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# Integrating Genetic and Metabolic Approaches to Uncover Heat Stress Tolerance in Wheat

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## Abstract

Wheat, scientifically known as Triticum aestivum, belongs to the *Poaceae* family. Pakistan aimed to achieve self-sufficiency in wheat production, but the increasing temperature has presented a huge challenge in the form of heat stress. The objective of this study was to characterize wheat genotypes those exhibit heat-tolerance and evaluate the impact of heat stress on metabolic processes. Wheat plants were exposed to high temperatures and phenotypic assessments were performed in a controlled environment. The study was aimed to predict the role of *DREB* genes associated with heat-tolerance, to investigate interaction and regulatory networks between these *DREB* and other stress tolerance genes using Bioinformatics techniques. Metabolic profile of wheat was generated using HPLC for significant metabolites related to heat stress were found. The identification of heat tolerance genes and wheat lines possessing more heat tolerance will be used in breeding resources to develop climate-resilient varieties.

## Abbreviations

ABA – Abscisic Acid
ABF2 – Abscisic Acid Responsive Element Binding Factor 2
AP2 – APETALA2 domain
ATAF – NAC transcription factor (Arabidopsis Transcription Activation Factor)
CABB – Centre of Agricultural Biochemistry and Biotechnology
CBF – C-repeat Binding Factor
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
CRT – C-repeat (cis-element)
CUC – CUP-SHAPED COTYLEDON transcription factors
DNA – Deoxyribonucleic Acid
DRE – Dehydration Responsive Element
DREB / DREB1 / DREB2 / DREB4 / DREB6 – Dehydration-Responsive Element-Binding proteins
ERF – Ethylene Response Factor
FASTA – Text-based format for nucleotide/protein sequences
HPLC – High Performance Liquid Chromatography
HSF / HSFA / HSFA3 – Heat Shock Factor (A subclass, A3 specific)
HSP / HSP100 / HSP17 / HSP60 / HSP70 / HSP90 – Heat Shock Proteins
LBD – Lateral Organ Boundaries Domain
MEGA – Molecular Evolutionary Genetics Analysis (software)
MH – Wheat genotype MH-21 (appears as abbreviation)
MYB – Myeloblastosis family transcription factor
NAC / NAM / ATAF / CUC – Plant-specific transcription factor family
NCBI – National Center for Biotechnology Information
PPI – Protein-Protein Interaction

## Objective

This study aimed to explore how wheat (Triticum aestivum L.) responds to heat stress by combining genetic and metabolic approaches. Wheat is a staple food for much of the world, yet rising temperatures threaten its productivity. Even a small increase in temperature can reduce yields, which makes the search for heat-tolerant varieties urgent.

We evaluated five wheat genotypes under controlled heat stress, focusing on traits such as relative water content, biomass, shoot and root growth, and chlorophyll levels. At the molecular level, we studied the role of DREB (Dehydration-Responsive Element-Binding) transcription factors, known regulators of plant stress responses. Using bioinformatics tools (NCBI, STRING, MEGA, wGRN), we examined their regulatory networks and interactions with other stress-related genes. At the same time, HPLC analysis was used to identify changes in key metabolites such as amino acids and sugars under heat conditions.

The objective was to connect genetic regulation with physiological and biochemical traits, providing insights into how some genotypes maintain growth and stability under stress. These findings can support breeding programs aimed at developing climate-resilient wheat varieties.

## Data Description

The dataset generated in this study contains morphological, physiological, metabolic, and bioinformatics information on five wheat genotypes exposed to controlled heat stress conditions. The data provide quantitative values of growth parameters, metabolite concentrations, and gene interaction networks related to heat tolerance.

Physiological and morphological data were recorded for shoot length, root length, biomass, relative water content (RWC), and chlorophyll content. Measurements were taken at different growth stages (seedling, consecutive days of stress, and alternative days of stress) under heat treatment and control conditions. Each data point represents the mean of five replications.

Metabolite profiling data were obtained using High-Performance Liquid Chromatography (HPLC). Identified compounds include amino acids, sugars, and stress-related metabolites such as proline and phenolics. Data files contain metabolite concentrations (in millimolar) for two contrasting wheat genotypes, presented in tabular format.

Bioinformatics data include gene sequences, regulatory networks, and phylogenetic relationships of DREB (Dehydration-Responsive Element-Binding) and heat shock protein (HSP) genes. FASTA sequences were retrieved from NCBI, while protein-protein interaction data were generated using STRING. Phylogenetic trees were constructed using MEGA and visualized in iTOL. Gene regulatory networks were predicted using the wheat Gene Regulatory Network (wGRN) platform. The processed outputs are provided as images, tables, and network files.

Data processing: Physiological and biochemical data were averaged and normalized prior to analysis. HPLC metabolite profiles were standardized against internal references. Bioinformatics analyses were performed using default parameters of the respective tools, and outputs were exported into image and text file formats for clarity.

The dataset as a whole provides a structured collection of raw and processed measurements that can support further analysis in plant stress biology, molecular breeding, and comparative genomics.

## Limitations

- **Sample size:** Only five wheat genotypes were tested, which may not capture the full genetic diversity of wheat germplasm. Broader screening could provide stronger conclusions.

- **Controlled conditions:** Heat stress was applied in a laboratory/growth room setting. Field environments involve multiple interacting stresses (heat, drought, soil variability) that were not replicated here.

- **Stress duration and intensity:** Heat treatments were applied for specific durations and temperatures. These conditions may not fully represent the fluctuating and prolonged heat events experienced in natural environments.

- **Metabolite profiling scope:** HPLC analysis identified key metabolites, but a more comprehensive metabolomic approach (e.g., LC-MS/MS, GC-MS) could have provided deeper insights into biochemical changes.

- **Focus on DREB genes:** While DREB transcription factors were central to this study, other stress-related genes and regulatory networks may also play important roles that were not fully explored.

- **Resource limitations:** Advanced omics technologies (transcriptomics, proteomics) were not employed due to time and funding constraints, which limited the breadth of molecular insights.

## Introduction

The Poaceae or Gramineae family, also known as the Grass family covers about 20% surface of the cropped land, it comprises a huge group of flowering plants highly evolved and ecologically significant [1]. Tetraploid pasta wheat and hexaploid bread wheat are the predominant polyploid varieties of wheat. Bread wheat is the result of the process of domesticating emmer wheat and then crossbreeding it with diploid goat-grass [2]. The nutritional requirements of 2.5 billion individuals in more than 89 nations are met by the consumption of wheat grains, which serve as a staple meal. Wheat constitutes 20% of the calories ingested by humans and 25% of their protein intake. Wheat bread is a good source of vitamins, minerals and proteins. The variability in the composition and quantity of dietary components can be attributed to environmental and genotypic factors [3].

The primary goal is to increase wheat crop productivity while reducing losses from both biotic and abiotic stresses. Biotic stress is caused by living organisms such as fungi, viruses, arachnids, insects, nematodes and weeds. Agricultural productivity is greatly reduced by extreme abiotic stresses such as drought, high temperature, and salinity, imposed worldwide. Given that agriculture is largely determined by climate (i.e., temperature, humidity, and rainfall), any finding of climate change will affect to crop production. an increase in temperature of each 1OC on average is expected to lower yields by 4-7% [4].

Abiotic stresses harm plants at cellular and tissue levels, impacting water retention and leading to significant production losses. Under heat stress, wheat experiences protein denaturation and the accumulation of ROS, causing physiological and biochemical changes. These changes result in reduced crop production and quality, disruption of plant metabolism, and alterations 2 in photosynthetic pathways [5]. Heat stress is responsible for membrane damage and also effects the activity and expression of antioxidant enzymes and genes i.e., glutathione reductase and Superoxide dismutase [6]. The cost of respiration rises, surpassing photosynthesis rates and further hindering growth. Combined drought and heat stress alter mitochondrial ultrastructure and increase oxygen radicals, as a result of which anti-stress proteins are produced [7].

Biotic stresses can be controlled by chemical methods using fungicides or pesticides up to some extent, but these solutions are not sustainable and costly. These methods of controlling are not effective in abiotic stresses and certain agronomic and genetic approach may be adopted. Plants produce high-temperature stress-specific proteins known as heat shock proteins (HSPs). The five principal plant HSPs families are HSP100, HSP90, HSP70, HSP60 and small HSPs. The majority of HSPs are activated by heat stress and act as molecular chaperones. They bind to partially unfolded or denatured proteins and hold them in a stable state that usually prevents thermal aggregation. Upon stress relief, HSPs refold these proteins to contribute to the recovery.

Tolerant varieties use their own defense mechanism to combat abiotic and biotic stresses. In order to get more insight to resistance and tolerance mechanism an inclusive study of genetics behind tolerance is required. For better identification of tolerance genes, modern sequencing technologies along with DNA macroarrays play an important role. Landraces play a crucial role in genomic research due to their extensive genetic diversity and their ability to tolerate stress. They are highly valued for their adaptable genetic resources that enable them to survive in different climatic conditions [8]. Traditional methods of plant breeding, combined with marker-assisted selection, genetic editing, and advanced phenotyping techniques, are being used to accelerate the development of desired plant traits.

Researchers are exploring the biochemical and genetic mechanisms behind enriching cereal crops with micronutrients, oils, phenolics, and other beneficial compounds, aiming to improve dietary health [9]. Among various genome-editing tools widely adopted technology CRISPR/Cas9 is used due to its simplicity and high efficiency. This system, along with its variations, holds great promise for creating new and better wheat varieties for good yield potential [10]. Tissue culture has been effectively utilized for years in improving plants. Recently, plant tissue culture-based techniques have been increasingly used to address various stresses in plants [11].

DREB (Dehydration-Responsive-Element-Binding) proteins possess single AP2 domains that exhibit a high degree of conservation and are capable of binding to cis-element DRE. Numerous plant species have been found to possess these proteins. DREB gene can be divided into six stress related subclasses i.e., heat stress, freezing stress, salt stress, ABA and sugar signaling, drought stress and cold stress [12]. Over past few years, the direction has been changed towards the manipulation of DREB sequences to work on better tackle to abiotic stresses. Furthermore, it has described that the DREB isolated from a plant can confer the tolerance to different abiotic stresses to related or another crop uses [13].

Eukaryotic transcriptional regulation is often mediated by large networks of protein-protein interactions in living organisms. This type of interaction further allows for positive and negative regulation of stress responses by DREB transcription factors leading to modulation of efficient expression of different and overlapping sets of downstream genes. In wheat, the expression patterns of DREB2 have been inherited from A. speltoids, a wild ancestor. Expression pattern of Heat Shock Protein 17 (HSP17) in Triticum aestivum is associated with heat tolerance and has been inherited from its progenitors, T. turgidum, and A. speltoids [14]. Heat Shock Transcription Factor A3 (HSFA3) shares similarities with DREB2A and DREB2C, which are activated in response to heat stress [15].

The upregulation of numerous transcription factors in wheat varieties have been observed in response to the combined effects of drought and heat. Transcription factors such as NAC6D and DREB are activated by prolonged exposure to high temperatures [16]. Heat tolerance is not only exhibited in bread wheat but also in other wheat genotypes such as Sears' wild wheatgrass and wild einkorn. Molecular and physiological changes in plants ensure survival at high temperatures. The heat tolerance traits are controlled by many genes and QTLs [17]. Advances in Bioinformatics and mass spectrometry has enabled comparative and comprehensive metabolic profiles of many crop genotypes [18]. Metabolomics profile the association between phenotypes and genotypes of plants under stress [19]. Plants produce variety of metabolites in stress conditions and expression of these metabolites depends on phenotypic and genotypic traits [20]. The aforementioned measures will greatly help in development of tolerant wheat cultivars.

## Materials and Methods

The proposed research was conducted at Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, during the year 2023-2024. This experiment was designed to check the role of DREB gene for heat tolerance in wheat genotypes.

### Plant Material

Mature dry seeds of wheat cultivars were collected from seed store, University of Agriculture, Faisalabad. Seeds of five wheat genotypes were selected for better yield and potential candidate for heat tolerance and leaves were used for experiment. Wheat genotypes are as follows in the table 3.1

| Sr. No. | Genotypes |
|---|---|
| 1 | Barani |
| 2 | Aas-11 |
| 3 | MH-21 |
| 4 | Subhani-21 |
| 5 | Accession 732 Genotype |

**Table 1: Wheat Genotypes**

### Growth Conditions

Using 5% sodium hypochlorite seeds were surface sterilized for 10 minutes and then washed with sterile distilled water. Seeds of certain wheat genotypes were incubated on paper towels at room temperature in labelled petri plates for two days in dark. After two days, following the germination seeds were transferred to pots filled with the mixture of compost and sand. The pots were kept in the laboratory at 25°C for a light duration time of 14 hours. Experiment was randomized using five replications. Heat stress was applied to the wheat genotypes.

**Figure 1: Seeds in Petri Plates**

Wheat seeds were surface-sterilized and germinated in Petri plates on moist paper towels under dark conditions. This step ensured uniform germination prior to transplantation

**Figure 2: Pots Filled with Compost and Sand**

After germination, seedlings were transferred to pots containing a soil mixture of compost and sand. This medium provided balanced aeration and nutrients for early plant growth.

### Heat Stress Treatment

Heat stress was applied using incubator. Heat stress treatment was divided into phases which are as follows. Seedling Stage, Wheat seedlings 10 days after sowing were heat treated at 45°C for 2 hours. Vegetative growth is highly affected by elevated temperatures. Vegetative period is shortened by high temperature that harms the photosynthesis. Recovery temperature after heat treatment was 25°C [21]. Vegetative Stage (2-4 Leaf Stage): 1. Wheat genotypes were subjected to 40°C heat treatment for 2 hours for 4 consecutive days. 2. Wheat genotypes were subjected to 40°C heat treatment for 2 hours for 8 days alternatively. Recovery temperature after heat treatment was 25°C. Control The wheat genotypes that were used as control were kept at 25°C.

**Figure 3: Seedlings in Incubator**

Young wheat seedlings were subjected to heat treatments in an incubator. Controlled temperature allowed precise application of heat stress at different growth stages.

### Physiological and Morphological Characteristics

Wheat genotypes following heat stress treatment were evaluated by considering following parameter: Relative Water Content (RWC) a crucial indicator for evaluating the hydration level of plant leaves. It quantifies the current water content of a leaf in comparison to its maximum water capacity when fully turgid [22], Biomass estimation is crucial for understanding crop growth and productivity [23], Root Length measuring is essential for understanding plant growth and resource acquisition [24], Shoot Length is an important agronomic trait in wheat, influencing lodging resistance and grain yield and Chlorophyll content in wheat leaves is an indicator of their growth and nutritional status, making it a valuable health index for field management [25].

### Metabolite Extraction and HPLC

Metabolite extraction in wheat involves identifying and isolating biochemical compounds produced normal and stressed conditions. Under normal condition plants produce amino acids, sugars and organic acids which play crucial role in plant defense and development. Under stress-condition plants produce stress-related compounds such proline and phenols. Metabolite extraction was conducted by following standard protocols. HPLC was done for the examination of differential expression of growth hormones to detect the metabolite and to analyze biochemical components in leave, stalk and seed under controlled conditions [26].

### Interaction and Regulatory Networks using Bioinformatics Tools

Genes associated with heat stress tolerance were identified, and the interaction between DREB genes and tolerance genes were analyzed using bioinformatics tools. The study also evaluated how transcription factors interact with each other during heat stress and examine the regulatory network involved. Tools that were used are as follow: National Center for Biotechnology Information (NCBI) is a valuable resource for biological and genomic data [27], Search Tool for the Retrieval of Interacting Genes (STRING) is a valuable resource for exploring protein-protein interaction networks and functional associations [28], Molecular Evolutionary Genetics Analysis (MEGA) is an advanced and user-friendly software suite designed for analyzing DNA and protein sequence data [29], A web base program called Interactive Tree of Life (iTOL) allows to explore and visualize genome wide data, comparing phylogenetic trees and hierarchical data [30] and to construct the interaction between heat tolerance genes and the DREB gene in wheat using the Wheat Gene Regulatory Network (wGRN) [31].

## Results

The study aimed to characterize wheat genotypes for heat tolerance, focusing on the involvement of DREB (Dehydration Responsive Element Binding) genes. The research primarily investigated the physiological parameters including water content, plant height, biomass, root development, and chlorophyll content in wheat seedlings subjected to heat stress.

### Morphological and Physiological Characteristics

### Shoot Length

Shoot length was measured for each treatment and statistically analyzed for significance, with data presented in Table 4.1.

| **Shoot Length (cm)** | | | | |
|---|---|---|---|---|
| **Treatment** | **Wheat Genotype** | | | |
| | | **Seedlings** | **Consecutive Days** | **Alternative Days** |
| **Stress** | **Barani** | 17 | 16 | 14.5 |
| | **Aas-11** | 22 | 23 | 21 |
| | **MH-21** | 16.5 | 17 | 14 |
| | **Subhani-21** | 15 | 13.6 | 14 |
| | **Accession 732** | 24 | 25.6 | 22 |
| **Control** | **Barani** | 17.6 | 17 | 16 |
| | **Aas-11** | 23.2 | 24 | 22 |
| | **MH-21** | 17 | 18 | 17 |
| | **Subhani-21** | 15.7 | 15 | 16 |
| | **Accession 732** | 22 | 23 | 21 |

**Table 2: Shoot Length of Wheat Genotypes**

The Accession 732 wheat genotype demonstrated varying performance in shoot length under different stress treatments compared to control conditions. During seedling stress, Accession 732 genotype showed a decrease in shoot length by 8.33%, contrasting with minor increases observed in Barani (+3.53%), Aas-11 (+5.45%), MH-21 (+3.03%), and Subhani-21 (+4.67%). In consecutive days stress, Accession 732 genotype exhibited a larger decrease in shoot length by 10.16%, while other genotypes showed increases or slight decreases. During alternative days stress, Accession 732 genotype also showed a decrease in shoot length by 4.55%, contrasting with increases observed in MH-21 (+21.43%) and Subhani-21 (+14.29%), and minor changes in Barani (+10.34%) and Aas-11 (+4.76%). These findings highlighted Accession 732 genotype's relative decrease in shoot length under stress conditions compared to other genotypes, indicating its response variability and potential challenges in maintaining shoot development under stress.

### Root Length

Root length from beneath the ground part of the plant for each treatment was measured using a measuring scale. Data is presented in Table 4.2.

| **Root Length (cm)** | | | | |
|---|---|---|---|---|
| **Treatment** | **Wheat Genotype** | | | |
| | | **Seedlings** | **Consecutive Days** | **Alternative Days** |
| **Stress** | **Barani** | 11 | 9 | 9.5 |
| | **Aas-11** | 12 | 10.7 | 11 |
| | **MH-21** | 10.7 | 8.5 | 8.6 |
| | **Subhani-21** | 7.8 | 8.4 | 8 |
| | **Accession 732** | 14 | 13.5 | 12 |
| **Control** | **Barani** | 8 | 8.6 | 8.5 |
| | **Aas-11** | 13.5 | 11.2 | 10.2 |
| | **MH-21** | 11.2 | 9 | 9.3 |
| | **Subhani-21** | 9 | 9.7 | 9 |
| | **Accession 732** | 13.4 | 13 | 11 |

**Table 3: Root Length of Wheat Genotypes**

The Accession 732 wheat genotype demonstrated varied performance in root length under different stress treatments. During seedling stress, Accession 732 genotype showed a slight decrease in root length by 4.29%, contrasting with Barani (-27.27%) and Subhani-21 (+15.38%) which showed notable decreases and increases, respectively. In consecutive days stress, Accession 732 genotype again exhibited a slight decrease in root length by 3.70%, whereas Subhani-21 (+15.48%) and MH-21 (+5.88%) showed increases and Barani (-4.44%) showed a slight decrease. During alternative days stress, Accession 732 genotype showed a decrease in root length by 8.33%, contrasting with slight decreases observed in Aas-11 (7.27%) and Barani (-10.53%), and increases seen in MH-21 (+8.14%) and Subhani-21 (+12.50%). These findings highlight Accession 732 genotype's moderate decline in root length under stress conditions compared to other genotypes, indicating its potential for maintaining stability in root development under varying stress durations.

### Biomass

Measuring the biomass of wheat at the vegetative stage involves assessing the plant's growth through various methods such as harvesting and weighing above-ground parts. Data is presented in Table 4.3.

| **Biomass (mg)** | | | | |
|---|---|---|---|---|
| **Treatment** | **Wheat Genotype** | | | |
| | | **Seedlings** | **Consecutive Days** | **Alternative Days** |
| **Stress** | **Barani** | 27 | 29 | 29.7 |
| | **Aas-11** | 37.6 | 35 | 36.4 |
| | **MH-21** | 38 | 35.6 | 37 |
| | **Subhani-21** | 24 | 23 | 24.2 |
| | **Accession 732** | 47 | 45.4 | 44 |
| **Control** | **Barani** | 39 | 41 | 40.4 |
| | **Aas-11** | 33 | 34 | 38 |
| | **MH-21** | 40 | 38 | 39.4 |
| | **Subhani-21** | 25 | 27 | 26 |
| | **Accession 732** | 42 | 44 | 44.8 |

**Table 4: Biomass of Wheat Genotypes**

Across different stress treatments, the performance of the Accession 732 wheat genotype in terms of biomass varied noticeably. Under seedling stress, the Accession 732 genotype showed a decrease in biomass by 10.64%, contrasting with significant increases observed in Barani (+44.44%), moderate increases in MH-21 (+5.26%) and Subhani-21 (+4.17%), while Aas-11 exhibited a decrease (-12.23%). During consecutive days of stress, the Accession 732 genotype displayed a slight decrease in biomass (-3.08%), whereas Barani showed a notable increase (+41.38%) and Subhani-21 also demonstrated a considerable rise (+17.39%). MH-21 showed a small increase (+6.74%), while Aas-11 decreased slightly (-2.86%). Moving to alternative days of stress treatment, the Accession 732 genotype exhibited a modest increase in biomass (+1.82%), aligning with increases in Barani (+35.92%) and MH-21 (+6.49%). Aas-11 remained stable (+4.40%), and Subhani-21 showed a slight increase (+7.44%). These findings highlight the Accession 732 genotype's ability to maintain biomass levels under stress conditions, demonstrating resilience compared to other genotypes across varying stress durations.

## Relative Water Content

RWC in wheat during the vegetative stage is a crucial indicator of the plant's hydration status and overall health. High RWC values signify optimal water availability and efficient physiological processes, contributing to increase growth and development. Data is presented in Table 4.4.

| **Relative Water Content (%)** | | | | |
|---|---|---|---|---|
| **Treatment** | **Wheat Genotype** | | | |
| | | **Seedlings** | **Consecutive Days** | **Alternative Days** |
| **Stress** | **Barani** | 40 | 39.5 | 39 |
| | **Aas-11** | 64.6 | 66 | 64.9 |
| | **MH-21** | 34 | 34.7 | 33 |
| | **Subhani-21** | 31.8 | 32 | 33.6 |
| | **Accession 732** | 76 | 77.5 | 75.6 |
| **Control** | **Barani** | 75 | 77 | 75.4 |
| | **Aas-11** | 81.2 | 82.4 | 83 |
| | **MH-21** | 74.2 | 75 | 74 |
| | **Subhani-21** | 45.7 | 46 | 44.9 |
| | **Accession 732** | 84.6 | 85 | 83.8 |

**Table 5: Relative Water Content of Wheat Genotypes**

During seedling heat stress treatment, the Accession 732 genotype exhibited a significant increase in relative water content (RWC) by 31%, surpassing Aas-11 (63%) and MH-21 (39%), while Barani decreased by 26% and Subhani-21 by 29%. In consecutive days of heat stress treatment, only the Accession 732 genotype showed an increase in RWC by 27%, contrasting with decreases observed in Barani (86%), Aas-11 (59%), MH-21 (80%), and Subhani-21 (76%). In alternative days of heat stress treatment, Aas-11 showed a modest increase in RWC by 14%, whereas the Accession 732 genotype maintained its resilience with only an 11% decrease in RWC, compared to decreases of 35% in Barani, 58% in MH-21, and 34% in Subhani-21. These results highlight the superior water retention capability of the Accession 732 genotype under different heat stress conditions, indicating its potential for maintaining higher RWC compared to other genotypes tested.

## Chlorophyll Content

Chlorophyll content in wheat leaves is an indicator of their growth and nutritional status, making it a valuable health index for field management. Data is presented in Table 4.5.

| **Chlorophyll Content (mg/g)** | | | | |
|---|---|---|---|---|
| **Treatment** | **Wheat Genotype** | | | |
| | | **Seedlings** | **Consecutive Days** | **Alternative Days** |
| **Stress** | **Barani** | 1.66 | 1.9 | 1.91 |
| | **Aas-11** | 2.14 | 2.46 | 3.39 |
| | **MH-21** | 2.2 | 1.96 | 1.81 |
| | **Subhani-21** | 2.37 | 2.5 | 2.42 |
| | **Accession 732** | 3.68 | 4.23 | 3.76 |
| **Control** | **Barani** | 1.89 | 2.18 | 2.66 |
| | **Aas-11** | 2.99 | 4.26 | 3.76 |
| | **MH-21** | 1.89 | 2.09 | 2.77 |
| | **Subhani-21** | 2.93 | 3.2 | 2.79 |
| | **Accession 732** | 3.99 | 4.3 | 4.18 |

**Table 6: Chlorophyll Content of Wheat Genotypes**

During seedling stress treatment, chlorophyll content increased significantly for Aas-11 by 40%, Subhani-21 by 24%, and Accession 732 genotype by 8%, while it decreased for Barani by 14% and MH-21 by 9%. In consecutive days stress treatment, Aas-11 exhibited a substantial increase in chlorophyll content by 73%, with smaller increases seen in Subhani-21 by 28% and Accession 732 genotype by 2%. However, Barani and MH-21 showed decreases in chlorophyll content by 15% and 7%, respectively. During alternative days stress treatment, MH-21 had the most significant increase in chlorophyll content by 53%, followed by Barani by 39%, while Aas-11 showed a moderate increase of 11%. Conversely, Accession 732 genotype and Subhani-21 exhibited decreases in chlorophyll content by 11% and 15%, respectively. These findings illustrate the varied responses of each wheat genotype to different stress conditions in terms of chlorophyll content, highlighting Aas-11's robust response across all treatment durations and MH-21's notable increase during alternative days stress treatment.

### Metabolomic Fingerprinting

The identification and quantification of metabolites present in wheat during the normal and heat stress conditions were done in growth room using the artificial lights. The quantification of metabolites was done by using HPLC. The most common identified and quantified molecules were sugars, amino acids, organic compounds, nucleotides and fatty acids. The class of metabolites is listed in table 4.6 and figure 4.1 as follows.

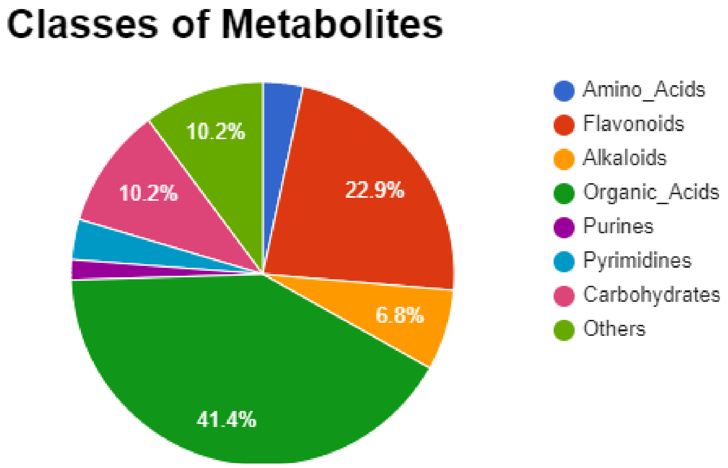

**Figure 4: Metabolites Classes**

Different classes of metabolites identified in wheat genotypes are shown. These include amino acids, sugars, and organic acids, which are central to plant responses under heat stress.

| Metabolites | Seedling Stage | | Consecutive Days | | Alternative Days | |
|---|---|---|---|---|---|---|
| **Millimolar (mM)** | **Genotypes** | | **Genotypes** | | **Genotypes** | |
| | **MH-21** | **Accession 732** | **MH-21** | **Accession 732** | **MH-21** | **Accession 732** |
| **Histidine** | 0.098 | 1.45 | 0.088 | 1.26 | 0.095 | 1.33 |
| **Alpha Aminodipate** | 0.206 | 0.232 | 0.309 | 0.236 | 0.403 | 0.334 |
| **Proline** | 0.0066 | 0.0098 | 0.006 | 0.0085 | 0.0071 | 0.087 |
| **Glyerol** | 0.0029 | 0.0056 | 0.0031 | 0.0059 | 0.0034 | 0.0062 |
| **Glutamine** | 0.108 | 0.014 | 0.209 | 0.035 | 0.103 | 0.016 |
| **Valine** | 0.0018 | 1.22 | 0.0029 | 1.32 | 0.0025 | 1.29 |
| **Lysine** | 1.45 | 1.045 | 1.34 | 1.041 | 1.39 | 1.036 |
| **Leucine** | 0.007 | 2.34 | 0.012 | 2.11 | 0.034 | 2.18 |
| **Glysine** | 1.81 | 0.223 | 1.78 | 0.225 | 1.74 | 0.229 |
| **Alanine** | 0.028 | 2.114 | 0.023 | 2.119 | 0.019 | 2.223 |
| **Tryosine** | 0.005 | 1.34 | 0.006 | 1.45 | 1.25 | 1.52 |
| **Tryptophan** | 1.033 | 2.202 | 1.041 | 2.205 | 1.055 | 2.209 |

**Table 7: Metabolites Concentrations in Wheat Genotypes during Heat Stress**

The table compares metabolite concentrations (in mM) between wheat genotypes MH-21 and Accession 732 at the seedling stage under two growth conditions: consecutive days and alternative days. It reveals genotype-specific variations in metabolites like Histidine, Proline, and Tryptophan, indicating distinct metabolic responses to different growth regimes. This data offers valuable insights into how these wheat genotypes adapt metabolically under controlled conditions, which is essential for understanding their potential for stress tolerance and guiding targeted improvement strategies through breeding or agricultural management practices.

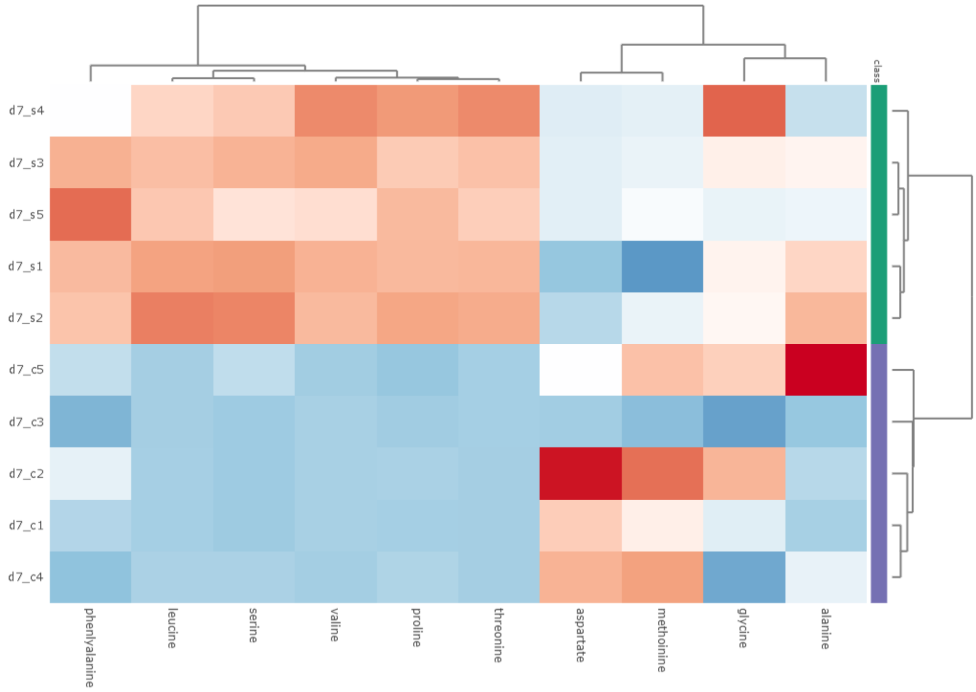

**Figure 5: Heatmap for Wheat Genotypes**

A heatmap illustrating the clustering of metabolites across wheat genotypes under stress. The color gradients represent variation in metabolite levels, highlighting genotype-specific metabolic responses.

The heatmap shows a heatmap with a dendrogram, used for hierarchical clustering to display the arrangement of clusters. The color gradient, ranging from light blue to dark red, represents varying data values across two axes labeled with different categories or variables, illustrating the interrelationships within the dataset.

## Interaction and Regulatory Networks using Bioinformatics Tools

In the field of bioinformatics, researchers explore gene regulatory interaction networks using various tools. These networks provide insights into how genes interact and influence each other's expression. One common approach is to construct protein-protein interaction (PPI) networks, which visualize connections between proteins.

## National Center for Biotechnology Information (NCBI)

NCBI was used to search for genes involved in heat stress and heat tolerance. FASTA sequence was downloaded to use for phylogenetic tree construction and to show the evolutionary relation among the genes. Data presented in table 4.7.

| Sr. No. | Gene | NCBI Reference Sequence |
|---|---|---|
| **1** | DREB2A | NC_003076.8 |
| **2** | DREB2B | NC_003074.8 |
| **3** | DREB2C | NC_003071.7 |
| **4** | DREB6 | NC_057801.1 |
| **5** | DREB1C | NC_057814.1 |
| **6** | DREB1 | NC_057802.1 |
| **7** | DREB4 | NC_057794.1 |
| **8** | hsp 16. 9A | NW_025225977.1 |
| **9** | Hsp90 | NC_057813.1 |
| **10** | Hsp90.2-A1 | NC_057803.1 |
| **11** | Hsp90.3-D1 | NC_057808.1 |
| **12** | Hsp90.1-A1 | NC_057797.1 |
| **13** | hsp16.9-12LC2 | NC_057803.1 |
| **14** | Hsp90.3-B1 | NC_057807.1 |
| **15** | Hsp90.1-D1 | NC_057799.1 |
| **16** | Hsp90.1-B1 | NC_057798.1 |
| **17** | Hsp90.3-A1 | NC_057806.1 |
| **18** | DREB1A | NC_003075.7 |

**Table 8: Genes Involved in Heat Stress Response**

## Genes Involved in Heat Tolerance

DREB2A, a stress-responsive transcription factor. It had a major role in enhancing tolerance to heat stress by regulating downstream genes responding to heat shock proteins (HSPs) and then protected the heat-sensitive proteins. Under heat stress, this gene was induced to be expressed, and promoted the activation of a cadre of heat-responsive genes. Meanwhile, DREB2A, a member of the DREB/CBF (C-repeat binding factor) family, and was known to bind to the DRE/CRT (dehydration-responsive element/C-repeat) motif in gene promoters.

DREB2B another DREB/CBF family member, similar to DREB2A. This gene responds to heat stress, and modulates the expression of heat tolerance related genes. DREB2B also physically interacts with other transcription factors to co-regulate stress-responsive pathways.

DREB2C another DREB/CBF transcription factor involved in the response to heat stress is DREB2C. It induces the expression of heat shock protein genes and genes encoding antioxidant enzymes that mediate heat tolerance. The gene is also upregulated under heat stress conditions, thus promoting thermotolerance in plants.

DREB6 belongs to the DREB subfamily and is involved in cold responsive as well as other abiotic stresses such as heat stress. The expression of heat-responsive genes are modulated, making the plant more resilient to high temperatures.

DREB1C belongs to the DREB1 subfamily. Acts as a transcriptional activator that the cold and heat stress response run: binds to the DRE/CRT cis-acting element. Various transcription factors need to co-ordinate with DREB1C to regulate an optimized plant stress tolerance.

HSPs (Heat Shock Proteins) during heat stress, heat shock proteins are necessary for cell protection. HSPs, including Hsp90, Hsp16. Some others, like 9, help the protein in folding, avoid aggregation and help maintain the structure of the cellular components. Under heat stress, their expression is also greatly induced.

## Interaction between Proteins using STRING

Most commonly proteins involved in heat tolerance in wheat in response to heat stress. Ranking provides information about the role of specific proteins in response to heat stress. Scores indicate interaction of a particular protein with other proteins and transcription factors in table 4.8 and Figure 4.3.

| Ranking | Protein Name | Score |
|---|---|---|
| 1 | HSP101c | 239 |
| 2 | Hsp90.1-B1 | 148 |
| 2 | Hsp90.1-A1 | 148 |
| 4 | HSP90-2 | 131 |
| 5 | Hsp90.3-A1 | 130 |
| 5 | Hsp90.2-B1 | 130 |
| 5 | Hsp90.2-D1 | 130 |
| 8 | A0A3B5XX47 | 96 |
| 8 | A0A3B5YUN2 | 96 |
| 8 | A0A3B6KEP5 | 96 |

**Table 9: Genes Mostly Involved in Heat Stress Response**

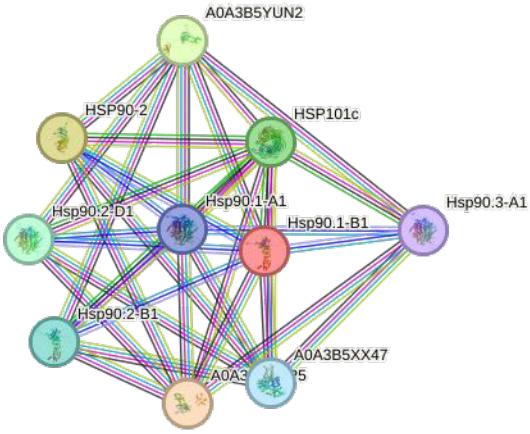

**Figure 6: Genes Mostly Involved in Heat Stress Response**

Proteins and genes most frequently associated with heat stress tolerance are ranked. High scores indicate stronger involvement in heat response pathways.

### Interaction between Dreb and HSFs

ABF2, DREB2A, and HSFA. ABF2, a transcription factor associated with abscisic acid (ABA) signaling pathways, contributes to overall stress responses, including heat stress. While not directly linked to DREB2A or HSFA3, ABF2 likely plays a role in coordinating stress responses. DREB2A, on the other hand, is a heat-responsive transcription factor that activates downstream genes, including heat shock proteins (HSPs), which protect cellular structures during heat stress. Co-expression analysis reveals a strong association between ABF2 and DREB2A, suggesting coordinated regulation under stress conditions. Similarly, HSFA3, a master regulator of heat shock proteins, also shows co-expression with ABF2, implying their joint involvement in heat stress adaptation as presented in figure 4.4.

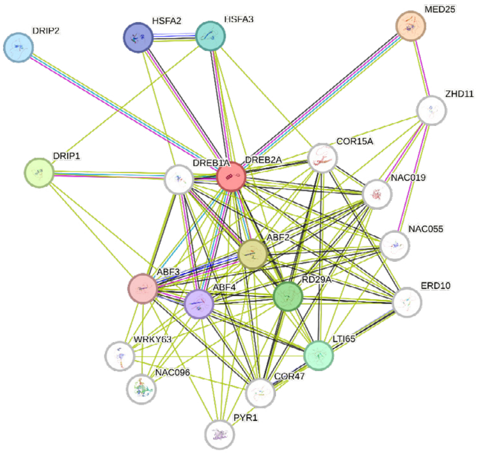

**Figure 7: Interaction between DREBs and HSFs**

Network diagram showing regulatory interactions between DREB transcription factors and Heat Shock Factors (HSFs). This suggests cooperative regulation in managing stress-responsive genes.

### Phylogenetic Relationship

Phylogenetic analysis is a powerful tool for elucidating the evolutionary relationships among different organisms. By comparing genetic sequences, researchers can construct a phylogenetic tree that visually represents these relationships, showing how different species or genotypes are related through common ancestors. The topology of the tree reveals distinct clades or groups, indicating evolutionary pathways and divergence events. High bootstrap values or posterior probabilities often support the reliability of these branches, providing confidence in the inferred relationships. This analysis can uncover both expected and novel relationships, offering insights into the evolutionary history, adaptation mechanisms, and genetic diversity of the organisms studied. Figure 4.5.

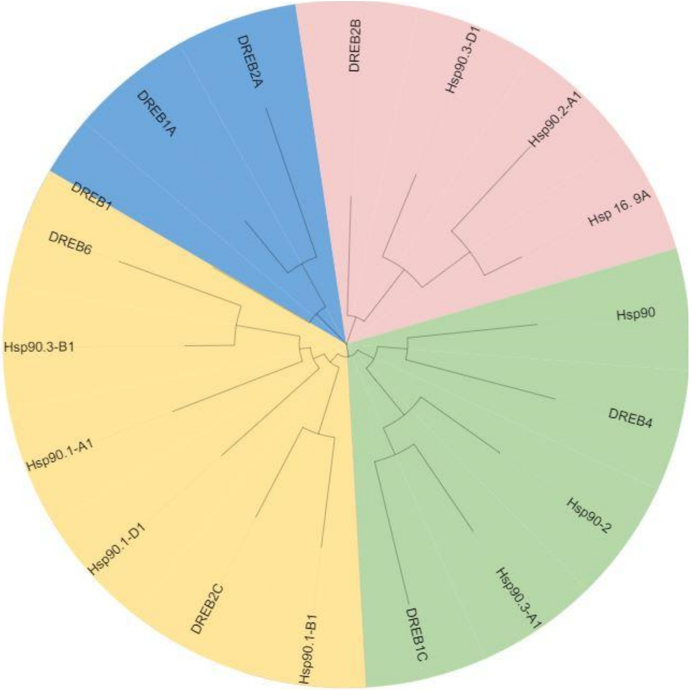

**Figure 8: Phylogenetic Tree**

Phylogenetic tree depicting evolutionary relationships among DREB and HSP genes in wheat. Clustering indicates conserved roles in abiotic stress responses.

### Major Clades

DREB clade includes genes such as DREB6, DREB2C, DREB1C, DREB4, DREB2A, DREB1A, and DREB2B. These genes are grouped together, indicating that they share a common ancestor and have diverged over time. The close grouping suggests functional similarities or shared regulatory mechanisms in response to dehydration and other stress conditions. Hsp90 clade includes genes such as Hsp90.3-B1, Hsp90.1-A1, Hsp90.1-D1, Hsp90.1-B1, Hsp90.3-A1, Hsp90-2, Hsp90.2-A1, Hsp90.3-D1, and Hsp16.9A. These genes also share a common evolutionary origin and are involved in the heat shock response, helping the plant to cope with elevated temperatures and other stress factors.

Notably, the presence of Hsp90 genes alongside certain DREB genes within the same clades underscores the intricate interplay between these families in orchestrating plant stress responses. This co localization suggests potential regulatory crosstalk or cooperative interactions between DREB transcription factors and Hsp90 molecular chaperones in modulating stress-related gene expression.

### Evolutionary Distance

The scale at the bottom of the tree indicates the evolutionary distance. The branch lengths show how much genetic change has occurred, with longer branches representing greater evolutionary divergence. For example, DREB2B appears to be more divergent from the rest of the DREB genes, indicating it might have evolved unique functions or adaptations.

### Biological Implications

The clustering of DREB and Hsp90 genes into distinct clades and subgroups highlights their functional diversity in stress responses. Understanding these relationships can help identify key regulatory genes involved in drought and heat tolerance, which are critical for plant survival and productivity. Insights from this phylogenetic analysis can inform breeding programs aiming to enhance stress tolerance in crops. By selecting genes from well-supported clades, researchers can target specific pathways and regulatory networks to improve plant resilience. The tree provides a snapshot of the evolutionary history of these gene families, showing how they have diversified to adapt to various environmental challenges.

### Regulator Prediction by wGRN

Regulator prediction using the wheat Gene Regulatory Network (wGRN) involves identifying key transcription factors and their target genes within the wheat genome. By analyzing the interactions and regulatory mechanisms, wGRN helps in pinpointing specific genes that play pivotal roles in stress responses, growth, and development. The wGRN database utilizes high throughput sequencing data and computational models to predict regulatory relationships, offering insights into how transcription factors like HSF, ERF, bZIP, NAC, and MYB show complex physiological processes. This predictive capability is crucial for understanding gene function and for breeding wheat varieties with enhanced resilience to environmental stresses. Data extracted from wGRN regulator predication is presented in table 4.8 and figure 4.6.

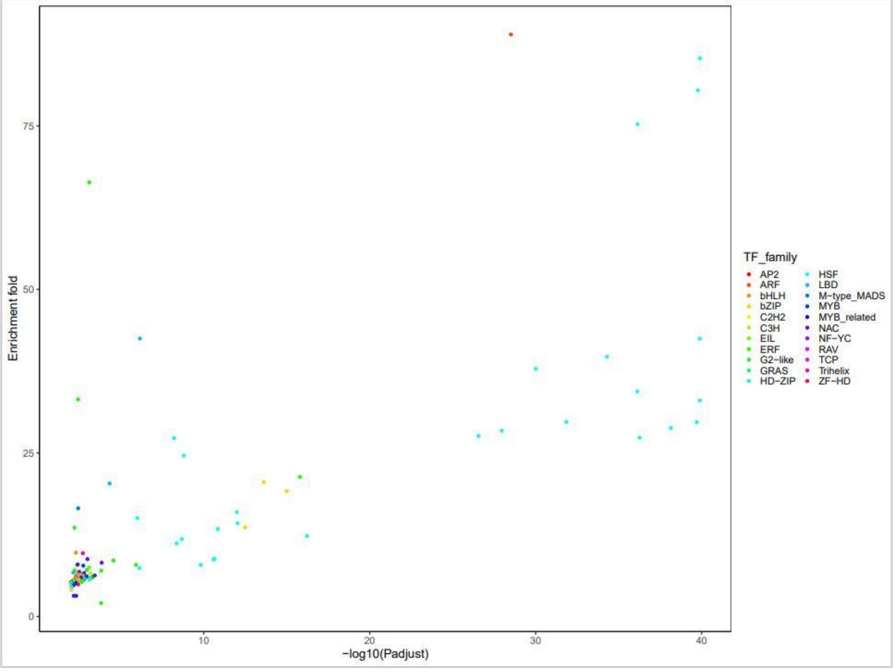

**Figure 9: Heat Stress Regulators**

Distribution of major transcription factor families identified as regulators during heat stress, with HSF and NAC families showing higher abundance.

| Transcription Factor | Count | Percentage |
|---|---|---|
| **HSF** | 33 | 29.46% |
| **ARF** | 1 | 0.89% |
| **ERF** | 15 | 13.39% |
| **bZIP** | 12 | 10.71% |
| **LBD** | 4 | 3.57% |
| **NAC** | 17 | 15.18% |
| **MYB** | 7 | 6.25% |
| **MYB_related** | 2 | 1.79% |
| **C3H** | 2 | 1.79% |
| **ZF-HD** | 2 | 1.79% |
| **Trihelix** | 1 | 0.89% |
| **M-type_MADS** | 1 | 0.89% |

**Table 10: Heat Stress Regulators**

Various genes identified in the wheat genome, primarily belonging to several transcription factor families such as HSF (Heat Shock Factor), ERF (Ethylene Response Factor), bZIP (Basic Leucine Zipper), NAC (NAM, ATAF, and CUC), MYB, LBD (Lateral Organ Boundaries Domain), and others. These genes have been evaluated based on their E-values and other metrics indicating the strength and significance of their matches to known transcription factor sequences.

Among the HSF family, genes such as TraesCS7D03G0854500LC, TraesCS2B03G1068900, and TraesCS1A03G0910400 exhibit very strong matches, as indicated by E-values ranging from 4.80E-44 to 1.40E-43. These genes have high identity scores and coverage, suggesting their critical roles in heat stress responses. In the ERF family, genes such as TraesCS7D03G1098600, TraesCS2D03G0694900, and TraesCS2A03G1251300 display strong homology, with E-values ranging from 1.50E-31 to 1.10E-05. These genes are likely involved in the ethylene signaling pathway, which is crucial for various plant stress responses and developmental processes. The presence of multiple ERF genes suggests a complex regulatory network in wheat that responds to ethylene and related stress signals.

## Gene Expression by wGRN

Gene expression analysis through the wheat Gene Regulatory Network (wGRN) involves deciphering the activity levels of genes within the wheat genome and understanding their regulatory mechanisms. By identifying key transcription factors like Heat Shock Factors (HSF), Ethylene Response Factors (ERF), and NAC domain proteins, wGRN offers insights into stress responses and developmental processes. This tool aids in unraveling the intricate networks governing wheat gene expression, crucial for enhancing crop resilience and agricultural productivity.

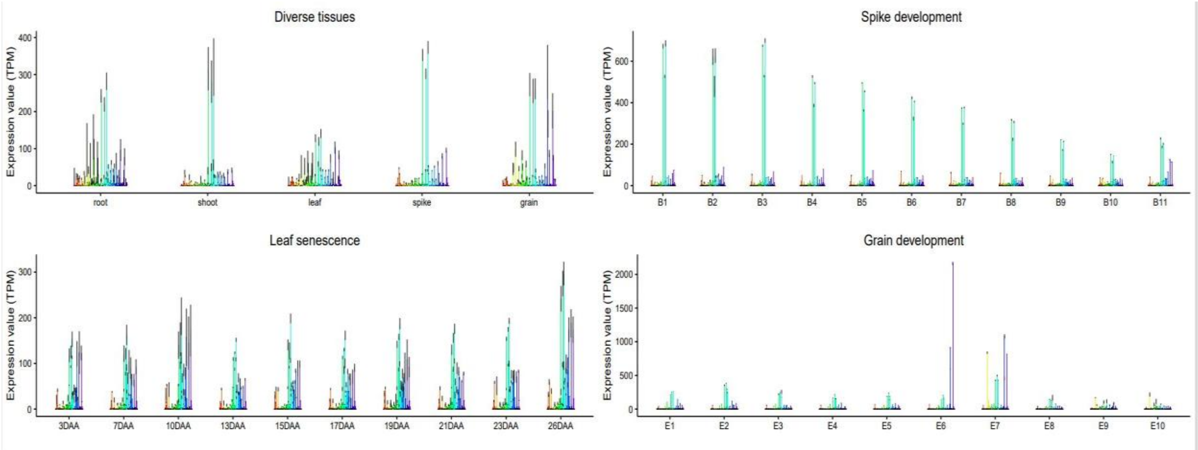

**Figure 10: Gene Expression of Heat Stress Genes**

Expression profiles of selected genes under heat stress. Increased expression of DREBs and HSPs highlights their role in enhancing tolerance.

## TF Enrichment by wGRN

TF Enrichment analysis by the wheat Gene Regulatory Network (wGRN) involves identifying transcription factors (TFs) significantly enriched within a set of genes of interest. By elucidating the regulatory relationships between TFs and their target genes, wGRN helps pinpoint key regulators influencing gene expression patterns across various conditions. This comprehensive analysis not only enhances our understanding of the regulatory mechanisms governing specific biological processes in wheat but also provides actionable insights for targeted genetic manipulation and crop improvement strategies. By leveraging enriched TFs and their regulatory networks, researchers can develop more resilient and productive wheat varieties tailored to thrive in diverse agricultural environments, thereby supporting sustainable food production in the face of global challenges.

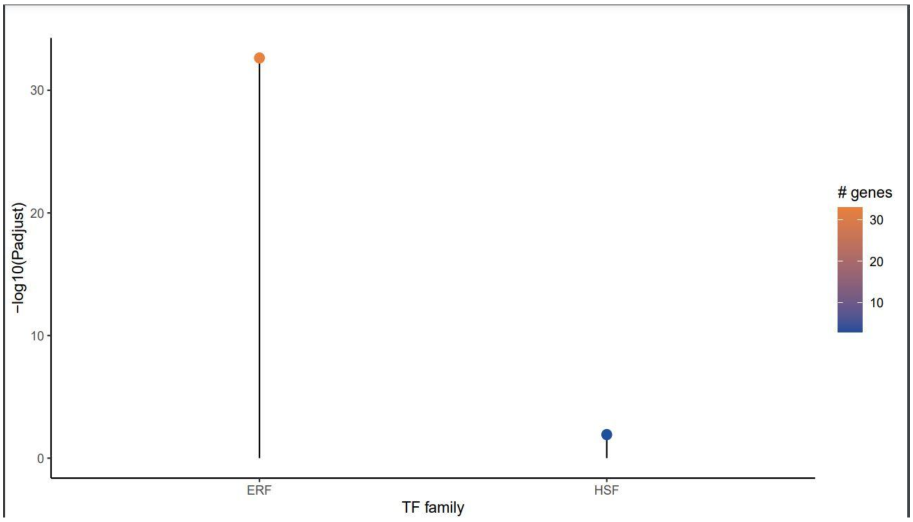

**Figure 11: ERF and HSF Transcription Factors**

Relative enrichment of Ethylene Response Factors (ERFs) and Heat Shock Factors (HSFs) under stress conditions. Their central positions in regulatory networks emphasize their significance.

## Pathway Networks

Network pathways analysis by wheat Gene Regulatory Network (wGRN) involves studying how genes in wheat interact with each other to control various biological processes. By creating a map of these interactions, scientists can understand the intricate regulatory mechanisms within wheat. This analysis helps identify key genes and pathways involved in important traits like yield, disease resistance, and nutritional content. By unraveling the complex network of gene interactions, researchers can develop strategies to improve wheat varieties, making them more resilient and nutritious to meet the challenges of food security and sustainable agriculture.

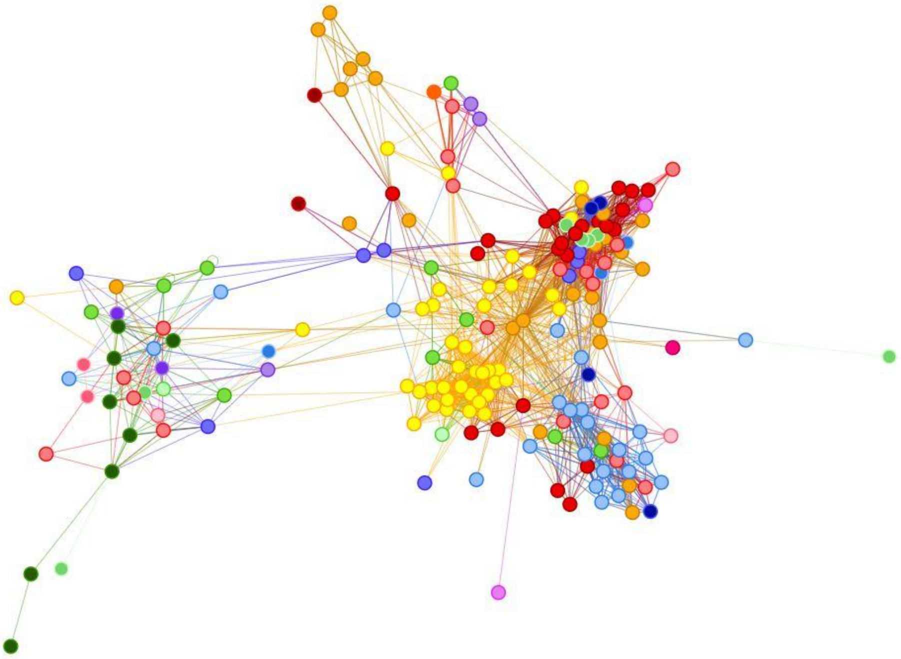

**Figure 12: Pathway Network during Heat Stress**

A pathway map summarizing transcription factor interactions during heat stress. Central hubs such as HSFs coordinate with other regulators to activate stress-protection mechanisms.

The interaction pathway map shows how different transcription factor families work together. HSF (Heat Shock Factors) are key players, found in the middle of the network with many connections to other transcription factors. This central position indicates that HSFs are crucial for responding to stress, like heat, and help regulate a variety of important processes in the cell. ERF (Ethylene Response Factors) also holds a central spot in the network, reflecting their significant role in managing responses to the plant hormone ethylene. Ethylene affects things like fruit ripening and flower development. The many connections ERFs have with other transcription factors show they are major coordinators in the network, helping manage a wide range of biological activities, much like HSFs.

## Discussion

One of the major stresses that reduces wheat production all around the global is heat stress, which can be increasingly intense worldwide due to global warming. The objective of the present study was to characterize wheat genotypes for heat stress tolerance at molecular level and exploring the role DREB homologs in heat tolerance. The DREB genes are key elements of the plant stress signaling network, especially in response to abiotic stress, such as drought and heat stress. Heat tolerant genotype maintained better growth parameters compared to sensitive ones, indicating that these traits can serve as reliable indicators of heat tolerance. Moreover, physiological traits such as chlorophyll and relative water contents were higher in heat-tolerant genotype, indicating better water retention and photosynthetic efficiency under stress conditions.

Interaction between DREB genes and heat shock proteins (HSPs) was also investigated using STRING which showed a highly complex network of interactions of DREB genes and HSPs which might play significant roles during heat stress response. The association with DREB genes hints at a collective response, with these DREB transcriptions factors representing possible new genes in HSP regulation in plants against heat stress. The construction and modification of the phylogenetic tree using MEGA and iTOL provided insights into the evolutionary relationships among DREB genes across different wheat genotypes. The phylogenetic analysis revealed clusters of DREB genes that were more prevalent in heat-tolerant genotype, suggesting a possible evolutionary adaptation to heat stress.

Using the wGRN online tool, key regulators involved in the heat stress response in wheat were identified. The regulatory network analysis highlighted several transcription factors (TFs) that are enriched in heat-tolerant genotypes. These TFs likely play pivotal roles in modulating gene expression in response to heat stress. Gene expression analysis further confirmed that these regulatory networks were activated under heat stress conditions, leading to the expression of genes associated with heat tolerance. This activation involved a cascade of regulatory events, where initial stress signals trigger the expression of DREB genes, which in turn regulated the expression of downstream genes involved in stress protection.

HPLC was employed to analyze the metabolite profiles of Accession 732 and non-transgenic wheat genotypes before heat stress treatment and after heat stress treatment. The results showed significant differences in the levels of certain metabolites, such as proline, sugars, and antioxidants, which are known to confer stress tolerance. Accession 732 genotype overexpressing DREB genes exhibited higher levels of these metabolites, correlating with enhanced heat tolerance. Profiling of differentially accumulated metabolites provided evidence of the metabolic changes in response to heat stress and the potential influence of DREB genes on these changes. It provides direct evidence of how genetic changes translate into physiological and biochemical responses, which can inform breeding and engineering strategies.

## Conclusion

In conclusion, the characterization of wheat genotypes for heat tolerance revealed that morphological and physiological traits, gene interactions, regulatory networks, and metabolite profiles are all critical components of the heat stress response. The information about the interaction of DREB genes with HSPs and the regulatory networks identified will be useful to understand the molecular basis of heat tolerance. These results were consistent with previously studies reported that DREB genes were important component in abiotic stress responses [32]. These results may help to identify useful genetic sources to improve heat-tolerance in wheat germplasm for future breeding programs and to add a trait for genetic change in wheat line for better food security under global climate change. The study enables a focus on key traits, gene and metabolite selection that could be used by breeders to devise ways to make wheat more resilient to higher temperatures.

## Declarations

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### Author Contributions

**Aamir Shehzad:** Conceptualization, methodology, data collection, analysis, writing original draft.

**Dr. Sehar Nawaz:** Supervision, validation, resources, writing review & editing.

**Muhammad Umar Ikram:** Data curation, visualization, editing.
(All authors read and approved the final manuscript.)

### Competing Interests

The authors declare that they have no competing interests.

### Ethics Approval

Not applicable.

### Consent to Participate

Not applicable.

### Consent to Publish

Not applicable.

### Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

### Code Availability

Not applicable.

### Ethics & Consent to Participate

The collection and use of wheat seeds and plant material complied with institutional, national, and international guidelines. No special permits were required for the collection of plant material. All experimental procedures adhered to relevant legislation and institutional regulations.

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