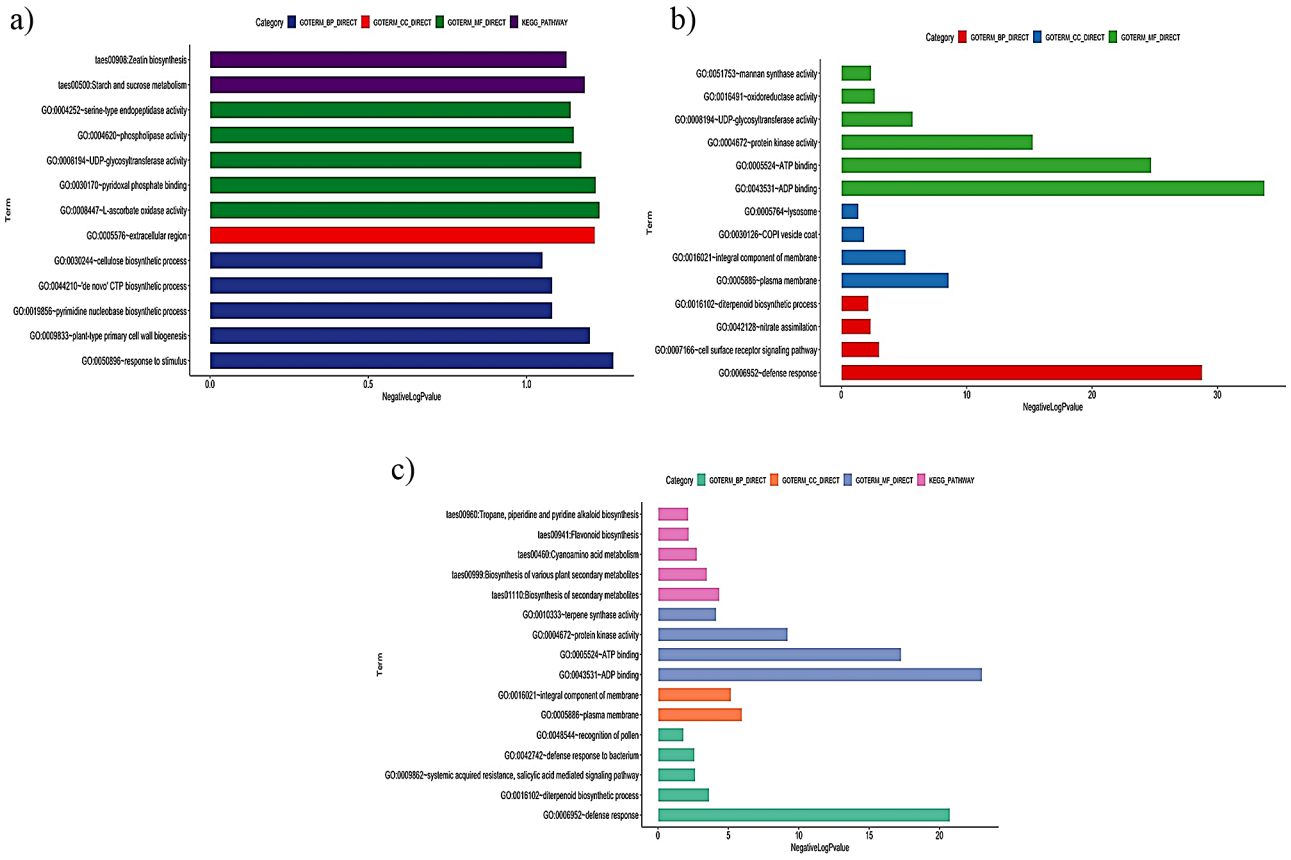

Fig. 4 Top GO terms in well-watered and drought exposed Dinanath leaves (**a**) Top GO terms in LT1 vs. LC, (**b**) Top GO terms in LT2 vs. LC and (**c**) Top GO terms in LT2 vs. LT1

binding (GO:0043531) and protein kinase activity (GO:0004672) and, the integral component of membrane (GO:0016021) was enriched in the largest number in CC followed by plasma membrane (GO:0005886). Under BP, defense response (GO:0006952), cell surface receptor signaling pathway (GO:0007166), nitrate assimilation (GO:0042128) and diterpenoid biosynthetic process (GO:0016102) were enriched in LT2 vs LC (Fig. 4b and Table S5). GO terms involved in DEGs of LT2 vs LT1 were mainly concentrated in BP and the most enriched was defense response (GO:0006952). For MF, ATP binding (GO:0005524) owned the most number, and the integral component of membrane (GO:0016021) was the most enriched in CC (Fig. 4c and Table S6).

To further understand the pathways that the DEGs were involved in, we performed KEGG enrichment analysis with the three experimental groups (Table S7). In LT1 vs. LC combination, starch and sucrose metabolism (taes00500) and zeatin biosynthesis (taes00908) were the pathways with larger number of DEGs (73 DEGs) but biological pathways namely plant-pathogen interaction (taes04626) and biosynthesis of secondary metabolites (taes01110) with 13 and 33 DEGs, respectively were found to be significantly enriched. Meanwhile, in LT2 vs. LC, 16 significantly enriched pathways were identified and, the most frequently associated pathways were metabolic pathways (taes01100), biosynthesis of secondary metabolites (taes01110), starch and sucrose metabolism (taes00500) and plant-pathogen interaction (taes04626) with 167, 133, 21, and 21 enriched DEGs, respectively. Other preferably enriched pathways were biosynthesis of various plant secondary metabolites (taes00999), cyanoamino acid metabolism (taes00460), glycerolipid metabolism (taes00561) and flavonoid biosynthesis (taes00941). The KEGG pathway enrichment analysis of LT2 vs. LT1 showed that the biosynthesis of secondary metabolites (taes01110) and phenylpropanoid biosynthesis (taes00940) were the top significantly enriched pathways with 52 and 12 DEGs, respectively and other notable enriched pathways were biosynthesis of various plant secondary metabolites (taes00999), starch and sucrose metabolism (taes00500), cyanoamino acid metabolism (taes00460) and flavonoid biosynthesis (taes00941). These results reveal that the biosynthesis of secondary metabolites play an important role in drought stress response in Dinanath grass leaves and furthermore, many KEGG pathways were common between LT1 vs. LC, LT2 vs. LC and LT2 vs. LT1 comparison groups indicating

that the drought-tolerance mechanism involves both common pathways and unique pathways to cope with different drought intensities.

Validation of RNA-seq results for selected DEGs by qRT-PCR

To confirm the results of the differential expression analysis from RNA-seq data, qRT-PCR was used to measure the transcriptional levels of ten selected DEGs that are potentially involved in drought tolerance in Dinanath grass. These genes, which were previously identified as participating in various biological processes related to abiotic stress—such as phenylpropanoid and fatty acid biosynthesis, and signal transduction pathways—were categorized based on their direct or indirect roles in plant drought tolerance or susceptibility. Detailed information about the selected genes and their primers can be found in Table S1. The expression patterns of all ten genes—phenylalanine ammonia-lyase, putrescine hydroxycinnamoyl transferase, abscisic acid 8'-hydroxylase 2, syntaxin-61, lipoxygenase 5, calcium-dependent protein kinase 7, and phospholipase D α1—were validated by qRT-PCR to be consistent with those observed in the RNA-seq data (Fig. 5 and Figure S7). This agreement suggests that these genes are likely critical for drought tolerance in Dinanath grass.

Global metabolomic analysis of Dinanath grass leaves under drought stress

To examine the impact of 48-hour and 96-hour drought stress on Dinanath grass, we conducted metabolite profiling of leaves using UHPLC-MS. A diverse array of metabolites was identified, including primary metabolites, predominantly lipids and derivatives, as well as secondary metabolites such as alkaloids, phenylpropanoids, flavonoids, flavones, flavanols, terpenoids, isoprenoids, and others.

PCA and PLS-DA (Figure S3) were conducted to determine the similarities and differences in metabolite profile of well-watered (LC) and drought-induced (LT1 and LT2) Dinanath grass. The PCA plots showed that samples under the same drought conditions clustered together, whereas samples from different drought groups were significantly separated. In both LT1 and LT2 groups, two main principal components were generated. In LT1, the principal components showed a total variation of 65.2%, with 48.8% of the variation explained by PC1 and 20% explained by PC2 (Figure S3a). The LT2 group showed 72.9% of the total variation, with the first and second PCs contributing 51.7% and 21.2%, respectively (Figure S3c). The PLS-DA score plot yielded similar categorization results as in the PCA and showed a clear separation of samples by their treatments (Figure S3b & S3d). Thus, both LT1 and LT2 group exhibited distinctive metabolic

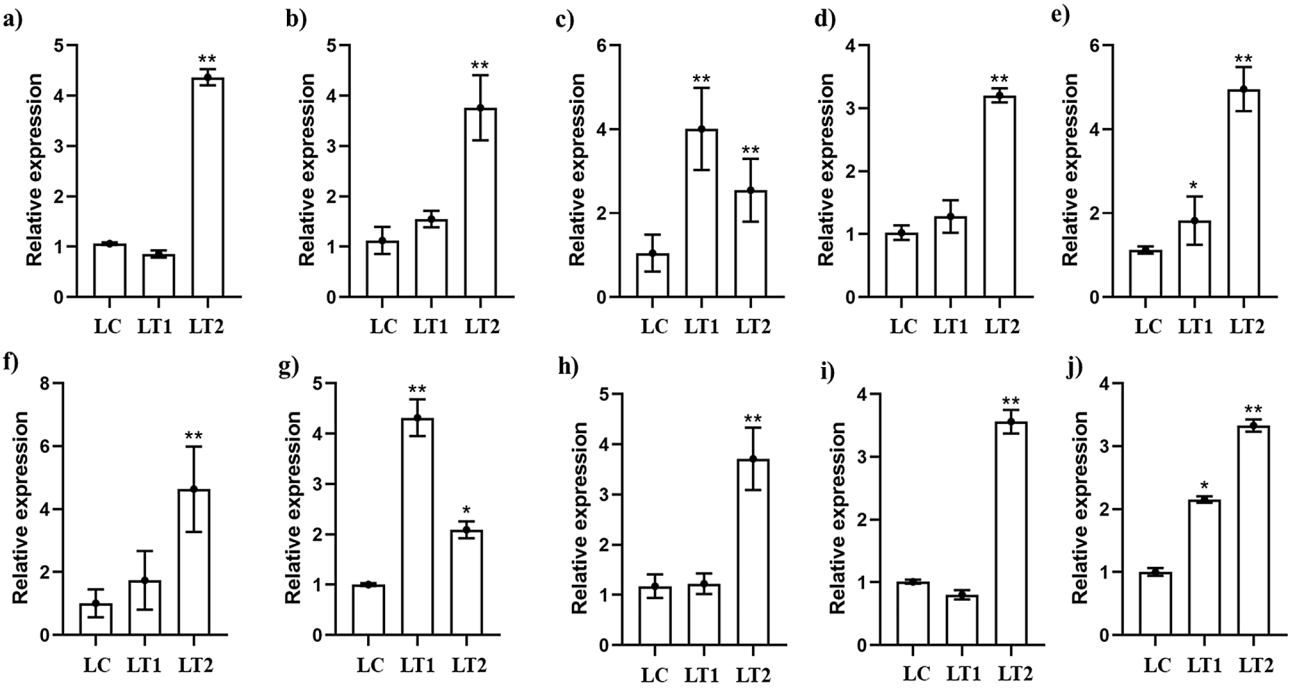

Fig. 5 Relative expression of genes by qRT-PCR Relative quantification of genes from Dinanath grass at 48 and 96 h after drought treatment was obtained through $2^{-(\Delta\Delta CT)}$ method. (**a**) Phenylalanine ammonia-lyase; (**b**) Putrescine hydroxycinnamoyl transferase; (**c**) Chalcone flavonone isomerase; (**d**) Chalcone synthase-2; (**e**) Flavonoid-3',5'-hydroxylase-1; (**f**) Abscisic acid-8'-hydroxylase-2; (**g**) Syntaxin-61; (**h**) Lipoxygenase-5; (**i**) Calcium-dependent protein kinase 7; (**j**) Phospholipase-D-alpha-1. Significant differences between resistant and susceptible plants were analysed through Dunnett test: * $p < 0.05$; ** $p < 0.01$. LC, well-watered; LT1 & LT2, 48-hour and 96-hour drought-exposed Dinanath leaves, respectively

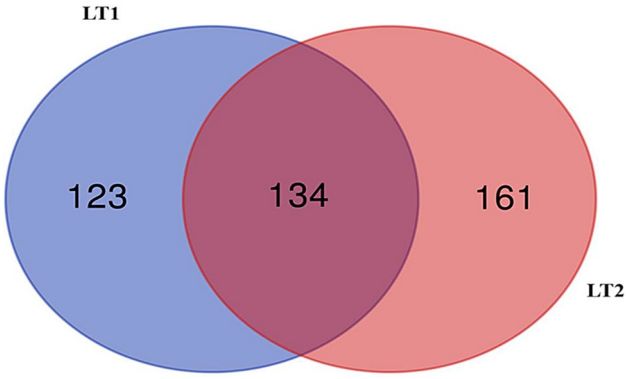

Fig. 6 Venn diagram showing the unique and shared metabolites in Dinanath grass at 48-hour (LT1) and 96-hour (LT2) after drought treatment

profiles compared to LC, indicating that changes in the metabolite profiles were caused by drought stress.

Volcano diagram analysis was performed to determine the number of accumulated and reduced metabolites (p-value<0.05 and log2FC>0.6 or < -0.6) in response to drought stress in Dinanath grass (Figure S4). A total of 1,143 metabolites were identified in drought and control leaf samples. In the LT1 group, the relative abundance of 436 metabolites was observed to change differentially, with 420 up-regulated and 16 down-regulated. In contrast, out of 559 altered metabolites in the LT2 group, 352 metabolites showed increased accumulation, while 207 exhibited decreased accumulation due to 96-hour drought stress. This suggests that Dinanath grass responds to both 48-hour and 96-hour drought conditions through accumulation of variety of metabolites, which in turn might be the crucial underlying mechanism responsible for drought tolerance in Dinanath grass.

Specific metabolomic changes in drought-exposed Dinanath grass leaves

The metabolic alterations in LT1 and LT2 groups were elucidated through KEGG enrichment pathway analysis, hierarchical clustering analysis, and Venn Diagrams. 256 and 293 metabolites were identified in LT1 and LT2 leaf samples, respectively (Table S8). Venn diagrams were generated to compare the commonalities and differences among altered metabolites in drought samples (Fig. 6). It was noted that LT1 and LT2 samples expressed 123 and 161 unique metabolites, respectively, and 134 were commonly altered under drought conditions. A comprehensive pathway involved for drought stress tolerance of Dinanath grass is shown in Fig. 7.

To explore the metabolic response of Dinanath grass under drought condition, we performed KEGG enrichment pathway analysis (Table S8). The analysis revealed that in LT1 samples differentially accumulated

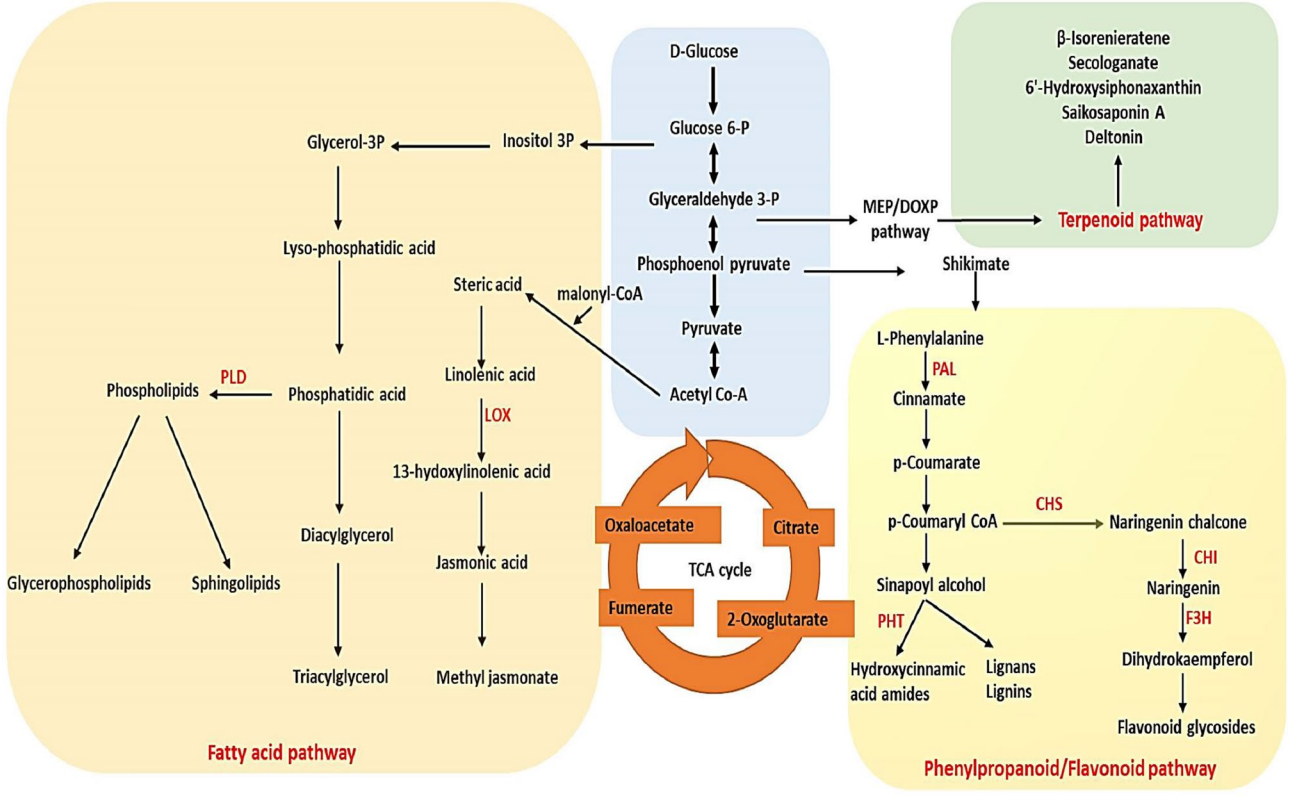

Fig. 7 Satellite metabolic pathways involved in the biosynthesis of resistance metabolites by Dinanath grass in response to drought

metabolites were significantly enriched in 13 pathways while, in LT2 group 20 biological pathways were highly represented. The significantly enriched pathways in LT1 and LT2 samples were enlisted in Table 4. Notably, the pathways related to phenylpropanoid biosynthesis (FC 8.14), flavonoids (FC 6.47) and terpenoids (FC 5.73) showed highest fold change in LT1 group whereas, in LT2 samples maximum fold change was exhibited by pathway associated with sterol lipids metabolism (FC 23.75) followed by glycerophospholipid metabolism (FC 14.86).

We found that the differentially accumulated metabolites related to drought response in LT1 and LT2 samples were majorly secondary metabolites including phenylpropanoids, flavonoids, terpenoids, and alkaloids, and lipid metabolites namely glycerophospholipids, fatty acids, sterol lipids and fatty acyls (Table S9 and S10). Both 48-hour and 96-hour drought-subjected Dinanath grass leaves exhibited increased contents of lipids descended by flavonoid contents and terpenoids. However, the comparison between the metabolites of LT1 and LT2 samples showed that the increase in lipid content was higher in 96-hour drought exposed LT2 samples (55.25%) than in LT1 samples (44.55%). On the other hand, the rise in secondary metabolites was higher in LT1 group (Flavonoids 36.02% and Terpenoids 15.64%) than in LT2 samples (Flavonoids 26.84% and Terpenoids 14.39%). No significant change has been observed in the panel of alkaloids and phenylpropanoids in both LT1 and LT2 samples.

Table 4 Significantly enriched pathways in LT1 and LT2 samples

In LT1	In LT2
Phenylpropanoid biosynthesis (ec00940)	Phenylpropanoid biosynthesis (ec00940)
Stilbenoid, diarylheptanoid and gingerol biosynthesis (ec00945)	Stilbenoid, diarylheptanoid and gingerol biosynthesis (ec00945)
Steroid hormone biosynthesis (map00140)	Flavone and flavonol biosynthesis (ec00944)
Metabolic pathways (map01100)	Biosynthesis of secondary metabolites (ec01110)
Carotenoid biosynthesis (map00906)	Purine metabolism (map00230)
Biosynthesis of secondary metabolites (map01110)	Histidine metabolism (map00340)
Flavone and flavonol biosynthesis (map00944)	Biosynthesis of plant secondary metabolites (map01060)
Biosynthesis of various plant secondary metabolites (map00999)	Biosynthesis of alkaloids derived from histidine and purine (map01065)
Teichoic acid biosynthesis (map00552)	Metabolic pathways (map01100)
Glycerophospholipid metabolism (map00564)	AMPK signaling pathway (map04152)
Nicotinate and nicotinamide metabolism (map00760)	Carotenoid biosynthesis (ec00906)
Biosynthesis of cofactors (map01240)	Biosynthesis of phenylpropanoids (map01061)
Degradation of flavonoids (map00946)	Teichoic acid biosynthesis (map00552)
	Glycerophospholipid metabolism (map00564)
	Steroid hormone biosynthesis (map00140)
	Ubiquinone and other terpenoid-quinone biosynthesis (map00130)
	Biosynthesis of cofactors (map01240)
	Monoterpenoid biosynthesis (map00902)
	Biosynthesis of various plant secondary metabolites (map00999)
	Porphyrin metabolism (map00860)

For better interpretation, the identified metabolites were clustered using a hierarchical analysis, and the expression clustering patterns were studied (Figure S5). Distinct metabolite patterns were discerned in the LT1 and LT2 groups. The heatmaps revealed that the relative abundance of fatty acid metabolites were consistently increased or decreased between LT1 and LT2 groups, indicating the involvement of fatty acid pathway in drought tolerance mechanism in Dinanath grass under both 48-hour and 96-hour water deficit conditions. Meanwhile, the intensity of secondary metabolites, particularly flavonoids and terpenoids were higher in LT1 group in comparison to LT2 samples. This variation in relative abundance of metabolites among LT1 and LT2 groups suggests that the intensity of drought has a stronger impact on the metabolic response associated with drought tolerance in Dinanath.

The screening of the top 10 enriched KEGG pathways showed that metabolites involved in amino acid biosynthesis were highly expressed in LT1 samples, and brassinosteroid biosynthesis pathway metabolites were represented more by LT2 samples (Figure S6). The increased relative content of metabolites involved in amino acid biosynthesis can be correlated with the increased content of secondary metabolites (flavonoids and terpenoids) in LT1 group. Meanwhile, the increased relative abundance of metabolites related to brassinosteroid biosynthesis pathway explains the increased lipid metabolites, especially sterol lipids and glycerophospholipids, in LT2 group. This result corroborates with the KEGG enrichment pathway analysis outcome.

The results of metabolomic analysis suggest that in Dinanath grass the biosynthesis of phenylpropanoids, flavonoids and terpenoids was highly stimulated in response to 48-hour drought stress, while sterol lipids and other fatty acid metabolites were the major metabolites involved in drought tolerance under 96-hour water deficit conditions. These defense metabolites may play a crucial role in the drought tolerance mechanism of

Dinanath grass, and the underlying metabolic reprogramming might depend on the intensity of drought exposure.

Discussion

Dinanath grass is a widely used high-quality forage and bio-energy crop [22]. It is well known for its drought tolerance and powerful recovery ability after watering, even under adverse drought conditions [3]. However, the underlying mechanisms responsible for the drought tolerance in Dinanath grass remain largely unexplored. Dinanath grass has displayed drought tolerance mechanism in terms of physiology and biochemistry. We noted an elevation in proline levels and canopy temperature, along with a reduction in RWC and SCMRs from well-watered conditions (LC) to LT1 and further to LT2. This pattern signifies an escalation in water stress as the treatment duration extends. The increase in MDA suggests the accumulation of ROS under drought stress, leading to damage to biomolecules and cellular structures [23]. Further the activities of SOD and POD increased from LC to LT2, contributing to the removal of excess ROS and the preservation of membrane stability under drought stress [24]. In our study, we observed an increase in the activity of antioxidant enzymes in response to the escalating drought stress. This indicates that Dinanath grass exhibits a physiological and biochemical drought tolerance response to water stress. Consequently, these stages, namely LC, LT1, and LT2, are deemed suitable for transcriptomic and metabolomic analyses. Hence, we investigated the transcriptomic and metabolic reprogramming in leaves of Dinanath grass exposed to two distinct drought periods (48-hour and 96-hour) and identified the drought-responsive genes and metabolic pathways associated with drought tolerance in Dinanath grass.

Transcriptome analysis and stress-responsive genes

Stress-mediated gene expression in Dinanath grass has been sparsely studied due to the absence of its complete genome sequence. Research suggests that transcriptome sequence data can be a reliable resource for assessing transcriptional changes at the genome-wide level in species without complete genome sequences [25]. Accordingly, in the present study, after establishing two different drought conditions (48-hour and 96-hour), the leaf samples of Dinanath grass were subjected to *de novo* RNA-seq based transcriptome analysis to identify the drought-responsive genes and associated metabolic pathways. We have identified 51,878 unigenes with an N50 length of 2,084 bp and the average length of 1,670 bp, suggesting high quality of assembly [26, 27].

Transcriptome sequencing unveiled a notably higher number of DEGs under 96-hour drought stress (704 DEGs) compared to 48-hour drought condition (218 DEGs), which implies a striking impact of drought intensity on the transcriptome changes associated with drought tolerance in Dinanath grass leaves. Previous studies reported similar link between the number of responsive genes and their association with the complexity and intensity of the applied stress treatments [28–30]. In addition, common and specific DEGs identified in well-watered (LC) and drought-exposed (LT1 and LT2) Dinanath grass leaves strengthen the fact that transcriptomic alterations corresponding to drought tolerance varies in accordance to the severity of drought stress. Alike, reports by Cao et al. (2014) and Robertson et al. (2022) showed that drought response and distinct patterns of gene expression were invoked depending upon the extent of drought stress [31, 32].

Gene expression analysis revealed that of the 214 DEGs identified under 48-hour drought conditions, 150 were downregulated, and 68 were upregulated. In response to 48-hour drought stress most of the upregulated core genes were RLKs, TFs and genes encoding key defense hormones and plant secondary products. Notably, among them RLKs namely LRR-RLK and RPK2/TOAD2 and, TFs DNA-binding WRKY and bHLH71 exhibited higher fold change. RLKs and TFs are known for their functional role in maintaining plant architecture and its growth and development under stress conditions including drought [33, 34]. Prior studies analogous to our result suggested that overexpression of RLKs (LRR-RLK and RPK2/TOAD2) genes and WRKY TFs promotes drought tolerance through their involvement in regulatory network of secondary cell wall formation and lignin deposition in leaves [35, 36]. These genes in coordination with abscisic acid (ABA)-dependent and reactive oxygen species (ROS)-dependent pathways are reported to be involved in secondary cell wall formation and lignin biosynthesis, which in turn contribute to stomatal closure and maintenance of turgor and cell wall extensibility and thus, enhances drought tolerance [34, 37–40]. Similarly, upregulated bHLH71 TFs has been reported to confer tolerance to drought stress through jasmonic acid (JA) pathway involving jasmonate ZIM domain (OsJAZ) protein [41, 42]. Other than this, genes encoding key defense hormones and plant secondary products majorly involved in lignin biosynthesis added up to the drought tolerance mechanism in the leaves of Dinanath grass [43].

Meanwhile, in the panel of downregulated DEGs, gene expression of AP2-like ethylene-responsive transcription factor CRL5 isoform X1 (AP2/ERF), protein kinase domain-containing protein, putative calcium-binding protein CML19, and probable serine/threonine-protein kinase At1g01540 showed higher fold change under 48-hour drought stress. Consistent with our findings, previous studies reported that these genes are negative regulators of plasma-membrane and stomatal

aperture and, their downregulation enhances drought tolerance [44–48]. AP2/ERF TFs and genes encoding protein kinases are known to exert their stress response mainly through ABA-mediated pathways. Suppression of AP2/ERF TFs and genes encoding protein kinases induces drought tolerance by enhancing sensitivity of guard cells to ABA and thus, decreasing transpirational water loss and, also by reducing stomatal aperture and increasing lignin content [49–52].

A total of 704 DEGs were expressed under 96-hour drought conditions, with 317 upregulated and 387 downregulated. The result showed that genes encoding disease-resistance proteins (R genes) and proteins related to defense compounds and secondary plant products were majorly regulated in 96-hour drought-exposed Dinanath grass leaves. Our results showed that in response to increased drought intensity (96 h) disease-resistance proteins RGA4, RGA5, and RPV1 were differentially regulated in Dinanath grass leaves. In consistent with our findings, Wu et al. (2020) and Shelake et al. (2022) demonstrated that RGA genes act as regulators of drought tolerance by modulating ABA-mediated stomatal closure and subsequently control water loss in response to a water deficit [53, 54]. Another study by Iquebal et al. (2019) reported that transcriptional alteration of disease-resistance proteins promotes physiological adaptation and improves tolerance to abiotic stress through the salicylic acid pathway [55]. R genes are known to activate downstream defense signaling involved in prevention of pathogen spread in host. R genes code for both intracellular receptor proteins and membrane associated proteins including RLK and LRR [56]. Abou-Elwafa (2017) have reported the implication of R genes in drought tolerance mechanism similar to our findings [57]. Besides, upregulation of genes encoding enzymes related to secondary metabolite biosynthesis implies the involvement of secondary metabolites in response to increased drought intensity in the leaves of Dinanath grass. For instance, tyrosine/DOPA decarboxylase 1 involves in diverting primary metabolites into secondary metabolite pathways. Its overexpression enhances drought tolerance by improving photosynthetic capacity, water use efficiency (WUE), antioxidant activity, dopamine content, and altering the levels of amino acids. (such as proline accumulation) [58].

The results from DEG analysis revealed that transcriptional regulation of genes involved in secondary cell wall thickening and lignin biosynthesis contributes significantly to the drought response in Dinanath grass leaves. Further, the comparative analysis of these drought-responsive genes showed that the regulatory pathways inflicting drought tolerance differed between 48- and 96-hour water deficit Dinanath grass leaves. Thus, the adaptation mechanisms in Dinanath grass to cope with drought stress varies in accordance to the intensity of drought.

Pathway analysis for drought tolerance

GO and KEGG enrichment analyses revealed the preferred pathways for the selected DEGs. Analysis of core drought responsive gene sets in 48-hour drought exposed leaves of Dinanath grass showed enriched GO terms related to response to stimulus, cell wall biogenesis, metabolic pathways of nucleotides, ROS-scavenging pathway and ABA-mediated stress response. While, in 96-hour drought exposed Dinanath grass leaves the enriched GO terms were defense response, cell wall biogenesis and membrane integrity, ABA-mediated stomata regulation, ROS-scavenging pathway, biosynthesis of secondary metabolites, photosynthesis and energy metabolism. Unique GO terms enriched between 48- and 96-hour drought exposed Dinanath grass leaves suggest specific drought response to varying drought intensities.

Functional enrichment of DEGs presented that 48-hour drought stress triggered KEGG pathways namely 'starch and sucrose metabolism', 'zeatin biosynthesis', 'plant-pathogen interaction' and 'biosynthesis of secondary metabolites' while under 96-hour drought condition the KEGG pathways enriched were 'metabolic pathways', 'biosynthesis of secondary metabolites', 'starch and sucrose metabolism', 'plant-pathogen interaction', 'cyanoamino acid metabolism', 'glycerolipid metabolism' and 'flavonoid biosynthesis'. Previous studies by Zhao et al. (2022) and Xiong and Ma (2022) have shown that plants accumulate several metabolites including sugars, amino acids, organic acids, nucleotides and their derivatives, and secondary metabolites like phenolics and flavonoids to regulate intracellular osmotic pressure, and scavenge ROS in response to drought [59, 60]. Our KEGG enrichment analysis results were inconsistent with these studies. Furthermore, comparative analysis of KEGG pathways between 48- and 96-hour drought exposed Dinanath grass leaves showed that enriched pathways were biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, starch and sucrose metabolism, cyanoamino acid metabolism and flavonoid biosynthesis. We found that in the leaves of Dinanath grass drought tolerance are implicated by carbohydrate metabolism, amino acid metabolism, fatty acid metabolism, secondary metabolite metabolism and antioxidant-related pathways. Interestingly, secondary metabolite metabolism and lipid metabolism played a crucial role in drought response to 48-hour and 96-hour water deficit conditions, respectively.

The present study in Dinanath grass leaves revealed that enrichment of secondary metabolites biosynthesis is the major drought response to 48-hour water deficit condition. The majority of the enriched secondary metabolites

were alkaloids, phenolic compounds, and terpenes. Specifically, flavonoids and terpenoids or isoprenoids were enriched during the 48-hour drought stress. [61, 62]. Drought induces oxidative stress in plants to produce ROS which in turn causes membrane lipid peroxidation, protein denaturation, and DNA damage. Secondary metabolites play a crucial role in defense-related activities in plants as they scavenge ROS and protect the plant cells from lipid peroxidation [63]. The phenylpropanoid pathway is a key metabolic pathway for secondary metabolites and the phenolics and flavonoids produced through this pathway are typical natural antioxidants. Studies have shown that drought increases the accumulation of phenolics and flavonoids, which helps to reduce ROS in plants during drought [64, 65]. Similarly, in our study we noted highest activity of metabolites of phenylpropanoid pathway, including 5-O-Feruloylquinic acid, sanggenon E, kaempferol 3-O-beta-D-glucosyl-(1->2)-beta-D-glucoside and kaempferol-3-O-galactoside. These metabolites of phenylpropanoid pathway are well known to be involved in biosynthesis of lignin, an important phenylpropanoid metabolite [39], that contributes to plant secondary cell wall thickening and maintains photosynthetic rate and osmotic balance under drought stress [66]. Additionally, lignin is deposited on specific tissue walls to enhance mechanical support and water impermeability [43]. Plant lignification reflects the degree of drought tolerance, and synthetic lignin content expresses the degree of lignification [67, 68]. In our study, tolerance to 48-hour drought stress is mainly through pathways associated with lignin biosynthesis in Dinanath grass leaves. Also, flavonoids are linked to ROS generation that contribute to drought tolerance. Our transcriptome analysis supported the metabolomic findings and indicated that the response to 48-hour drought stress involves regulation of genes associated with secondary cell wall thickening and lignin biosynthesis through secondary metabolite metabolism, especially phenylpropanoid pathway.

The results obtained from analysis of 96-hour drought exposed leaves of Dinanath grass revealed that lipid metabolism is the main contributor of drought response. Our findings showed accumulated fatty acid metabolites including sterol lipids, glycerophospholipids, and fatty acyls were the primary contributors to the defense mechanism against increased drought intensity in Dinanath grass leaves. Among the enriched metabolites, androsterone glucuronide, a sterol lipid, showed higher activity followed by glycerophospholipid (phosphorylcholine), flavonoids (kaempferol 3-O-beta-D-glucosyl-(1->2)-beta-D-glucoside, kaempferol-3-O-galactoside and lutein 5,6-epoxide) and terpenoids (deltonin and saikosaponin A). Intense drought stress causes the destruction of plant cell membranes by disintegration of membrane lipids. Sterol lipids are integral components of the membrane lipid bilayer and studies have shown that increased sterol lipid levels are involved in maintaining the integrity of plasma membrane during water deficit [69, 70]. In addition, sterols, flavonoids, terpenoids along with long chain aliphatic lipids constitutes to the composition of cuticular wax. Cuticular wax layer protects the plants against environmental stresses including drought. Many plants exhibit drought response by increasing the deposition of cuticular wax which in turn is believed to influence the barrier properties of cuticular transpiration [71]. ABA-mediated cuticular wax biosynthesis has been reported to involved in regulation of stomatal aperture and thus, enhance drought tolerance [72]. Similarly enriched sterol lipids, flavonoids and terpenoids in response to 96-hour drought stress indicates their involvement in maintaining membrane integrity and fluidity mainly through cuticular wax formation in the leaves of Dinanath grass.

Moreover, sterol lipids are the biosynthetic precursors of steroid hormones including brassinosteroids in higher plants. Brassinosteroids are important regulators of the secondary cell wall deposition and synthesis of lignin in plants [73]. Studies have shown that brassinosteroids promote stress tolerance by positively modulating multiple cellular processes such as CO_2 assimilation, photoprotection, antioxidant defense, redox balance, ROS scavenging and signaling, defense response, detoxification, secondary metabolism, and autophagy in plants [74]. Recent report by Yaqoob et al. (2022) suggested that crosstalk between brassinosteroid signaling, ROS signaling and phenylpropanoid pathway might mediate tolerance to stress including drought [75]. Taking into account our results and the previous studies we suggest that sterol lipids also participate in lignin biosynthesis through brassinosteroid signaling and contribute to increased tolerance to intense drought stress. In addition, brassinosteroid signaling has a potential role in mediating defense response in plants by regulating R genes like protein [76]. Similarly, our study showed transcriptional alteration of R genes that were correlated with increased plant-pathogen interaction in Dinanath grass leaves exposed to intense 96-hour drought stress. Our transcriptomic findings corroborate with metabolomic analysis and indicating that lipid metabolism metabolites, particularly brassinosteroid signaling and flavonoids, play a role in enhancing drought tolerance in Dinanath grass leaves under 96-hour drought conditions.

In this study, biliverdin emerged as a crucial metabolite, particularly under drought stress conditions. The potent antioxidant biliverdin, which is catalysed by biliverdin reductase [77], plays a pivotal role in protecting plants from oxidative stress [78–80]. Furthermore, porphyrin metabolism is intricately involved in the tetrapyrrole biosynthetic pathway. Notably, its product, protoporphyrin IX (Proto IX), channels into both the magnesium (Mg)

and iron (Fe) branches for chlorophyll and heme biosynthesis, respectively [81].

In our investigation, the associated metabolites under drought stress conditions were linked to the highly expressed DEGs. For instance, increased expression of the phenylalanine ammonia-lyase gene resulted in increased levels of drought-responsive flavonoids, such as kaempferol-3. Similarly, Jan et al. (2022) reported that the resistance of transgenic rice plants to drought stress was enhanced by increased accumulation of kaempferol and quercetin [82]. Additionally, there was a correlation between the metabolite and transcriptome data obtained in the present study, and the increase in carotenoids was due to the overexpression of carotenoid cleavage dioxygenase-7, which prevents oxidative stress in rice varieties [83, 84]. Furthermore, phosphoryl choline accumulation has been positively correlated with increased expression of glycerophosdiesterase genes, which are involved in glyceropholipid metabolism as a source for lipid synthesis to activate signal transduction that protects the cell membrane from drought-induced oxidative stress [85].

Conclusion

The present investigation describes the molecular and metabolic mechanisms responsible for drought tolerance in the leaves of Dinanath grass by utilizing transcriptomic and metabolomic analyses. In this study, we comprehensively analyzed the global and specific changes in transcript and metabolic profiles in Dinanath grass leaves under two different drought intensities (48- and 96-hour). The results show a correspondence between changes in gene profiles and changes in metabolite profiles. The DEGs and differentially accumulated metabolites triggered by both 48- and 96-hour drought stress were commonly enriched in KEGG pathways associated mostly with secondary cell wall thickening and lignin biosynthesis. These pathways, in turn, play a role in maintaining membrane integrity and promoting stomatal closure in Dinanath grass leaves. Combined transcriptomic and metabolomic analysis suggested that drought tolerance in Dinanath grass leaves was found to be linked to (1) increased ABA levels and activated ABA signaling pathway, (2) enhanced enzymatic activity or metabolic pathways associated with ROS scavenging, (3) enhanced secondary metabolites metabolism and accumulated secondary metabolites such as phenolics and flavonoids, and (4) enhanced lipid metabolism and accumulated lipid metabolites mainly sterol lipids. Further, the comparative analysis of these drought-responsive genes showed that tolerance to 48-hour drought stress was inflicted by secondary metabolites biosynthesis, especially phenylpropanoid pathway, while with increased drought intensity to 96-hour the tolerance was imparted by lipid metabolism, mainly brassinosteroid signaling. And this shift in drought response indicates that the adaptation mechanism in Dinanath grass is tightly linked with the intensity of drought stress. The present study provides valuable insights into the molecular regulatory network underlying drought tolerance in Dinanath grass. These findings offer useful clues for future molecular studies and molecular breeding for drought tolerance in grasses and food crops.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

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Author contributions

SP designed and conceived the project; PKY, BKM, SA, SP, HS, and YK performed the research; AR, AC and CNS analyzed the data; PE and YK interpretated and validated the results. PE, SP and YK wrote the manuscript. All authors contributed to writing the manuscript. PE edited and organized the manuscript. All authors commented, discussed, and provided input on the final manuscript.

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Data availability

All data presented in this manuscript are included in the supplemental tables and raw data of RNA sequencing has been deposited in NCBI database under BioProject: PRJNA1107114. (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1107114).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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