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Cryptic Resilience: Decoding Molecular Networks in Pearl Millet for Enhanced Heat Stress

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Abstract

Pearl millet (*Pennisetum glaucum*), a vital cereal crop renowned for its drought tolerance, is a cornerstone for smallholder farmers in arid and semi-arid regions, ranking as the fifth most significant cereal globally. Despite its resilience, the molecular mechanisms underlying its tolerance to heat stress remained elusive. To address this knowledge gap, we subjected ten-day-old pearl millet seedlings to an unprecedented temperature of 50°C for 60 seconds. Subsequent next-generation RNA sequencing aimed to unravel differential gene expression in heat-stressed seedlings compared to control conditions. Our analysis revealed a remarkable 29.8% differential expression in the genome sequence in response to heat stress. Heat-stressed pearl millet leaves exhibited differential expression in 11,483 genes, with fold changes ranging from 2 to 18.6 compared to the control group. Of these, 3,612 genes displayed upregulation, while 7,871 genes exhibited downregulation. These genes play roles in diverse biological processes involving crucial enzymes such as aminoacyl-tRNA synthetases, ligases, methyltransferases, oxidoreductases, and DNA-directed RNA polymerases. The Photosystem II Type I Chlorophyll-a/b-binding protein and heat shock proteins displayed the most significant fold changes in heat-stressed leaves. Moreover, various transcription factor families, including bHLH, ERF, NAC, WRKY, MYB-related, C2H2, bZIP, MYB, FAR1, and B3, vital in controlling pearl millet's response to heat stress, were linked to over 100 differentially expressed genes. The dataset generated through this research, shedding light on the

molecular processes enabling pearl millet to withstand heat, holds immense value given the crop's role in food security and resilience to extreme weather. In the context of climate change and global warming, this knowledge lays the foundation for further studies on metabolic engineering and selecting crops resilient to high temperatures. Our transcriptomics approach provides comprehensive gene expression profiles of heat-stressed plants. It elucidates pearl millet's response to heat stress, offering a crucial resource for future investigations into crop adaptation strategies.

Keywords
Heat stress; plant productivity; global climate change; arid and semi-arid regions; RNA next-generation sequencing; transcription factor family

1. Introduction

Global plant health is significantly impacted by heat stress, an abiotic stressor whose intensity is expected to rise due to climate change, rising temperatures, and erratic weather patterns [1-3]. Heat stress, brought on by temperatures over a plant's ideal range, causes several physiological and biochemical alterations, such as increased transpiration, decreased photosynthesis, denaturation of proteins, and oxidative damage. Plant growth, development, and tolerance to other stresses-particularly drought-are all negatively impacted by heat stress [4]. Stress throws off cellular homeostasis, including water absorption and nutrients. This throws off metabolism, delays the end of photosynthesis, interrupts the creation of energy, and eventually kills the cell. As a result, plants under stress experience an increase in reactive oxygen species (ROS) and lipid peroxidation [5]. Plant biologists and crop breeders must comprehend how plants react to heat stress and develop improved temperature and drought tolerance through breeding in light of the current worldwide water crisis. Similarities exist between pearl millet (*Pennisetum glaucum*) and other C4 plants, such as sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), and maize (*Zea mays*). Because of the effective carbon dioxide fixation mechanism used by C4 plants, they can survive in hot, dry climates. C4 plants, such as pearl millet, are essential for food security because their grains provide substantial nutrition. They are members of the Poaceae family, share a common ancestor, and have undergone genetic diversity. It is crucial to comprehend how high temperatures affect plant gene expression, a side effect of global warming [6].

Pearl millet is becoming increasingly important as a climate-resilient crop to combat global warming and ensure food and nutrition security for a growing population. Understanding its molecular networks is crucial to enhancing the resilience of pearl millet to changing climate conditions [6]. Owing to its exceptional tolerance to drought, pearl millet presents a significant chance to investigate the molecular processes that underlie the reactions to heat and drought [7-9]. Pearl millet's stress resilience is controlled by a variety of molecular pathways that have been investigated. For example, Chardin et al. [7] reported that RWP-RK proteins, characterized by a conserved RWP-RK domain, are involved in various biological processes related to nitrogen signaling and metabolism. This explains how the RWP-RK gene family enhances the ability of elephant grass to withstand heat, as demonstrated by Jin et al. [8]. Sun et al. [9] also identified shared differentially

expressed genes in pearl millet under heat and drought conditions. Thus, we aimed to explore how these genes might contribute to pearl millet tolerance.

Abiotic stress can take various forms, including soil pollutants, salt, and heat stress [1-9]. Despite these challenges, when supplemented, pearl millet displays adaptability and a positive response to stress factors [10]. For instance, the application of melatonin has been shown to reduce cadmium toxicity in pearl millet, fostering development and enhancing antioxidant defense, as demonstrated by Awan et al. [10]. Additionally, our previous study [11] delves into the pearl millet (*Pennisetum glaucum*) response to salt stress, a significant concern in arid and semi-arid environments. In this experiment, 14-day-old seedlings were subjected to seven days of daily irrigation before being categorized into control, 75 mM NaCl, and 150 mM NaCl. Saline treatment notably impacted weight and chlorophyll content, particularly at 150 mM NaCl, compared to the control and 75 mM NaCl groups. RNA sequencing of leaves unveiled 3246 upregulated and 7408 downregulated genes, accounting for 27.6% of differentially expressed genes. In the investigation outlined in this study [11], pathway analysis uncovered associations between downregulated genes and the synthesis of coumarin and cholesterol. Conversely, upregulated genes were linked to crucial processes such as lysine degradation and phytyl-PP biosynthesis.

Pearl millet is highly vulnerable to drought stress, resulting in diminished seedling elongation, biomass, and chlorophyll content, and elevated levels of reactive oxygen species (ROS) and lipid peroxidation [12]. Also, high-temperature stress, especially during reproductive stages, can lead to reduced seed yield, individual seed weight, increased ROS content, and diminished antioxidant enzyme activity in pollen and pistils [13]. Transcriptional changes in pearl millet leaves, with the highest number of differentially expressed genes (DEGs) observed after 96 hours of exposure to high temperatures, further accentuate the impact of this stress [14]. These findings collectively indicate that drought and high-temperature stress induce ROS generation and decrease antioxidant enzyme activity, crucial factors in apoptosis induction [12-14]. Previous studies on pearl millet's heat tolerance have identified specific hybrids and genotypes displaying significant resilience to heat and drought stress [15, 16]. These studies underscore the relevance of physiological and morphological traits in enhancing tolerance to these stressors, emphasizing the potential of molecular breeding techniques to improve this trait further [16]. Various screening methods for assessing heat tolerance, including membrane thermo-stability tests and field-based screening techniques, have been proposed [17].

Our study aimed to delve into the intricate molecular networks operating within pearl millet plants to understand better how they cope with heat stress. To mimic acute heat stress conditions, we subjected pearl millet leaves to a short burst of high temperature (1 minute (60 sec) at 50°C), a novel approach that has not been previously explored to the best of our knowledge. Through RNA sequencing, we aimed to unravel the subtle changes in gene expression that occur in response to this brief but intense heat exposure. This innovative method allowed us to decipher the cryptic mechanisms underlying heat stress resilience in pearl millet, shedding light on the genetic pathways that enable plants to withstand and adapt to extreme environmental conditions.

Our study examined how pearl millet responds at the molecular level to severe heat conditions. By subjecting plants to a temperature of 50°C for 60 seconds, which exceeds temperatures utilized in earlier research [9, 10], we aimed to induce a highly acute heat stress response. This strategy enabled us to scrutinize brief heat exposure's immediate and temporary impacts on gene expression, thereby circumventing potential complications such as prolonged heat-induced cellular damage [12-

14]. Utilizing transcriptome analysis, we comprehensively examined changes in gene expression patterns induced by this brief but intense heat treatment. The present study identified a notable shift in heat shock proteins and Photosystem II Type I Chlorophyll-a/b-binding protein, indicating their potential roles in alleviating heat stress. Our research contributes substantially to understanding crop adaptation to abiotic stress, providing insights for enhancing heat tolerance in cereal crops, including pearl millet. Moreover, our work highlights the practicality of genomic adaptation signatures in germplasm characterization. These signatures can improve the efficiency of marker-assisted selection (MAS) and other crop improvement programs. Integrating these markers into breeding initiatives, particularly in regions prone to heat stress, is expected to streamline the selection of heat-tolerant cultivars, ultimately improving crop productivity and yield. This research sets the stage for future investigations aimed at bolstering drought resilience in pearl millet and other cereals and represents a significant advance in understanding crop adaptation to abiotic stress. The emphasis on genomic adaptation signatures underscores their potential in MAS and germplasm characterization, offering a promising avenue for improving crop performance in regions susceptible to heat or drought stress.

2. Materials and Methods

2.1 Plant Materials and Stress Treatment

As previously mentioned in our earlier investigation [11], the seeds utilized in the current study belong to the classification of pearl millet *[Pennisetum] glaucum subsp. monodii*. These seeds are cataloged under reference number 1319 in the Center of Genetic Resources database at the Saudi Arabian Ministry of Environment, Water, and Agriculture. The seeds were first rinsed with distilled water and then 5% sodium hypochlorite solution before starting the germination procedure on damp sheets. The germinated seedlings were moved into a potting soil mixture made up of dirt and vermiculite in a 1:1 ratio after 48 hours. Every day, 20 ml of distilled water was put into each pot (which measured 6 by 7 inches) to hydrate these seedlings. These growing circumstances were kept at 25°C in a greenhouse. After ten days of growth, seedlings of pearl millet were subjected to heat stress at 50°C for 60 seconds, using COSORI Smart 12-in-1 Air Fryer Toaster Oven Combo, Airfryer Convection Oven Countertop, China). The plant pots were placed inside the oven, and the timer was set for one minute, with the preset temperature set to 50°C (122°F).

Following a 60-second heat exposure, leaf samples were collected, fixed in RNAlater Stabilization Solution in accordance with Lader's technique [18, 19], and then kept at 4°C. The Trizol RNA isolation technique, which was adapted from Chomczynski and Mackey [20], was used to extract leaf RNA. Fourteen seedlings were planted under standard settings as the control group in the experiment, while fourteen seedlings received heat shock treatment as the stress group.

2.2 Validation of RNA-Seq Analysis

The RNA-seq analysis underwent validation through the following steps:

2.2.1 Quality Check of Raw Sequencing Reads

The quality of raw sequencing reads was evaluated using FastQC, focusing on sequencing depth and per-base sequence quality. Results are summarized in Table S1 and Table S2.

2.2.2 Library Preparation

For library preparation, high-quality RNA samples with a RNA Integrity Number (RIN) above 7 were selected. Approximately 2 ng or 10 nm of each sample was utilized. Qubit concentration and RNA quality were assessed to ensure suitability for library construction. The libraries met predefined sequencing criteria, with qubit concentrations exceeding 2 ng or 10 nm and peak sizes ranging between 400 and 500 nm [21].

2.2.3 RNA-Seq Library Preparation

The Illumina TrueSeq RNA library preparation kit was employed for RNA-seq library construction. Low-quality reads, identified by Phred scores below 33, were removed using Trimmomatic [22]. Subsequently, a second round of quality control was conducted to confirm adherence to specified standards.

2.2.4 Validation via Visualize Alignment

Reads filtering and data processing were performed, followed by alignment to the Pearl millet gene assembly v.1.1 reference genome using HISAT2 [23]. SAM files were converted to BAM format using Samtools [24]. The DESeq2 package in RStudio was used to normalize the combined count matrix [25]. All RNA sequences for pearl millet leaves were deposited in the NCBI's Sequence Read Archive (SRA) database with accession number PRJNA991076.

2.2.5 Biological Significance Validation

Differential expression analysis was performed using DESeq2 to identify genes exhibiting significant expression changes. A comprehensive list of differentially expressed genes was generated, including upregulated and downregulated genes. Additionally, a comparative assessment of Pearl millet stress-related gene IDs was conducted using the methodology outlined by Ghatak et al. [26], utilizing a set of 545 heat- and drought-responsive genes from Arabidopsis. High-throughput analysis was facilitated by categorizing proteins and corresponding genes using the Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System [27], ensuring an organized and efficient analysis.

3. Results

3.1 Heat Stressed Differentially Expressed Genes (DEGs), Protein Classification, and Transcription Factors in Pearl Millet

In the RNA sequences of heat-treated pearl millet seedlings, 11,483 differentially expressed genes (DEGs) were identified. The preliminary whole-genome sequence of pearl millet spans approximately 1.79 giga bases (Gb) and encompasses an estimated 38,579 genes [28]. Notably,

during heat stress, 29.8% of this genomic sequence exhibited differential expression. Among these DEGs, 3,612 genes were upregulated compared to the control group, with 2,388 genes having associated information and 1,224 genes lacking such data. Additionally, 7,871 genes were downregulated compared to the control group, with 3,544 genes lacking related information and 4,327 genes having supporting details (Figure 1). It is essential to clarify that "corresponding information" refers to crucial data found in publicly accessible databases, including EC numbers, gene names, protein families, UniProt accession, KEGG Cross-reference (KO), KEGG-pathways, Transcription Factor Family, and Gene Ontology IDs (GO). Compared to the control, the fold change values for the differentially expressed genes ranged from 2 to 18.57 (Figure 2 and Table S3). A heatmap (Figure 3) was utilized to visually represent the expression levels of multiple genes across various samples, facilitating the identification of potential variations or parallels in gene expression profiles. Additionally, Principal Component Analysis (PCA) effectively distinguished heat-stressed leaves from unstressed ones, indicating substantial changes in gene expression in Figure 4.

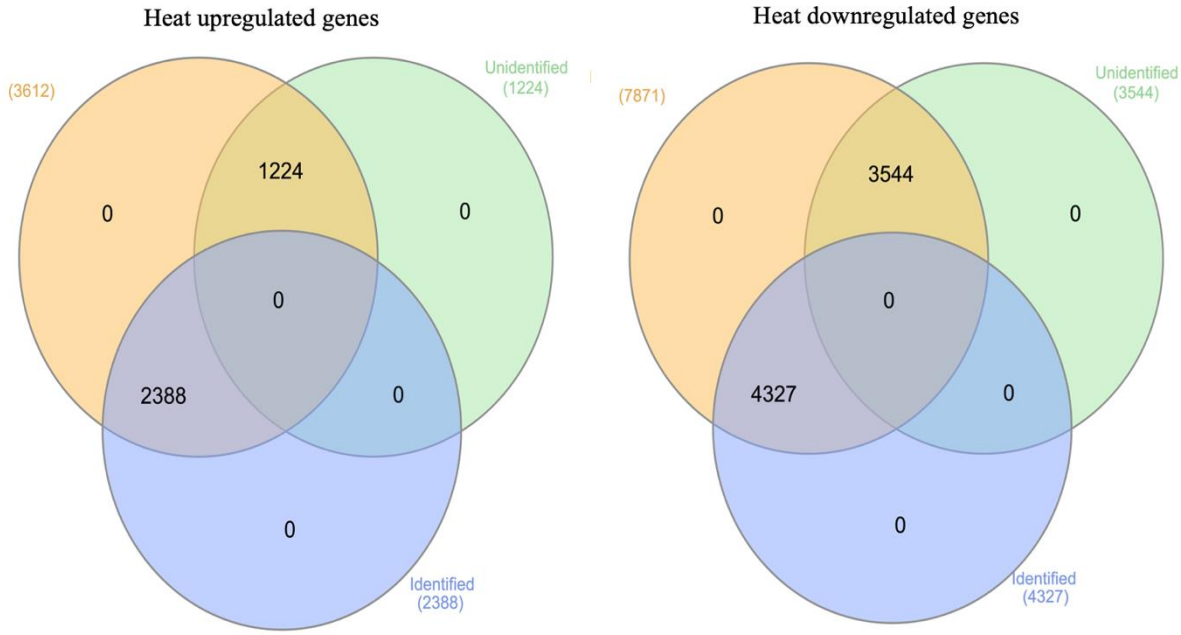

Figure 1 Gene expression difference in pearl millet leaves under heat stress compared with the control.

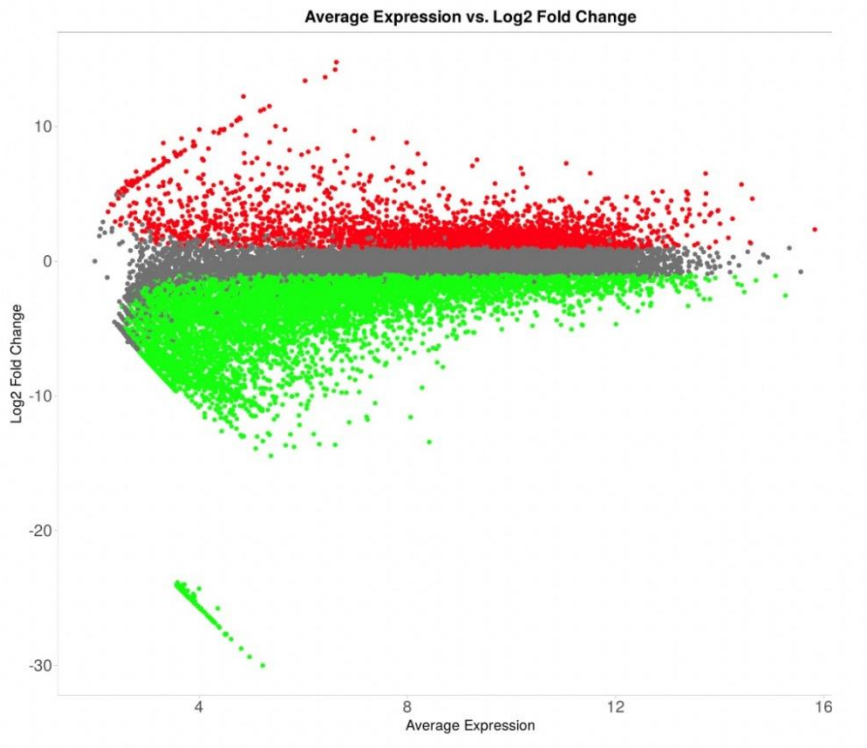

Figure 2 Fold change in differentially expressed genes in heat-stressed leaves compared with the control.

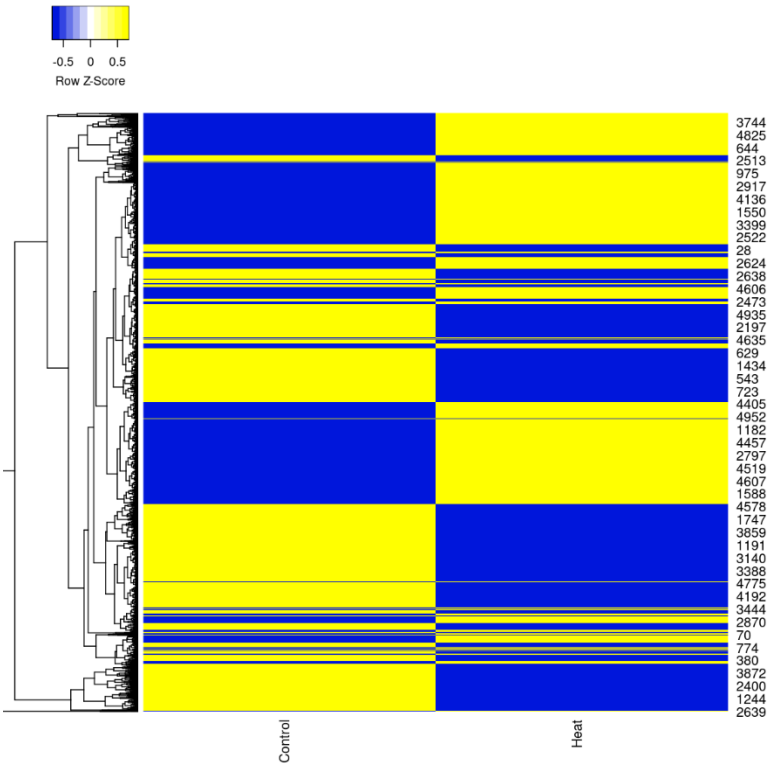

Figure 3 Heat map showing the expression levels of multiple genes across different samples of the heat-stressed leaves compared to control. The heat map visually represents the gene expression patterns within each sample.

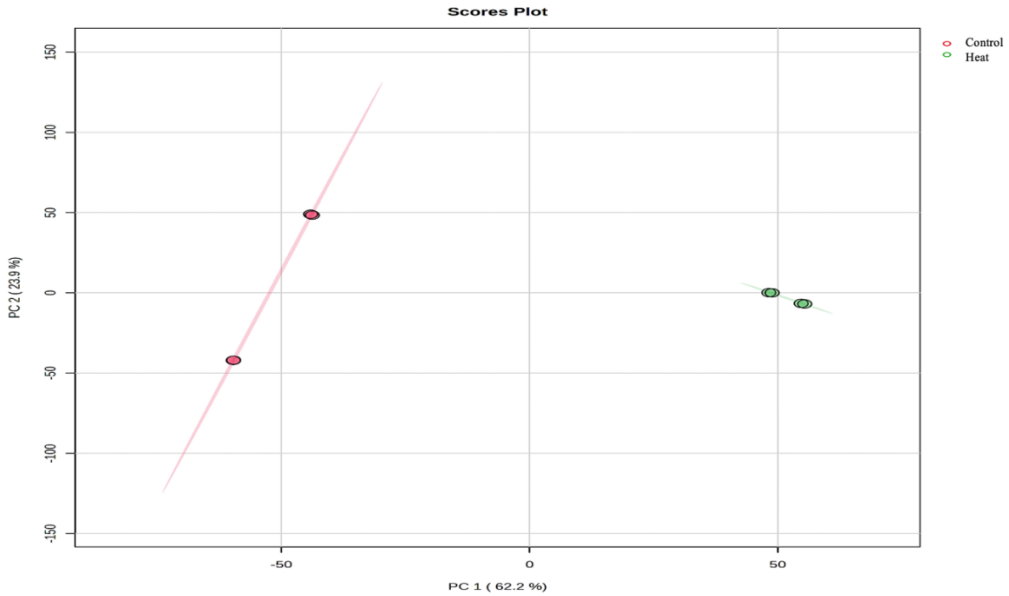

Figure 4 Principal component analysis of leaves under control and heat stress conditions.

In analyzing differentially expressed genes (DEGs) under heat stress in pearl millet, a notable convergence was observed in the protein class associated with translational proteins among both upregulated and downregulated genes. The classification of DEGs sheds light on proteins' crucial roles in various biological functions during heat stress in pearl millet. This investigation focused on enzymes that ensure cellular longevity and function by catalyzing essential chemical events. The main topics explored include ligases, methyltransferases, oxidoreductases, aminoacyl-tRNA synthetase, and DNA-directed RNA polymerases. Their functions and significance in various cellular activities are comprehensively detailed in Table 1. This analysis underscores the intricate molecular responses and adaptations occurring in pearl millet under heat stress, providing valuable insights into the regulatory mechanisms governing cellular processes. Notably, the upregulated genes exhibited increased fold changes (16-11 fold) in proteins associated with photosynthesis and chlorophyll function. Heat shock proteins displayed fold changes ranging from 13 to 9, and peroxidases showed fold changes from 12 to 11 (Table 1).

Table 1 Classification of the pearl millet gene family of differentially expressed genes and their PANTHER family using (*Sorghum bicolor*) as a reference gene.

Gene ID	Mapped ID	Gene Name	Gene Symbol	Persistent id	PANTHER Family/Subfamily	PANTHER Protein Class
SORBI|EnsemblGenome = SORBI_3005G143500|UniProtKB = C5Y3V8	C5Y3V8	Glutamyl tRNA amidotransferase subunit B, chloroplastic/mitochondrial	GATB	PTN004285524	GLUTAMYL-TRNA(GLN) AMIDOTRANSFERASE SUBUNIT B, MITOCHONDRIAL (PTHR11659:SF0)	ligase
SORBI|EnsemblGenome = SORBI_3005G224400|UniProtKB = A8QW51	A8QW51	Probable O-methyltransferase 2	OMT2	PTN004300477	O-METHYLTRANSFERASE 2-RELATED (PTHR11746:SF180)	methyltransferase
SORBI|EnsemblGenome = SORBI_3001G126300|UniProtKB = C5WNV2	C5WNV2	Adenylosuccinate synthetase 2, chloroplastic	PURA2	PTN004313098	ADENYLOSUCCINATE SYNTHETASE 2, CHLOROPLASTIC (PTHR11846:SF12)	ligase
SORBI|EnsemblGenome = SORBI_3002G384400|UniProtKB = C5X2M4	C5X2M4	Thiamine thiazole synthase 2, chloroplastic	THI1-2	PTN005163102	THIAMINE THIAZOLE SYNTHASE 1, CHLOROPLASTIC (PTHR43422:SF16)	oxidoreductase
SORBI|EnsemblGenome = SORBI_3003G169400|UniProtKB = Q01923	Q01923	DNA-directed RNA polymerase subunit beta	rpoC2	rpoC2	DNA-DIRECTED RNA POLYMERASE SUBUNIT BETA'' (PTHR19376:SF54)	DNA-directed RNA polymerase

SORBI|Gene_OrderedLocus Name = Sb10g008780|UniProtKB = C5Z7K4	C5Z7K4	AlaninetRNA ligase, chloroplastic/mitochondrial	Sb10g008780	PTN004304977	ALANINE-TRNA LIGASE, CYTOPLASMIC (PTHR11777:SF9)	aminoacyl-tRNA synthetase
SORBI|EnsemblGenome = SORBI_3003G281800|UniProtKB = C5XIF2	C5XIF2	CASP-like protein 3A1	Sb03g033320	PTN004277018	CASP-like protein 3A1 (PTHR33573:SF48)	-
SORBI|Gene = psbA|UniProtKB = A1E9Q4	A1E9Q4	Photosystem II protein D1	psbA	PTN002108170	PHOTOSYSTEM II PROTEIN D1 (PTHR33149:SF40)	-
SORBI|EnsemblGenome = SORBI_3010G034100|UniProtKB = C5Z3W1	C5Z3W1	Translation factor GUF1 homolog, mitochondrial	Sb10g003070	PTN005166714	translation initiation factor	TRANSLATION FACTOR GUF1 AND MITOCHONDRIAL (PTHR43512:SF7)
SORBI|EnsemblGenome = SORBI_3003G234800|UniProtKB = C5XEK4	C5XEK4	CASP-like protein 4U1	Sb03g029220	PTN005012543	-	CASP-like protein 4U1 (PTHR33573:SF35)
SORBI|EnsemblGenome = SORBI_3005G224400|UniProtKB = A8QW51	A8QW51	Probable O-methyltransferase 2	OMT2	PTN004300477	methyltransferase	O-METHYLTRANSFERASE 2-RELATED (PTHR11746:SF180)
SORBI|EnsemblGenome = SORBI_3008G164900|UniProtKB = C5YRU8	C5YRU8	CASP-like protein 1B1	Sb08g021090	PTN004277710	dehydrogenase	CASP-like protein1B1 (PTHR11615:SF244)
SORBI|EnsemblGenome = SORBI_3004G093800|UniProtKB = C5XY39	C5XY39	CASP-like protein 2D1	Sb04g007720	PTN004277927	dehydrogenase	CASP-like protein 2D1 (PTHR11615:SF143)

SORBI|EnsemblGenome = SORBI_3003G437400|UniProtKB = P84516	P84516	Cationic peroxidase SPC4	Sb03g046810	PTN004786712	peroxidase	CATIONIC PEROXIDASE SPC4 (PTHR31235:SF255)
SORBI|EnsemblGenome = SORBI_3006G007900|UniProtKB = A8QW53	A8QW53	5-Pentadecatrienyl resorcinol O-methyltransferase	OMT3	PTN004300658	methyltransferase	ACETYLSEROTONIN O-METHYLTRANSFERASE 3 (PTHR11746:SF272)
SORBI|EnsemblGenome = SORBI_3001G416700|UniProtKB = C5WPC2	C5WPC2	Arginine biosynthesis bifunctional protein ArgJ, chloroplastic	Sb01g039230	PTN004606241	acetyltransferase	ARGININE BIOSYNTHESIS BIFUNCTIONAL PROTEIN ARGJ, MITOCHONDRIAL (PTHR23100:SF0)

To comprehend the functions of differently expressed genes in pearl millet, we compared them with Arabidopsis orthologous genes, identifying 66 out of the 545 genes in Arabidopsis. These genes were shared between pearl millet and Arabidopsis (Table S4). Conversely, downregulated proteins exhibited a distinct correlation with biological regulation and the response to stimuli, as detailed in Table S5.

The correlation between the number of differentially expressed genes (DEGs) in pearl millet leaves under heat stress and protein families highlights the dynamic changes in gene expression levels in response to heat stress conditions. The range of DEGs varies from 39 genes associated with the DNA-binding with one finger family protein to 404 genes related to the basic helix-loop-helix (bHLH) family protein. These findings suggest that the bHLH family is more sensitive to heat stress, which is evident in the significant up- or down-regulation of gene expression in pearl millet leaves. Conversely, the protein belonging to the Dof family displays a comparatively lower quantity of DEGs, suggesting a more subdued reaction to heat stress circumstances (Figure 5).

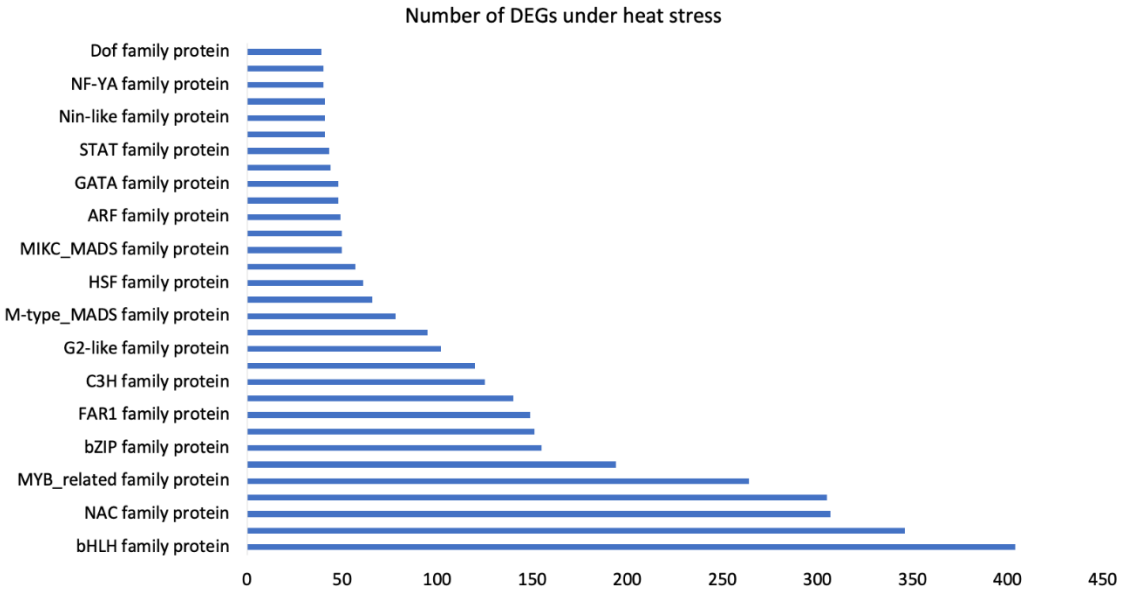

Figure 5 Protein family associated with the number of differentially expressed genes in pearl millet leaves under heat stress. The number of DEGs ranged from those related to the bHLH family protein to 39 related to the Dof family protein.

The pathways associated with the number of differentially expressed genes in pearl millet leaves under heat stress in the Kyoto Encyclopedia of Genes and Genomes (KEGG) highlight the variety of pathways impacted by heat stress and the differing numbers of differentially expressed genes associated with each path. Notably, 59 genes are linked to the ribosome pathway, while 15 genes are associated with the peroxisome pathway among the DEGs. This implies that specific pathways, like the ribosome pathway, undergo substantial changes in the expression of their genes in response to heat stress, potentially indicating their crucial role in plant responses to such stressors. Conversely, the tiny number of DEGs in the peroxisome pathway indicates a relatively minor role in the plant's reaction to heat stress (Figure 6).

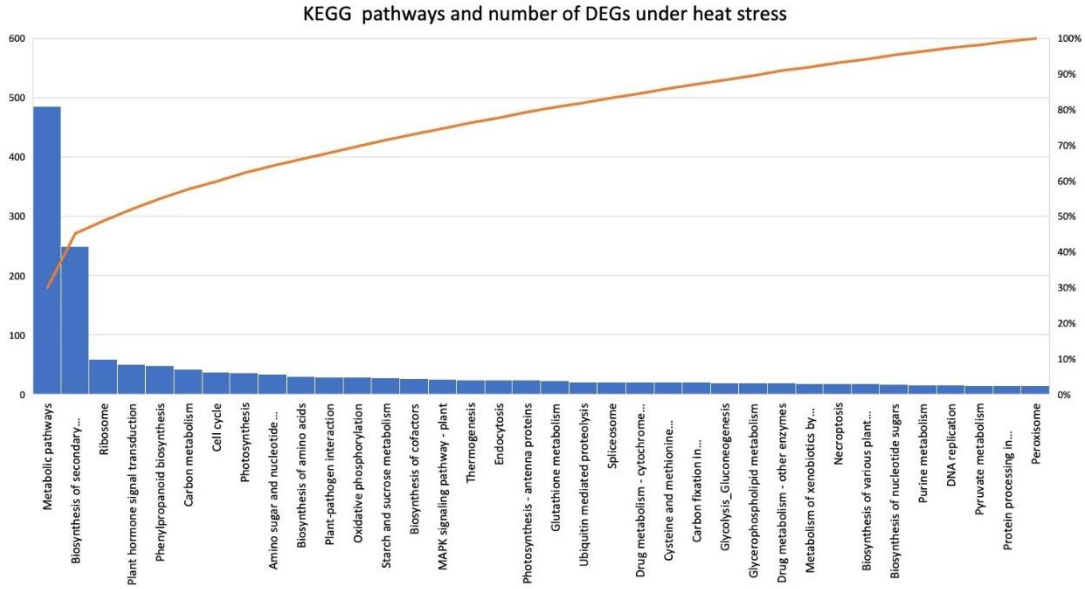

Figure 6 KEEG pathways associated with the number of differentially expressed genes (DEGs) in pearl millet leaves under heat stress. The number of DEGs ranged from 15 to 59 genes related to peroxisomes.

4. Discussion

4.1 Differentially Expressed Genes (DGs) under Heat Stress

Comparing the differentially expressed genes in heat-stressed pearl millet leaves with 545 heat- and drought-responsive genes from Arabidopsis, 66 genes were identified. Notably, Pgl_GLEAN_10027569, a Photosystem II Type I chlorophyll a/b-binding protein, exhibited a fold change value of 16, while Pgl_GLEAN_10022809, a subtilase family protein, had a fold change value of 3.4 (Table S4). These results align with the findings of Awan et al. [10], indicating a consistent increase in the fold change of the Photosystem II Type I chlorophyll a/b-binding protein. Compared to pearl millet subjected to only cadmium, Awan et al. [10] demonstrated that treating cadmium-stressed plants with exogenous melatonin led to an increased chlorophyll content in the plant. Several studies have investigated differentially expressed genes in pearl millet subjected to heat treatment. Sun et al. [9] utilized Pacbio sequencing to assess the heat and drought stress responses, identifying 6920 and 6484 differentially expressed genes, respectively. Focusing on drought tolerance, Dudhate et al. [29] identified 6799 and 1253 differentially expressed genes in two pearl millet inbred lines, while Choudhary et al. [30] pinpointed ten differentially expressed genes responsive to drought stress. These studies collectively underscore the intricate genetic response of pearl millet to diverse stressors, encompassing both heat and drought conditions. Our investigation focused on specific proteins, including CASP-LIKE PROTEIN 4U1, O-METHYLTRANSFERASE 2-RELATED, TRANSLATION FACTOR GUF1, MITOCHONDRIAL, CASP-LIKE PROTEIN 1B1, CASP-LIKE PROTEIN 2D1, and CATIONIC PEROXIDASE SPC4. These proteins share similarities in their involvement in essential cellular functions within plants, particularly within mitochondria or chloroplasts. Their roles span various metabolic pathways, energy production, and protein synthesis [20-25].

Moreover, several of these proteins serve as specific catalytic enzymes, encompassing synthetases, ligases, and methyltransferases. The significance of particular proteins in translating genetic information into functional proteins is highlighted by their close association with protein synthesis, exemplified by alanine tRNA ligase and the translation factor GUF1 homolog. Proteins such as arginine biosynthesis bifunctional protein ArgJ and adenylosuccinate synthetase 2 play crucial roles in metabolic pathways, specifically in purine and arginine biosynthesis, respectively [27-31].

CASP-like proteins may participate in various cellular processes through regulatory functions. The photosystem II protein D1, as a component of the essential photosynthesis complex in plants, plays a crucial role in this context. Additionally, cationic peroxidase SPC4 might be involved in peroxidase activity, a function of potential significance in responding to oxidative stress conditions, such as the heat stress observed in our investigation. These proteins exhibit diverse functions critical to fundamental cellular processes in plants, including energy production, protein synthesis, metabolic pathways, DNA and RNA processing, and photosynthesis. When combined, they collectively support overall plant development, growth, and adaptation to environmental challenges [29-31].

These findings contribute to our understanding of plant adaptation processes and provide insights into identifying critical regulatory components and pathways involved in the response to heat stress [31-46].

4.2 Unraveling the Intricate Molecular Mechanisms of Heat Tolerance

Pearl millet holds vital importance as a staple crop, especially in semi-arid regions characterized by high temperatures and drought. In our research, we devised an efficient experimental framework to simulate heat stress situations and study gene expression changes in response to this stress. The leaves of pearl millet plants, known for their resilience, were subjected to 50°C for 60 seconds, and subsequent analyses revealed significant alterations in gene expression profiles under heat stress. Surprisingly, a unique class of downregulated genes, termed "TRANSLATION FACTOR GUF1-RELATED (PTN000563148)," was identified, revealing a distinctive biological regulation mechanism. These proteins play a crucial role in protein synthesis, coordinating translation initiation, elongation, and termination, ensuring accurate translation of genetic information. The study also highlighted an intriguing correlation between the increase in heat shock proteins and the rise in TRANSLATION FACTOR GUF1-RELATED proteins during heat stress. This sheds light on the complex effects of heat shock on gene expression, including the suppression of protein synthesis, mRNA sequestration, and the subsequent translation during the recovery stage. Proteins with the most substantial negative loadings were more prevalent in stressed samples, particularly heat shock proteins of the 70-kDa family (Hsp 70) and peroxidases. These proteins play active roles in crucial processes related to plant cellular metabolism and stress responses [31, 36, 38]. The analysis identified three protein kinase orthologs, showing fold alterations between 13 and 10, consistent with earlier investigations into pearl millet's responses to drought stress. The study also uncovered upregulated genes encoding essential chaperone proteins and various heat shock proteins crucial for intracellular functions [38].

4.3 The Role of Photosystem Binding and Heat Shock Proteins in Mitigating Heat Stress

Recent research highlights the role of the Photosystem II Type I Chlorophyll-a/b-binding protein in mitigating drought stress in pearl millet. The notable overexpression of genes, exhibiting a 16-

fold difference compared to the control, underscores the protein's potential significance in addressing challenges posed by elevated temperatures leading to drought stress. This issue substantially impacts agricultural systems, impeding crop growth, development, and output [47-50]

The chlorophyll-binding protein is an indispensable component of the Type I Photosystem II, crucial for energy transfer and light absorption. Recent studies suggest its potential involvement in stress response, particularly in the context of drought tolerance [51, 52].

Limited water availability during droughts can reduce photosynthetic activity in plants, potentially leading to oxidative stress and damage to the photosynthetic system. The Type I Chlorophyll-a/b-binding protein, with its broad-spectrum light absorption capacity, plays a crucial role in maintaining photosynthetic efficiency. This ensures effective energy acquisition in water-deficient conditions [53], enhancing the plant's ability to sustain photosynthesis and promote growth even amid drought [54].

Plants often generate elevated levels of reactive oxygen species (ROS) under drought stress due to an imbalance in light absorption and utilization. ROS can induce oxidative stress and cellular damage. A photoprotective mechanism linked to the Type I chlorophyll a/b-binding protein dissipates excess light energy as heat, reducing the likelihood of ROS generation. Emerging data suggests that this protein scavenges ROS and enhances the plant's tolerance to oxidative stress during drought conditions [55]. Its interaction with stomatal control implies a role in facilitating effective stomatal closure under water scarcity, a pivotal adaptive response contributing significantly to pearl millet's drought tolerance by preserving cellular hydration and conserving water.

Pearl millet employs various adaptation strategies to enhance drought tolerance, with the Photosystem II Type I Chlorophyll-a/b-binding protein playing a crucial role. This protein efficiently harvests light, provides photoprotection, mitigates ROS, and contributes to stomatal control. These mechanisms prove essential in maintaining photosynthetic activity and cellular homeostasis in water-scarce conditions [56].

Under heat stress, plants also experience increased levels of reactive oxygen species (ROS) due to various cellular processes being disrupted. ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals, are generated as byproducts of metabolic reactions in chloroplasts, mitochondria, and peroxisomes. The accumulation of ROS can lead to oxidative stress, causing damage to lipids, proteins, and DNA within plant cells [55-57].

To counteract the harmful effects of ROS, plants have evolved antioxidant defense systems, including enzymes like superoxide dismutase, catalase, and peroxidases, as well as non-enzymatic antioxidants such as ascorbate, glutathione, and tocopherols. These antioxidants help scavenge ROS and maintain cellular homeostasis under heat-stress conditions [55-57].

Moreover, plants also employ heat shock proteins (HSPs) as stress response mechanisms. HSPs act as molecular chaperones, assisting in protein folding, assembly, and degradation, thereby protecting cellular structures from heat-induced damage. Additionally, some HSPs have been shown to directly regulate ROS levels and enhance plant tolerance to heat stress [57].

Heat shock proteins (HSPs) constitute a highly conserved class of proteins present in all living organisms, playing a pivotal role in maintaining cellular integrity and orchestrating stress responses, particularly in challenging conditions like extreme heat. The exceptional tolerance of pearl millet to heat stress provides a unique opportunity to delve into how heat shock proteins contribute to heightened heat tolerance [57].

Functioning as molecular chaperones, heat shock proteins actively facilitate intricate processes such as protein folding, assembly, and degradation, thereby preserving the delicate balance of cellular homeostasis. Systematically categorized based on their molecular weight, these proteins include small HSPs (sHSPs), HSP90, HSP70, HSP60, and HSP100. Typically present at low constitutive levels under normal conditions, their expression significantly increases in response to various stressors, such as elevated temperatures [58].

High temperatures subject pearl millet cells to proteotoxic stress, leading to the unfurling or misfolding of proteins, disrupting vital cellular processes. The prompt upregulation of heat shock protein expression serves as a protective response, preserving cellular components and facilitating precise protein folding. Acting as sentinels, these HSPs prevent denatured proteins from aggregating, overseeing their refolding, or aiding in their targeted degradation if irreversibly damaged, thereby maintaining cellular architecture integrity [14]. Our findings unequivocally demonstrate that, in response to high temperatures, pearl millet enhances the expression of specific HSPs, particularly HSP70 and small HSPs. These HSPs are crucial in reducing heat-induced protein degradation, preserving membrane integrity, and initiating cellular recovery post-stress exposure [14, 57].

The primary determinant of pearl millet's heat tolerance lies in its ability to sustain photosynthetic efficiency even under severe heat stress. Heat shock proteins contribute significantly to this process by ensuring photosynthetic proteins' correct folding and assembly, guaranteeing uninterrupted photosynthesis in adverse conditions. HSPs are essential for maintaining energy generation and promoting plant development under heat stress by preventing the denaturation of photosynthetic enzymes and safeguarding photosystem complexes. Additionally, they serve as potent scavengers of reactive oxygen species (ROS) and enhance the activation of antioxidant defense systems, playing a dual role in combating oxidative stress. Their presence equips pearl millet plants to combat oxidative damage caused by heat, thereby increasing the plant's tolerance to high temperatures [59, 60].

Heat shock proteins are intricately regulated by heat-shock transcription factors (HSFs), key players in the heat-stress response pathway. In normal circumstances, HSFs remain inactive, but in response to heat stress, they become active, initiating the expression of several HSP genes. The well-coordinated HSF-HSP regulatory nexus in pearl millet is essential for ensuring the crop's remarkable heat tolerance.

Heat shock proteins are indispensable components of pearl millet's stress response apparatus, diligently maintaining cellular balance, upholding photosynthetic efficiency, and fostering redox equilibrium during heat stress. The crop's outstanding heat tolerance is primarily attributed to the swift induction and meticulous management of HSPs under the guidance of HSFs. Understanding the intricate interactions between heat shock proteins and their regulatory systems in pearl millet provides valuable insights for developing strategies to enhance heat tolerance across various crops. This knowledge holds promise for improving agricultural sustainability and bolstering global food security in the face of the escalating challenges posed by climate change.

4.4 A Glimpse into the Varied Enzymes in Differentially Expressed Genes of Heat-stressed Pearl Millet

Our research provides a comprehensive understanding of the essential enzymes expressed by pearl millet under heat conditions, shedding light on their crucial roles in maintaining regular cellular

processes and ensuring plant survival. These enzymes play pivotal roles in various biological processes, including cellular regulation, genetic expression, energy generation, and defense against oxidative stress. Our study, mainly focuses on enzymes such as TRANSLATION FACTOR GUF1, MITOCHONDRIAL; CASP-LIKE PROTEIN 4U1; O-METHYLTRANSFERASE 2-RELATED; CASP-LIKE PROTEIN 1B1; CASP-LIKE PROTEIN 2D1; CATIONIC PEROXIDASE SPC4, significantly advances our understanding of the intricate molecular mechanisms that underlie the maintenance of cellular health and the resilience of pearl millet against high temperatures.

The enzymes identified in the study are detailed in Table S4 and Table S5, each categorized into different classes with unique functional roles:

4.4.1 Ligase

Mediating ligation, ligases covalently link two molecules, maintaining the integrity of genetic material crucial for protein synthesis, DNA repair, and replication [9].

4.4.2 Methyltransferases and O-Methyltransferases

These enzymes contribute to DNA methylation, RNA modification, and gene expression regulation by catalyzing the transfer of methyl groups. They play essential roles in cellular signaling pathways and epigenetic changes [56, 57].

4.4.3 Oxidoreductase

Involved in redox reactions transferring electrons between molecules, oxidoreductases include CATIONIC PEROXIDASE SPC4 (PTHR31235:SF255). These processes are vital for metabolism, cellular respiration, and toxic chemical detoxification. Oxidoreductases support energy production and maintain the redox balance of cells. CATIONIC PEROXIDASE protects against oxidative damage by catalyzing the reduction of hydrogen peroxide and other reactive oxygen species [58].

4.4.4 Aminoacyl-tRNA Synthetase

Ensuring the right amino acids are added during protein synthesis, this enzyme attaches specific amino acids to matching transfer RNA (tRNA) molecules. A reduction in its activity prevents protein synthesis in pearl millet under heat stress, as observed in a prior study by DE PINTO et al. [59].

4.4.5 CASP-LIKE PROTEIN Group

In heat-stressed pearl millet, differentially expressed genes associated with enzymes like CASP-LIKE PROTEIN 1B1 (PTHR11615:SF244), CASP-LIKE PROTEIN 2D1 (PTHR11615:SF143), and CASP-LIKE PROTEIN 4U1 (PTHR33573:SF35) were identified. These proteins, with caspase-like activity, play roles in maintaining tissue homeostasis and participating in programmed cell death (apoptosis) [59].

4.4.6 Mitochondrial Arginine Biosynthesis Bifunctional Protein ArgJ (PTHR23100:SF0)

Pearl millet exhibited an increase in this enzyme under heat stress, emphasizing its importance [60-64].

Out of the six pearl millet cultivars previously studied in the region [62], this specific cultivar

displayed greater nucleotide diversity in the tRNALeu intron. This highlights the genetic diversity within pearl millet cultivars and underscores the importance of preserving and understanding this diversity. Despite the challenging climate of the Arabian Peninsula, these regionally adapted cultivars prove resilient, making them invaluable for agricultural sustainability in a dynamically changing global environment.

4.5 The Top Transcription Family Expressed in Pearl Millet under Heat Stress

Due to an ongoing evolutionary arms race with environmental stresses, plants have developed sophisticated signaling networks and adaptive mechanisms that empower them to recognize and respond to diverse threats. One such response involves the generation of stress-inducing phytochemicals, enhancing the plant's immunological defense. The plant's immunological defense responses to heat stress involve a coordinated network of antioxidant systems, molecular chaperones, and osmolytes to mitigate oxidative damage, maintain cellular function, and enhance heat tolerance. At the core of these intricate defensive mechanisms are transcription factors (TFs), acting as primary regulators of plant stress responses. These TFs function as sentinels, discerning stress signals and orchestrating the activation of genes associated with defense reactions. The accumulation of defense-related metabolites relies heavily on the interplay between transcription factors and stress-signaling pathways [62, 63]. In a previous study, Dhawi [64] utilized enrichment analysis to unveil critical biological processes crucial for pearl millet's response to heat. Among 36,041 genes, 10 exhibited substantial fold changes previously unidentified in such reactions. Computational techniques unveiled domains of conserved amino acid transporters, and genes displayed distinct grouping patterns based on phylogenetic analysis, indicating potential evolutionary links and functional commonalities. Molecular function analysis revealed evidence of both protein kinase and phosphorelay sensor kinase activity in heat-treated leaves.

In Figure 5 and Figure 6, presented in this work, we comprehensively analyzed the transcriptional responses of pearl millet to heat stress conditions. Among the various transcription factor families examined, some exhibited significant elevation in response to heat stress, aligning with findings reported by Meraj et al. [63].

4.6 Heat Stress is Orchestrated by a Complex Network of Transcription Factors

The transcription factor families that have been found, such as bHLH, ERF, NAC, WRKY, MYB-related, C2H2, bZIP, MYB, FAR1, and B3, are essential controllers of pearl millet's response to heat stress. Understanding their roles and interconnections can help us understand the mechanisms underlying plants' ability to respond to heat stress. With the ability to adapt to changing environmental conditions, this understanding creates new opportunities for developing stress-tolerant pearl millet varieties, which could lead to improved agricultural sustainability and food security. The intricate response of plants to heat stress is orchestrated by a complex network of transcription factors, playing a pivotal role in adapting to harsh environmental conditions. Among these regulators, the heat stress transcription factor (Hsf) family stands out for its diverse expression patterns and multifaceted functions, acting as a critical orchestrator in the heat stress response [64]. This family coordinates a transcriptional reaction, activating genes crucial for cellular protection and adaptation under elevated temperatures. Another significant contributor to regulating genes responsive to abiotic stress, especially heat stress, is the AP2/ERF family, focusing on the DREB

subfamily [65]. This family of transcription factors significantly contributes to the activation of stress-responsive genes, playing a crucial role in enhancing the plant's ability to cope with heat challenges. The intricate balance the AP2/ERF family maintains allows plants to modulate gene expression patterns, influencing their heat stress tolerance.

Within this network of transcription factor families, the WRKY family, notably WRKY25, has been identified as a participant in the heat stress response, contributing partially to thermotolerance [66]. WRKY transcription factors are molecular switches regulating gene expression in heat stress adaptation. Their involvement underscores the complexity of the plant's regulatory network in fine-tuning responses to varying degrees of heat stress.

The bHLH transcription factors emerge as critical mediators in plant responses to diverse stressors, including heat, cold, and drought [67]. BhHsf1, a heat shock factor from the resurrection plant Boea hygrometric, showcased enhanced thermotolerance in transgenic Arabidopsis and tobacco, albeit with concurrent growth retardation [68]. This highlights the intricate balance between stress response and growth regulation controlled by bHLH transcription factors, portraying their multifaceted roles in plant adaptation to heat stress. Similarly, FtbHLH2, a bHLH gene from Tartary buckwheat, exhibited increased tolerance to cold stress in transgenic Arabidopsis, showcasing the versatility of bHLH transcription factors across different stress conditions [69]. In tea plants, Camellia sinensis, the upregulation of several bHLH genes under heat and drought stress further emphasizes their potential role in orchestrating responses to these environmental challenges [70]. These studies underscore the pivotal and versatile role of bHLH transcription factors in shaping plant responses to heat stress.

Furthermore, the WRKY family, particularly WRKY25, continues to be implicated in enhancing thermotolerance in plants [71]. The AP2/ERF family and other transcription factor families, such as WRKY, bHLH, bZIP, MYB, NAC, and C2H2, extend their influence to enhance cold stress tolerance in plants [72]. Identifying DREB/ERF, MYB, NAC, and WRKY transcription factors as potential candidates for improving tolerance to various stresses, including abiotic and biotic, positions them as key players in engineering stress-resilient transgenic plants [73].

Lastly, the splicing of bZIP60, a transcription factor containing a primary leucine-zipper domain, plays a crucial role in plants' unfolded protein response (UPR). This response is especially significant during stress conditions such as heat or endoplasmic reticulum (ER) stress in Arabidopsis seedlings. Through its splicing, bZIP60 activates ER-associated transcription factors, which help mitigate stress damage by coordinating cellular responses to restore ER homeostasis and ensure proper protein folding [73]. This underscores the interconnectedness of stress response pathways and the intricate regulatory mechanisms governing plant adaptation to heat stress. The wealth of information derived from the studies on these transcription factors collectively contributes to our understanding of the molecular intricacies that govern pearl millet's heat stress tolerance, offering avenues for future research and crop improvement strategies.

5. Conclusion

The current study delved into the molecular responses of pearl millet to heat stress, a pressing concern in agriculture, especially in semi-arid regions susceptible to drought and high temperatures. Our controlled experimental setup aimed to minimize tissue injury while simulating extreme heat stress conditions, subjecting pearl millet plants to a brief yet intense heat treatment of 60 seconds

at 50°C, a temperature range not explored in previous research. Employing cutting-edge next-generation RNA sequencing technology, we conducted an in-depth analysis of gene expression profiles. Our findings revealed significant variations in the gene expression patterns of pearl millet leaves grown under normal conditions and those subjected to heat stress. Notably, a cluster of genes associated with crucial biological functions, including photosynthesis, chlorophyll functioning, peroxidases, and heat shock proteins, exhibited remarkable upregulation during heat stress, with fold changes ranging from 9 to 16.

A particularly intriguing discovery was the unique group of downregulated genes labeled "TRANSLATION FACTOR GUF1-RELATED (PTN000563148)", encoding proteins essential for the intricate regulation of protein synthesis. These findings underscored the pivotal role of these proteins in controlling protein synthesis under heat stress conditions. Abundant levels of peroxidases and heat shock proteins in stressed samples indicated their active involvement in vital cellular functions and responsiveness to drought and high heat stresses.

Our study also shed light on the functions of chaperone proteins, protein kinases, and other heat-stress-responsive genes in mitigating the adverse effects of elevated temperatures. Significant changes in the expression of transcription factor families such as bHLH, ERF, NAC, WRKY, MYB-related, C2H2, bZIP, MYB, FAR1, and B3 genes were observed. These transcription factors play a pivotal role in regulating pearl millet's response to heat stress, and understanding their roles and interactions is crucial for deciphering the fundamental processes governing plant responses to elevated temperatures. The current study contributes substantial insights into the intricate chemical reactions of heat-stressed pearl millet. The identified alterations in gene expression associated with diverse cellular functions provide valuable knowledge to enhance crop resilience, particularly in the face of challenges posed by climate change. This more profound understanding of chemical reactions opens avenues for developing solutions to safeguard crops and enhance yields under heat-induced stress conditions. The comprehensive insights from this study pave the way for informed strategies to address the evolving challenges in agriculture.

6. Proposed Study in the Future

In future recommended studies, this research lays the groundwork for several promising avenues to delve deeper into understanding plant responses to heat stress and fortifying crop resilience. First and foremost, there is a call to conduct detailed investigations unraveling the specific functions of upregulated genes, especially those linked to photosynthesis, chlorophyll functioning, peroxidases, and heat shock proteins. This knowledge can serve as a foundation for targeted strategies to enhance crops' heat resilience. Additionally, exploring translational regulation mechanisms, particularly those controlled by the downregulated genes, presents an opportunity to gain insights into how these genes modulate protein synthesis under heat-stress conditions. The study suggests a need for a comprehensive exploration into the functions of identified transcription factor families and their interconnections, paving the way for potential genetic manipulation to enhance heat stress response. The validation of observed physiological changes, such as antioxidant activity, H_2O_2, and MDA levels, through diverse experimental approaches is emphasized to bolster data reliability. Long-term impact studies are proposed to discern the enduring consequences of gene expression alterations on plant growth, development, and crop yield, which are crucial for formulating sustainable strategies. Extending the research to include multiple pearl millet cultivars

with varying degrees of heat tolerance facilitates comparative analyses, revealing cultivar-specific responses and contributing genetic factors. The integration of multi-omics approaches, including transcriptomics, metabolomics, and proteomics, is suggested for a holistic understanding of molecular responses to heat stress. Field-based studies are recommended to validate laboratory findings under natural growing conditions, considering the complexities of the natural environment. Furthermore, the exploration of genetic variation within natural populations of pearl millet for heat stress responsiveness is proposed, aiming to identify genetic markers that can contribute to marker-assisted breeding programs for developing heat-tolerant cultivars.

List of Abbreviations

DEGs	Differentially expressed genes
HS	heat shock proteins
ABA	Abscisic acid (ABA or abscisin II) is a plant hormone
TFs	transcription factors

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Author Contributions

The author was entirely responsible for the conceptualization of the manuscript, data analysis, explanation, and final production.

Competing Interests

The author declares no competing interests.

Data Availability Statement

The RNA sequences of the pearl millet leaves used in the current study for all samples were submitted to the National Center for Biotechnology Information (NCBI) database under the Sequence Read Archive (SRA) data, with accession number PRJNA991076.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1: RNA Qubit concentration and average size for each group, where C is the control and H is the heat-stressed leaves.
2. Table S2: Library preparations and sequencing description for control (C)and heat treated samples (H).
3. Table S3: Statistical analysis of differentially expressed genes (DEGs) in pearl millet leaves exposed to heat stress.

4. Table S4: Pearl millet gene fold change in differentially expressed genes (DEGs) in heat-treated leaves compared with control samples matched by Arabidopsis orthologs.
5. Table S5: Description and classification of upregulated and downregulated gene protein functions and biological roles.

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