Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas

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(2020-975-D)

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Thesis

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DEDICATION

This thesis is Dedicated to my beloved "Parents"

(My Lord, have mercy on them, as they raised me when I was a child)

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Certificate– I

This is to certify that the thesis entitled, **"Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas"** submitted in partial fulfilment of the requirements for the award of the degree of **Doctorate of Science in Agriculture (Genetics and Plant Breeding),** to the **Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Sofora Jan (Regd. No. 2020-975-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

(Dr. Reyazul Rouf Mir)
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We, the members of the Advisory Committee of **Ms**. **Sofora Jan (Regd. No. 2020-975-D)**, a candidate for the degree of **Doctor of Philosophy in Agriculture (Genetics and Plant Breeding)** have gone through the manuscript of the thesis entitled, **"Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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Certificate – III

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ABSTRACT

Wheat is one the most important cereal crops in the world. Cold stress is a major constraint affecting wheat production, particularly in regions with low temperatures. In this study, we conducted a comprehensive assessment of cold stress tolerance in wheat genotypes through field screening, cell membrane stability through electrolyte leakage assay and biochemical profiling. A core set comprising of 4,560 genotypes was evaluated for two years (2020-21 and 2021-22), revealing substantial genetic variation for cold stress tolerance. The majority of genotypes exhibited moderate tolerance, while a smaller proportion showed susceptibility to cold. Based on the analysis of cold screening data in the field, a mini-core set of 350 genotypes was selected for membrane stability analysis using electrical conductivity assays. Significant differences were observed in membrane stability among the genotypes, indicating the presence of genetic variation for this trait. Furthermore, a mini-core set was narrowed down to 50 diverse candidate genotypes which were subsequently profiled for various biochemicals including reactive oxygen species (ROS) like lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2), osmoprotectant *viz;* proline and enzymatic antioxidants including Ascorbate Peroxidase (APX), Superoxide Dismutase (SOD), Guaiacol Peroxidase (GPX), Catalase (CAT). Correlation analysis of the biochemicals revealed negative associations between antioxidants and reactive oxygen species (ROS), highlighting their role in mitigating oxidative damage under cold stress conditions. This study

enhances our understanding of the physiological and biochemical mechanisms underlying cold stress tolerance in wheat. The identified genotypes with superior cold stress tolerance can serve as valuable genetic resources for wheat breeding efforts. Based on the results of cold screening in the field and biochemical screening, we selected two contrasting genotypes, SKUA_52 and SKUA_4301, for further metabolomic and lipidomic analysis. SKUA_52 exhibited cold tolerance, while SKUA_4301 was susceptible to cold stress. The metabolomic investigation into the responses of wheat genotypes SKUA_52 (cold tolerant) and SKUA_4301 (cold susceptible) to cold stress provided valuable insights into the activation of defense mechanisms, particularly highlighting the modulation of key defense hormones including Salicylic Acid (SA), Jasmonic Acid (JA), Abscisic Acid (ABA), JA-Isoleucine (JA-ILE), and cis-Octadecanoid-derivative and flavonoids in cold-tolerant genotypes. Untargeted GC-MS metabolomic analysis provided insights into the metabolic dynamics, showcasing the upregulation of osmoprotectants and stress-responsive amino acids in the cold-tolerant genotype. Lipidomic analysis demonstrated the importance of upregulating unsaturated lipids and downregulating saturated lipids in maintaining membrane fluidity under cold stress in cold tolerant genotype. Additionally, gene expression analysis of the ICE-CBF-COR pathway through qRT-PCR revealed the coordinated upregulation of specific COR and CBF genes in the cold-tolerant genotype. Furthemore transcriptomics analysis identified highly upregulated genes involved in cold tolerance, including TraesCS1D03G0703900 (*Wcor18*), TraesCS2A03G1018600, and TraesCS2B03G1138100, associated with cold-responsive protein *WCOR15-2B*, TraesCS3D03G0862400 (*DHN33*), TraesCS5A03G1181100 (*Wcor615*), and TraesCS5B03G1052800 (*RAB15*) demonstrated infinite fold changes in our cold tolerant genoype. Likewise, proteomics analysis uncovered differential expression of proteins associated with antioxidative defense, osmotic adjustment such as late embryogenesis abundant (LEA) proteins, and signal transduction pathways, providing insights into wheat's cold stress adaptation. The study concludes with an exploration of the inheritance of cold tolerance traits through hybridization experiments, affirming the alignment of observed results with Mendelian principles. Overall, this multiomics approach enhanced our understanding of the complex molecular landscape of cold stress adaptation in wheat, informing strategies for breeding resilient crop varieties capable of thriving in challenging environments like Jammu and Kashmir, India.

Keywords: Wheat, reactive oxygen species (ROS), Cold tolerance, enzymatic antioxidants, biochemical, metabomics, defense harmones, flavonoids, lipidomics, ICE-CBF-COR, transcriptomics, proteomics.

Signature of Student

Dated: _____

Signature of Major Advisor

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Sofora Jan

Place: Faculty of Agriculture, Wadura.

Dated:

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Chapter -1

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) belongs to the family Poaceae is an important staple cereal crop throughout the world. In general, wheat refers to either the bread wheat (*Triticum aestivum),* which covers 95% of wheat cultivated area worldwide, or pasta wheat (*T. durum*) which covers about 5% of cultivated wheat (Arzani and Ashraf, 2017). Both are polyploids, where durum wheat is a tetraploid with AABB genome (2n=4x=28) and bread wheat is a hexaploid with AABBDD genome (2n = 6x = 42). Wheat is one of the globally important crops valued for its nutritional values with high demand for bread and starch production and as a livestock feed. Wheat grains are a rich source of essential proteins, carbohydrates, vitamins and minerals and bread alone constitutes the fifth of the average human daily consumption. Due to its high demand as a food source for humans, often inferior wheat cultivars with lower nutritional quality are used as a livestock feedstock.

Globally, wheat covers an area about 217 million hectares, the most of any crop, with an annual yield of around 764 million tonnes (USDA, 2021). The major wheat-producing countries are European union, China, India, Russia, USA, France, Canada, Australia, Pakistan, Turkey, UK, Argentina, Iran and Italy, etc (Statista, 2021-2022). India contributes about 14% to the total world wheat area and produced all time high output of 107.59 million tonnes of wheat with a record average productivity of 358 kg/ha production (Gupta *et al.*, 2021). In India, it is grown in Uttar Pradesh, Madhya Pradesh, Punjab, Haryana, Rajasthan, Bihar, Gujarat, Maharashtra and Jammu and Kashmir. The crop thrives well in the tropical and subtropical, temperate and cold zones even beyond 67°N of Jammu and Kashmir.

The production and productivity of wheat crops experience significant declines due to a combination of abiotic and biotic stresses. These reductions in

crop productivity pose a major threat to global food security, placing populations at the risk of starvation. This concern is particularly pertinent given the world's rapid population growth, projected to reach 9 billion by 2050, coupled with an anticipated 85 percent increase in food demand (FOA STAT, 2017). The escalating climatic fluctuations further compound these challenges, acting as primary drivers of abiotic and biotic stresses that impose limitations on agricultural production (Rosenzweig *et al.,* 2014). Abiotic stresses such as episodes of excessive cold or heat, precipitation or drought and soil salinity or sodicity represent some of the most common types of stresses that plants experience in response to climate change (Barmukh *et al.,* 2022). Extreme weather events are becoming more common as a result of climate change, causing severe episodes of freezing injury in our modern crop cultivars, exposing them to low temperature conditions for which they were not bred or for which native plants had not time to adapt through selection pressures (Solanke *et al.,*2008; Kumar *et al.,*2013). At sub-zero temperatures, intracellular or extracellular ice crystal formation, change in permeability of bio-membranes and generation of reactive oxygen species (ROS) occurs. These changes result in a combination of symptoms like poor germination, reduced seedling vigor or stunted growth, reduced leaf size, leaf yellowing and withering, reduced tillering, poor root proliferation, disturbed plant water relations, impeded nutrient uptake, premature heading, increased seed abortion and reduced seed size leading to reduced yield (Li *et al.,* 2015; Hassan *et al.,* 2021).

Freezing damage to such crop is a major world-wide problem. Every year, 85% of the wheat sown area in the world is affected by spring frost and it usually takes place during March and April at the early booting stage (Yue *et al.,* 2016). In the spring season, when wheat canopy temperature falls 0°C or below, severe frost damage occurs (Frederiks *et al.,* 2015). Winter wheat initially suffers low-temperature stress when tillering begins and when photosynthate assimilation and nutrient absorption sites are under development (Rinalducci *et al.,* 2011).

In India, wheat is the second most important cereal crop after rice, as it contributes about 14% to the total world wheat area and produced all time high output of 107.59 million tonnes of wheat with a record average productivity of 358 kg/ha production (Gupta *et al.*,2021). In India, it is mainly grown in the northern plains like Uttar Pradesh, Madhya Pradesh, Punjab, Haryana, Rajasthan, Bihar, Gujarat and Maharashtra during the winter season (October to March) when the average winter temperature ranges between 10-15°C. However, it can be well grown in the tropical and subtropical, temperate and cold zones even beyond 67°N of Jammu and Kashmir. In Jammu and Kashmir wheat growing areas has remained confined to sub-tropical area of Jammu division, however, temperate climate of Kashmir valley and higher hills are more conducive for realizing higher yields of wheat crop because the valley of Kashmir and higher hills receive most of the annual precipitation during the months of December to May, which coincides with the critical growth period of the crop in the valley. But the occurrence of low-temperature (0°C or below) during the winter months significantly reduces germination and subsequent seedling emergence thus significantly affects wheat production in the temperate region of Kashmir valley.

The harmful effects of cold stress on wheat growth and development highlights the need to develop wheat varieties that can tolerate cold stress, which will lead to more stable crop yields in regions with frequent cold weather. To achieve this, researchers search for genetic markers that are associated with cold stress tolerance and use them to identify plants with the desired trait. Understanding the molecular and biochemical mechanisms that underlie the response of wheat to cold stress is crucial for improving wheat productivity and yield under cold stress conditions. The last two decades have witnessed an increased use of omics approaches in studying and dissecting complex traits and their integration in breeding programs (Varshney *et al*., 2021; Roorkiwal *et al*., 2020). The methods to deploy these procedures into plant breeding have improved

at lightning speed and their costs have plummeted, which has made their deployment easy.

Cold tolerance particularly in cereals represents one of the most important traits in plant breeding and various omics approaches have been utilized in studying this complex trait (Jan *et al.*, 2023). Metabolomics is a generally new methodology for improved comprehension of metabolic systems and the resulting biochemical organization of plants and other biological organisms. The imposition of low-temperature stress on a plant leads to the modification of metabolism. First, cellular metabolism must adjust to the consequences that rising or falling temperatures have on metabolic processes and metabolism overall. The second aspect of the modifications of metabolism in response to changing temperatures would be those linked with enhanced tolerance mechanisms. The active reconfiguration of the metabolome seems to be achieved in part by changes in cold-regulated gene expression initiated by low-temperature-activated signalling, such as the CBF cold response pathway in Arabidopsis (Cook *et al*., 2004; Guy *et al*., 2007), changes in transcript abundance and regulatory processes, independent of transcript abundance (Kaplan 2004, 2007).

Lipidomics, a branch of metabolomics, investigates the comprehensive profile of lipids within biological systems. In the context of cold stress in wheat, lipidomics plays a pivotal role in unravelling the dynamic changes in lipid composition and metabolism. By employing techniques such as GC-MS, lipidomics provides detailed insights into the alterations of various lipid classes, including phospholipids and glycolipids, under cold stress conditions. Understanding these lipidomic changes is crucial for deciphering the molecular mechanisms associated with cold tolerance, as lipids are integral to membrane fluidity and cellular integrity, key factors in plant adaptation to cold environments. In this research, we planned to use these techniques to understand the molecular and biochemical mechanisms underlying the response of wheat to cold stress by identifying changes in the physiology, biochemical activities,

metabolome and lipidome, in response to cold stress. Thus, the objectives of this study were:

Objectives

1. Screening of wheat germplasm for vegetative cold tolerance under in vitro and in vivo conditions.
2. Biochemical analysis of diverse wheat genotypes in response to cold stress under controlled condition
3. Comparative metabolome and lipidome profiling in contrasting wheat genotypes.
4. Characterization and differential gene expression study of cold tolerance genes using qRT-PCR.
5. Study of inheritance pattern of cold tolerance in bread wheat.

In addition to above approved objectives, we have also done additional work including:

1. Transcriptomics to study differentially expressed transcripts/genes between two contrasting genotypes for cold tolerance.
2. Proteomics to study differentially expressing proteins in contrasting wheat genotypes for cold tolerance.

Chapter- 2

REVIEW OF LITERATURE

A brief review of the available literature on the objectives of the present study is presented under the following headings.

2.1 Effect of cold stress on morphological processes of wheat and evaluation for cold resilient wheat genotypes under natural conditions in the field

2.2 Cold signal perception and transduction in plants

2.3 Biochemical response of wheat plants to cold stress

2.4 Metabolomics for underpinning cold tolerance in cereals

2.5 Lipidomics to understand lipid turnover in response to cold stress in wheat response of plants for cold stress

2.6 Genetic regulation of cold acclimation in cereals

2.7 Inheritance pattern for cold stress tolerance

2.1 Effect of cold stress on morphological processes of plants and evaluation for cold resilient genotypes under natural conditions in the field

Wheat is a cool-season crop that is adapted to grow in regions with relatively low temperatures, but exposure to cold stress can cause damage and reduce yield. To ensure consistent wheat yields and minimize the adverse effects of sudden cold weather events, it is crucial to implement appropriate agricultural and management practices (such as the time of sowing, fertilization and planting methods), as well as to develop cold-tolerant wheat varieties. Researchers have highlighted the importance of selecting wheat genotypes that are able to withstand cold temperatures, with screening methods that range from simple and large-scale to more advanced and focused methods that incorporate various omics techniques.

Limin and Fowler (2006) emphasized the significance of effective management and breeding strategies to mitigate the risk of frost damage in wheat crop. Sinclair (2011) also emphasized the need for sophisticated screening techniques that involve a tiered approach with decreasing numbers of genotypes in each tier, which allow for the evaluation of a large number of genotypes in a cost-effective and time-efficient manner. The process of multitier screening for phenotyping involves a systematic evaluation of wheat genotypes, aiming to decipher and enhance crop resilience. The initial tier comprises assembling a diverse set of genotypes, subjected to phenotypic evaluation. The diverse 10% of phenotypically diverse genotypes are then selected for the second tier, where more precise phenotyping for physiological traits takes place. This tier involves advanced assessments such as various antioxidant activity assays. Finally, in the third tier, a subset of candidate genotypes is identified based on refined physiological assessments and subjected to sophisticated analyses like metabolomics, transcriptomics, lipidomics and genomics. These high-cost techniques provide in-depth insights into biochemical composition, gene expression, lipid profiles and genetic makeup, culminating in a comprehensive understanding of the relationships between genetic factors, physiological responses and environmental influences. This multitier approach ensures a nuanced comprehension of wheat crop traits, informing strategic decisions in crop improvement and breeding programs.

Morphological screening provides a cost-effective and relatively simple approach for identifying wheat germplasm with potential cold stress tolerance, which is a critical trait for maintaining wheat productivity in regions affected by cold weather events. There are various studies highlighting the importance of cold stress tolerance in wheat varieties, especially in regions with harsh winter conditions. The evaluation of cold stress tolerance through various methods such as natural evaluation, freezing chamber exposure and visual scoring can provide valuable information to breeders and researchers to develop more resilient wheat

varieties. Ruzgas and Liutkevicius (2001) studied 900 winter wheat varieties from various regions for cold stress tolerance at the Lithuanian Institute of Agriculture, Lithuania. The study evaluated the plants in natural conditions using a scale of 1–9. Score 1 means that all plants were dead while as score 9 indicated that all plants survived. The study found that winter hardiness was a major limiting factor. Winter hardiness positively correlated with plant height and grain yield and a significant portion of distant origin varieties showed resistance to Lithuanian winter conditions. In another study, Li *et al*. (2010) screened 677 genotypes of wheat for cold stress tolerance at different agro-ecological locations of china. After winter, wilting degree was scored in 5 scales as grades of cold tolerance. The seedlings leaves of the material without wilting was classified in cold tolerance grade 1 and with leaf top wilting, half leaf wilting and whole leaf wilting was classified in grade 2, grade 3 and grade 4, respectively. Whole seedlings death was classified in grade 5. The materials with equal or lower wilting degrees than the check (wilting degree of the check 'Dongfanghong 3' was grade 3) were regarded as cold tolerant genotypes. Results revealed that seedling wilting grades due to cold of 115 accessions were equal to that of the check Dongfanghong 3 (wilting degree 3). Likewise, Chipilski and Uhr (2014) investigated the cold tolerance of 23 Bulgarian winter common wheat varieties during the four years period (year 2009-2012). Hardening under natural conditions and direct plant freezing test in a controlled frost chamber were applied for the evaluation. The genotypes were compared with each other based on the average data received for survival of plants in the frost chamber imposed to temperature levels -20°C. Among the all studied genotypes, the variety "Fermer" was distinguished by the highest cold tolerance. The applied analysis of variance revealed that genotype and environment conditions during the hardening of plants have significant influence on the variation of the studied trait. Similarly, Mohammadi *et al*. (2015) assessed cold stress tolerance in 380 durum wheat entries in Iran. They used visual scores to evaluate foliage damage due to frost in early April, finding significant variation in cold stress tolerance among the

landraces. Cold stress also led to reductions in 1000-grain weight (by 21.6%) and grain yield (by 91.9%). Another study conducted by Zhao *et al*. (2019) evaluated cold stress tolerance in 543 wheat accessions across different environments and recorded low-temperature tolerance traits like mortality rate of tillers and frost damage grades using 0 to 4 scale wherein 0: leaves had no frozen parts; 1: only tips of leaves were frozen, with older leaves rarely or not frozen at all and the overall field was predominantly green; 2: the majority of leaf areas, especially of young leaves, were unfrozen and dead yellow leaves were laying on the ground; 3: the majority of leaf areas were frozen and whole dead leaves constituted the bulk of intact leaves visible on the ground; 4: all leaves were frozen and dead, with even whole plants having died. They found significant differences in phenotypic traits among genotypes and environments and these traits were positively correlated.

In another study, Ahmadi *et al*. (2019) conducted an assessment of cold stress tolerance in 28 bread wheat lines from both temperate and cold agro-climatic zones of Iran. The study employed a regulatable freezer to determine LT50, which is the temperature at which 50% of plants die. Results indicated that eight temperate-adapted lines and cv. Baharan had a lower LT50 than Parsi, with LT50 at -6°C. The line M-94-7 had the lowest LT50 of -12°C. Similarly, among the cold-adapted lines, eight lines had a lower LT50 than Mihan, with LT50 at -12°C. The lines C-94-11 and C-94-13 had the lowest LT50 of -15°C. Heydari (-9.5°C) and Orum (-11.5°C) had lower cold stress tolerance than cv. Mihan, while Zarea (-13°C) had higher cold stress tolerance. Six lines from the temperate agro-climatic zone and nine lines from the cold agro-climatic zone showed both greater cold tolerance (lower LT50) and higher grain yield than Parsi and Mihan, respectively.

Overall, these studies demonstrate that evaluating wheat germplasm for cold stress tolerance using various methods can help us to identify and select wheat lines better adapted to cold-prone regions. This, in turn, can help us to

develop new wheat varieties that are more resilient to cold stress, ultimately improving wheat production and yield in regions with frequent cold weather.

Most cereal crops tend to survive and continue their life cycle by developing their tolerance ability under increasing freezing degrees (Dubcovsky and Dvorak, 2007; Thomashow, 2010) by exhibiting a wide range of genetic expressions; such behavior is termed cold acclimation (Monroy *et al.,* 2007). It is stated that the counter action of plants to low-temperature stress is carried out through detection (sensing) of stress followed by signal perception, transduction and induction of cold-tolerant gene expression (Ganeshan *et al.,* 2008). During cold tolerance, multiple gene expression levels (ICE-CBF-COR, COR and Wheat, WCS) are generated and subsequently initiate the cascade of transcriptional, biochemical and physiological events vital for cold tolerance in the plant (Kosová *et al.,* 2008). Various omics approaches have been utilized in studying this complex trait to gain a better understanding of the molecular and biochemical mechanisms underlying the plant's response to cold stress. This knowledge can be used to develop more cold-tolerant wheat varieties and ultimately improve wheat production and yield in regions with frequent cold weather.

2.2 Cold signal perception and transduction in plants

Uncovering the mechanism through which plants perceive and transduce cold signals is crucial for understanding how plants avoid/minimise injury caused by chilling temperatures. In plants, the plasma membrane serves as the central hub for perceiving cold stress, playing a pivotal role in maintaining membrane fluidity and integrity when exposed to low temperatures (Orvar *et al.,* 2000; Solanke *et al.,* 2008; Yadav *et al.,* 2010). It acts as the frontline sensor, detecting changes in temperature and orchestrating adjustments to ensure the plant's adaptability and survival in challenging cold conditions. This intricate process involves a network of molecular responses and signaling pathways that are finely tuned to safeguard the plant's overall health and functionality. Cold stress reduces membrane fluidity, which affects membrane-associated cellular functions, thereby making the plasma

membrane (PM) the primary sensor of low-temperature stress (Knight *et al.*, 2012). Microdomains with lipid raft formation and composition, such as sphingolipids in the PM, are utilized by plants to detect specific temperature ranges. Calcium channels, as well as receptor-like protein kinases, such as two-component histidine kinases, RLKs and G-protein associated kinases, play crucial roles in the perception of cold stress. However, calcium channels are major class of cold stress sensors that allow calcium to enter the cell (Chen *et al.*, 2021). Through the membrane rigidification-activated mechanosensitive or ligand-activated Ca^{2+} channels, cold stress induces a transient Ca^{2+} influx into the cytosol. Following cold stress perceptions, the cytosol and nucleus of plant cells undergo cold stress signal transduction. Ca^{2+} and reactive oxygen species (ROS) are examples of second messengers that transmit extrinsic cold signals to intracellular signaling systems. The major components underlying cold signal-sensing/transduction in cereals include calcium signaling, reactive oxygen species (ROS), protein kinase [the Mitogen-Activated Protein Kinase (MAPK) signaling], plant hormone signaling and lipid signaling cascade (Yadav *et al.*, 2010) (Figure 1). Here, we discuss the mechanisms underlying some of these key components involved in cold signal sensing and transduction in further detail.

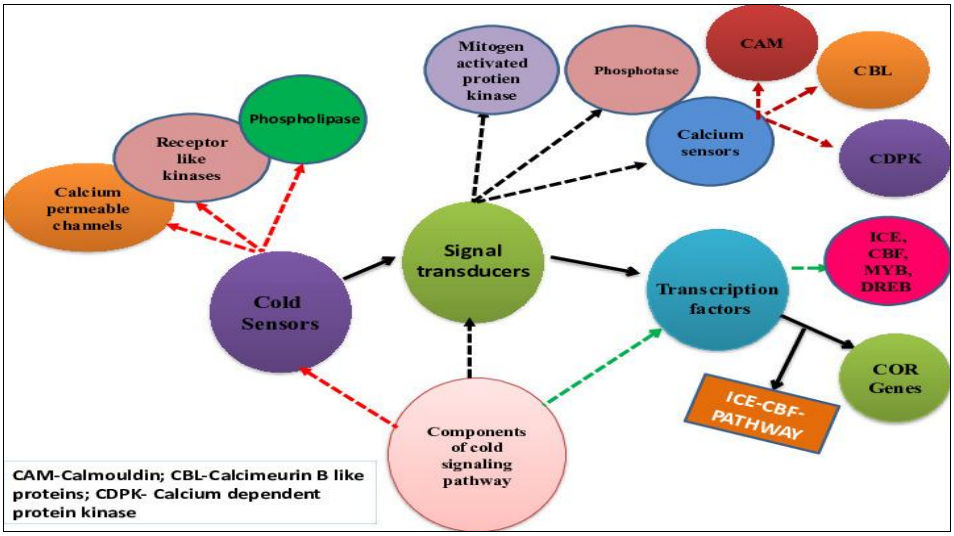


Figure 1: Schematic model showing different components involved in signalling pathway for acquisition of cold tolerance in Plants.

2.2.1 Calcium channels

Calcium (Ca^{2+}) is a universal secondary messenger that responds to cold stimuli. The fluctuation in cytosolic Ca^{2+} levels is sensed by calcium-dependent protein kinases (CDPKs), which modify the phosphorylation status of substrate proteins and mediate the cold stress signaling pathway (Knight *et al.,* 2001; Ray *et al.,* 2007; Solanke *et al.,* 2008; Asano *et al.,* 2012; Thoday *et al.,* 2015; Wilkinis *et al.,* 2016; Zhu *et al.,* 2016). The CDPKs produced under cold stress target significant cold stress-responsive genes, leading to the adaptation of cereals to unfavorably low temperatures (Table 2.1). Occasionally, CDPKs confer simultaneous multiple stress tolerance. For instance, overexpression of the rice *OsCDPK7* gene conferred cold, salt and drought tolerance in transgenic rice plants (Saijo *et al.,* 2000, 2001; Komatsu *et al.,* 2007). In addition to calcium-dependent protein kinases (CDPKs), other calcium sensors like calmodulins, calmodulin-like proteins (CMLs) and calcineurin B-like (CBL) proteins also act as calcium sensors that capture transient calcium signals in the cytoplasm and transfer these signals to downstream components, causing physio-biochemical changes necessary for cold stress tolerance (Albrechet *et al.,* 2003).

2.2.2 MAPK signaling

MAPKs regulate cell division, development, metabolism and stress responses, including cold stress in plants (Moustafa *et al.,* 2014). The proteins of different MAPK subfamilies [MAPKs, MAPK kinases (MKKs) and MAPK kinase kinases (MKKKs)] interact sequentially in the signaling cascade (Goyal *et al.,* 2018). Precisely, MKKKs get phosphorylated in response to environmental or developmental signals and subsequently activate MKKs, which in turn activate MAPKs (or MPKs). MAPKs have been examined as signaling molecules in cold stress adaptation in rice, wheat and maize (Table 2.1).

2.2.3 Phytohormones

Plant hormones like auxin, abscisic acid (ABA), ethylene (ETH), cytokinins (CKs), gibberellins (GAs), jasmonic acid (JA) and brassinosteroids (BRs) modulate cold tolerance via CBF (C-repeat/DRE-Binding Factor)-dependent and CBF-independent pathways. In the ABA-independent pathway/CBF-dependent pathway, low temperature boosts CBF transcription factors, which activate downstream cold-responsive genes that increase freezing tolerance in rice, wheat and maize (Xu *et al.,* 2008; Sun *et al.*,2009; Lv *et al.,* 2018; Verma *et al.*,2019). On the other hand, in the ABA-dependent pathway, endogenous ABA activates basic leucine zipper (bZIP) transcription factors (Uno *et al.*,2000; Xiong *et al.*,2002), WRKY transcription factors (Ramamoorthy *et al.,* 2008; Tao *et al.,* 2011), dehydration responsive element-binding factors (DBFs) (Xu *et al.,* 2008) and then regulate ABA-dependent cold-responsive genes that modulate cold stress in cereals. In addition to ABA, ethylene, jasmonates (JA) and salicylic acid (SA) also induce cold stress tolerance (Zhao *et al.,* 2019) by rapidly raising endogenous JA levels via stimulation of JA biosynthesis genes This enables ICE1 and ICE2 to activate CBFs by binding to the DRE/CRT box promoter sequence element found in the ICE regulon promoters (Kazan *et al.,* 2013; Wang *et al.,* 2020).

2.2.4 Lipid molecules

The lipid bilayer membrane separates the cell contents from the outside environment, making them vital in cold signal transmission (Hou *et al.,* 2016). The plasma membrane senses environmental stimuli like cold stress and generates signaling lipids via enzymes, including phospholipases, lipid kinases and phosphatases. Signaling lipids such as phosphatidic acid (PA), phosphoinositides (PIs), sphingolipids, lysophospholipids, oxylipins, N-acylethanolamines and free fatty acids (FFAs) make up less than 1% of total lipids and their concentrations increase with biotic and abiotic stresses, including cold stress in rice, wheat and maize (Munnik and Vermeer, 2010; Meijer and Munnik, 2003; Testerink and Munnik, 2005; Bargmann *et al.,* 2009). These signaling lipids cause lipid-dependent cascade

reactions in cold-exposed plants, activating downstream genes that stimulate adaptation to cold stress (Hou *et al.*, 2016). These genes encode proteins involved in osmolyte biosynthesis, regulation of ion channels, mediation of receptors, participation in calcium signaling components and the facilitation of various signaling factors or enzymes, which help the plants to tolerate cold stress (Tuteja, 2007).

Table- 2.1: List of some key sensors and their corresponding genes that regulate signal transduction during cold acclimation in rice, wheat and maize.

Senor family	Crop	**Gene**	**Reference**
Calcium dependent protein kinase (CDPK)	Rice	*OsCDPK7*	Saijo *et al.* (2000), Saijo *et al.* (2001)
	Rice	*OsCPK4*	Ray *et al.* (2007)
	Rice	*OsCPK17*	Almadanim *et al.* (2017)
	Rice	*OsCPK24*	Liu *et al.* (2018b)
	Rice	*OsCPk13*	Ray *et al.* (2007)
	Maize	*Zm*cpk5	Trzcinska-Danielewicz *et al.* (2009)
	Maize	*Zm*cpk1	Weckwerth *et al.* (2015)
	Maize	ZmCK1	Wang *et al.* (2013)
	Wheat	*TaSnRK2.8*	Zhang *et al.* (2010)
Calcineurin B-like (CBL) protein	Wheat	*TaCBL*	Sun *et al.* (2015)
CBL-interacting protein kinases (CIPK)	Wheat	*Ta CIPK*	Sun *et al.* (2015)
CIPK	Rice	*OsCIPK3*	Xang *et al.* (2007)
Mitogen activated protein kinase (MAPK)	Wheat	*TaMPK3*	Goyal *et al.* (2018)
MAPK	Wheat	*TaMPK6*	Goyal *et al.* (2018)
MAPK	Wheat	*TaMPK4*	Goyal *et al.* (2018)
MAPK	Rice	OsMPK3	Zhang *et al.* (2016)
MAPK	Maize	*ZmMPK17*	Pan *et al.* (2015)
MAPK	Maize	*ZmMKK4*	Kong *et al.* (2011)
MAPK	Maize	*ZmMPK5*	Mira *et al.* (2021)
Phospholipase C	Wheat	*TaPLC1, TaPLC2*	Khalil *et al.* (2011)
Inositol polyphosphate 5-phosphatases (5PTases)	Rice	*Os5PTase3a, Os5PTase3b, Os5PTase10b, Os5PTase11, Os5PTase12, Os5PTase13 and Os5PTase15*	Faraji *et al.* (2020)

2.3 Biochemical response of wheat plants to cold stress

Cold stress is a significant environmental factor that triggers oxidative processes in plant cells. Reactive oxygen species (ROS) are the primary initiators of these processes, arising from disrupted electron transport chains and leading to macromolecular damages to DNA, proteins, lipids and carbohydrates. These damages at the whole-plant level translate into reduced photosynthesis, assimilate translocation and carbon gain, ultimately affecting growth and reproduction. The biochemical response of wheat to cold stress involves a complex network of pathways controlled by various genes and proteins. Cold tolerance is regulated by a large number of genes, each making a small positive or negative contribution to the final response. Genetic variability of tolerance is influenced by plant developmental stage and physiological status. Membrane damage index, solute and electrolyte leakage index (ELI), can be increased under cold stress, providing a means to study genetic variability. Membrane damage can also result from lipid peroxidation caused by ROS, with malondialdehyde (MDA) serving as an evaluation factor for membrane damage. H_2O_2 content changes in response to cold stress are crucial as it promotes the formation of hydroxyl radical and other toxic ROS. Wheat plants produce antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) to scavenge ROS and protect cells from oxidative damage. SOD serves as the vanguard, neutralizing superoxide radicals by catalyzing their dismutation into molecular oxygen and hydrogen peroxide. Meanwhile, CAT takes centre stage in peroxisomes, safeguarding cellular components by converting hydrogen peroxide into water and oxygen, thereby averting potential oxidative damage. Peroxidases, encompassing variants like guaiacol peroxidase and ascorbate peroxidase, contribute significantly by detoxifying hydrogen peroxide and other peroxides through diverse electron donors. Among them, APX specifically utilizes ascorbate to mitigate oxidative stress. This ensemble of antioxidant enzymes is strategically distributed across various cellular compartments,

including the cytoplasm, chloroplasts and peroxisomes, collectively fortifying wheat plants against reactive oxygen species (ROS). Their coordinated actions not only neutralize ROS generated during environmental challenges, such as cold stress, drought or pathogen attacks but also maintain redox balance essential for cellular homeostasis (Ritonga *et al.*, 2020). Also, the Proline, which acts as a compatible solute, plays a dual role in osmoprotection and cellular stabilization. It helps to regulate osmotic balance by preventing water loss and maintaining turgor pressure, crucial for cellular integrity during cold-induced dehydration. Simultaneously, proline serves as a cryoprotectant, shielding cellular structures from freezing-induced damage (Wang *et al.*, 2022).

Numerous studies have been conducted to investigate the biochemical response of wheat to cold stress. These studies have utilized various assays to measure changes in the levels of specific compounds and enzymes involved in the response to cold stress. One of the early research was conducted Janda *et al*. (2007) to study the effects of frost hardening on freezing survival rate, lipid composition, antioxidant activity and salicylic acid content in wheat. The study found that low temperature hardening and high light intensity increased the activity of antioxidant enzymes and salicylic acid content, while decreasing the saturation level of hexadecanoic acid. The greatest induction of glutathione reductase and ascorbate peroxidase occurred with cold treatment in normal light, while the highest activity of catalase and guaiacol peroxidase occurred under high light and low light conditions, respectively. Another study conducted by Nejadsadeghi *et al.* (2014) compared the biochemical responses to cold stress in three wheat genotypes. They found that tolerant genotypes had lower levels of electrolyte leakage, H_2O_2 and malondialdehyde during cold stress. They concluded cold acclimation induced oxidative stress tolerance by modulating antioxidative systems. Likewise, Kolupaev *et al.* (2015) studied the response of antioxidant enzyme activity and osmolyte content in winter rye, soft and durum wheat and barley seedlings to cryostress. Rye showed higher activity of guaiacol

peroxidase (GPX) and high content of proline which may determine its increased frost tolerance. Hardened seedlings of all cereals showed increased activity of GPX and catalase (CAT), as well as the contents of proline and soluble carbohydrates. After freezing, rye and soft wheat showed higher activities of all tested antioxidant enzymes compared to durum wheat and barley, while hardened soft wheat displayed increased content of low-molecular-weight protectors. In conclusion, a variety of biochemical assays have been used to study the response of wheat to cold stress. These assays have provided insights into the complex network of pathways involved in the response to cold stress and have identified specific compounds and enzymes that play important roles in the response.

2.4 Metabolomics for underpinning cold tolerance in cereals

Metabolomics is a powerful analytical approach used to study the comprehensive set of small molecules, or metabolites, present in a biological system. The primary goal of metabolomics is to provide a snapshot of the metabolic status of an organism under specific conditions, offering insights into its physiological and biochemical responses. Metabolomics tools and techniques encompass a range of analytical methods that can be broadly categorized into non-targeted and targeted approaches. In non-targeted metabolomics, a global analysis is conducted without a predefined set of metabolites. Techniques like Nuclear Magnetic Resonance (NMR) spectroscopy, Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) are commonly employed (Kim *et al.,* 2010; Zhang *et al., 2*012). Contrastingly, targeted metabolomics focuses on the quantitative analysis of a predefined set of metabolites. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and triple quadrupole mass spectrometry are often used for targeted studies (Zhang *et al.*, 2012).

Metabolomics is a generally new methodology for improved comprehension of metabolic systems and the resulting biochemical organization of plants and other biological organisms. Exposure to freezing situations results in

genuine harm to the plant cell owing to ice formation and dysfunction of cell membranes. Many plant species increase their freezing resilience amid their presentation to non-freezing low temperatures by a procedure known as cold acclimation (Zuther *et al.,* 2018). The imposition of low-temperature stress on a plant leads to the modification of metabolism. First, cellular metabolism must adjust to the consequences that rising or falling temperatures have on metabolic processes and metabolism overall. The second aspect of the modifications of metabolism in response to changing temperatures would be those linked with enhanced tolerance mechanisms. The active reconfiguration of the metabolome seems to be achieved in part by changes in cold-regulated gene expression initiated by low-temperature-activated signalling, such as the CBF cold response pathway in Arabidopsis (Cook *et al.,* 2004; Guy *et al.*,2007), changes in transcript abundance and regulatory processes, independent of transcript abundance (Kaplan, 2007). Many metabolites contribute to cold stress tolerance, which generally functions as osmolytes, compatible solutes, chelating agents and energy sources and in the retailoring of membrane lipid composition to optimize the liquid/crystalline physical structure necessary for proper membrane function (Guy *et al.,* 2007). Many stress-responsive metabolites, including soluble sugars, amino acids, organic acids, polyamines and lipids, increase in the cold acclimation of wheat (Zhao *et al.,* 2019). In addition to these sugar metabolite profiles, the abscisic acid (ABA), jasmonic acid (JA), phytohormone signalling and proline biosynthesis pathways are significantly modulated under cold acclimation and freezing treatments (Zhao *et al.,* 2019). At the young microspore development in spikes of two contrasting genotypes of wheat for cold stress, quantitative and qualitative differences in primary metabolites involved in osmoprotection were observed after cold exposure. In a cold-acclimated genotype, maltose, fructose-6-phosphate, beta-alanine, sucrose, glutamate, proline and lipids were significantly increased (Cheong *et al.,* 2020). The metabolomic results for the tillering nodes in the overwintering period showed that disaccharides had a strong protective effect on winter wheat and mino acid metabolism (*i.e.*, proline, alanine and GABA)

changed significantly throughout the whole wintering process, whereas organic fatty acid metabolism changed significantly only in the late stage of overwintering (Bao *et al.,* 2021). During cold exposure of Tibetan hulless barley (*Hordeum distichon* L.) in sensitive and tolerant cultivars have differential responses to cold stress, eight metabolites, including monoacylglycerol, deoxyadenosine, 6-methylmercaptopurine and coniferin, were significantly altered, which are mainly related to glutathione metabolism and thus can be regarded as candidate biomarkers (Yang *et al.,* 2020).

2.5 Lipidomics to understand lipid turnover in cereals in response to cold stress

Lipidomics, the comprehensive study of lipid profiles, plays a crucial role in understanding the molecular mechanisms underlying cold tolerance in wheat as it provides insights into how lipids, essential components of cellular membranes, respond to and contribute to cold stress. Lipid species, including phospholipids, glycolipids and lysophospholipids, are key components of cell membranes, influencing membrane fluidity, stability and functionality (Hou *et al.,* 2016). Under cold stress conditions, lipidome analysis can reveal dynamic changes in the composition and abundance of various lipid classes. For instance, alterations in phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), lysophosphatidylcholines (LPC) and other lipid species may occur in response to cold stress. The identification of specific lipids, their up- or down-regulation and the overall lipidomic profile contribute to a comprehensive understanding of how the wheat plant adjusts its lipid metabolism to cope with cold conditions (Zhao *et al.,* 2021). Moreover, unsaturated lipids, characterized by double bonds in their fatty acid chains, are of particular interest. Up-regulation of unsaturated lipids, such as digalactosyldiacylglycerol (DGDG), PC, monogalactosyldiacylglycerol (MGDG), PE and PG species, suggests an active response to cold stress. These lipids contribute to membrane flexibility,

critical for maintaining cell integrity in low-temperature environments (Cheong *et al.*, 2019).

The plasma membrane senses external stimuli such as cold stress and is generally a source of signalling lipids that are generated by modifying enzymes such as phospholipases, lipid kinases or phosphatases. Signalling lipids such as phosphatidic acid (PA), phosphoinositides (PIs), sphingolipids, lysophospholipids, oxylipins, N-acylethanolamines and free fatty acids (FFAs) constitute less than 1% of total lipids and their concentrations significantly increase upon biotic and abiotic stress, including cold stress (Testerink and Munnik, 2005). These signalling lipids upon cold exposure in plants trigger lipid-dependent cascade reactions, which control the expression of gene clusters and activate plant adaptation processes (Hou *et al.*, 2016). The products encoded by these genes include osmolytes, ion channels, receptors, calcium signalling components and other regulatory signalling factors or enzymes (Tuteja, 2007). Signalling lipids are able to recruit protein targets transiently to the membrane and thus affect the conformation and activity of intracellular proteins and metabolites (Huo *et al.*, 2016). Phosphatidic acid is the precursor of phospholipid (PL) biosynthesis, acts as the main lipid signal in eukaryotes, binds to specific proteins and activates the MAPK signalling pathway, Ca2+-dependent protein kinase, NADPH oxidase and ion channels (Testerink and Munnik, 2011). The biosynthesis of PA involves two different pathways: one is the direct hydrolysis of PL by phospholipase D (PLD) and the other is phospholipase C (PLC), which catalyzes the hydrolysis of poly-phosphatidylethanolamine (PPI) together with diacylglycerol kinase (DGK) and synthesis of PA from diacylglycerol (DAG) (Arisz *et al.*, 2009). Under cold stress, the PLD/DGK and PLD pathways are both responsive (Chen *et al.*, 2015). Phospholipase D can catalyze the hydrolysis of phosphodiester bonds and produce inositol triphosphate (IP3), diester glycerol (DAG), acetylcholine (Ach) and PA. As second messengers in cells, IP3, DAG, PA and Ach can cause a series of secondary reactions by changing intracellular Ca2+ and protein kinase K (PRK)

levels, thus completing the process of the cell response to cold signals (Hong *et al.,* 2016). Moreover, the activity of PLD is closely related to the response of plants to low temperature (Muzi *et al.,* 2016). It has been reported that the Short-term chilling stress (0–180 min) in barley causes rapid and transient increases in PLD activity in young leaves, while long-term chilling stress (24–36 h) causes significant decreases in PLD activity in young leaves and roots (Margutti *et al.,* 2017). The involvement of the phospholipase C and phospholipase D pathways in cold-induced expression of the cold-responsive genes CBF9 and CBF 14, which play a key role in cold acclimation, was proven in barley (Marozsán-Tóth *et al.,* 2015). However, in another study, PLD contributed to signalling pathways in response to short-term chilling stress in barley seedlings through regulation of the balance between proline and ROS levels (Margutti *et al.,* 2017). Microarray-based expression analysis and expression profiles of the entire set of PLD genes in the rice genome revealed that seven PLD genes are expressed significantly under abiotic stresses, including cold stress and they also exhibited an overlapping expression pattern and were also differentially expressed during developmental stages, thus suggesting the role of PLDs in abiotic stress (Singh *et al.,* 2013). Several studies have demonstrated the important role of the phosphoinositide signalling pathway at multiple developmental stages and in response to environmental stress in plants (Munnick *et al.,* 2009). Phosphoinositide-specific phospholipase C (PI-PLC, PLC) is an essential enzyme in phosphoinositide signalling. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) upon activation, generating inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), both of which are secondary messengers in the phosphoinositide signal transduction pathway. DAG activates ion channels and protein kinases to generate phosphatidic acid, a typical plant second messenger molecule, whereas IP_3 activates receptors on the endoplasmic reticulum membrane, which release Ca2+ into the cytoplasm (Gierczik *et al.,* 2017). Cerebroside C increases tolerance to chilling injury and alters the lipid composition of wheat roots, possibly due to a partial reduction in lipid peroxidation and alterations in lipid composition,

including inhibition of the activities of PLC and phospholipase D (PLD) (Li *et al.*, 2013). There are two PLC genes in the wheat genome: TaPLC1 and TaPLC2. TaPLC1 was recently shown to interact with Gα and to be involved in the response to cold stress in wheat (Khalil *et al.*, 2011). In barley, the involvement of PI signalling components in plant abiotic stresses, such as low-temperature tolerance, was ruled out. In transformant barley lines PITP and PI4K, upon frost exposure, the overexpression of phosphatidylinositol transfer protein (PITP) and phosphatidylinositol 4-kinase (PI4K), which are very upstream elements of the phospholipid signalling pathway in the signal transduction network, was found to regulate many cold-responsive genes, including the HvCBF regulon (HvCBF4B, HvCBF9, HvCBF12 and HvCBF14) (Gierczik *et al.*, 2019).

A small group of membrane lipids, so-called phosphatidylinositides, exists in eukaryotic cell membranes. Phosphatidylinositol (PtdIns) and its phosphorylated compounds are considered important signalling molecules (Lin *et al.*, 2022). The signalling process managed by PtdIns is adjusted via phosphorylation and/or dephosphorylation of the hydroxyl group of the inositol ring through the specific kinases and phosphatases that induce an interaction with PtdIns-binding proteins (Meijer and Munnik, 2003). As previously mentioned by Meijer and Munnik (2003), the regulators among the PtdIns-changing proteins are PtdIns 3-kinases and PtdIns 5- phosphatases. During plant phosphatidylinositide metabolism, PtdIns 3-kinase (PI3K) was proven to be a signalling kinase molecule under stress (Xue *et al.*, 2009). Inositol polyphosphate 5-phosphatases (5PTases) are considered a class of phosphatases that hydrolyze the inositol ring 5-position phosphate in water-soluble phosphoinositides and inositol polyphosphates (Meijer and Munnik, 2003). 5PTases act as essential players in various cellular processes and support cellular responses during development and stressful conditions.

2.6 Genetic regulation of cold acclimation in cereals

The cold acclimation process requires a variety of genes to be activated. Many cold-responsive genes have been found in plants (Guo *et al.,* 2019) and are recognized as dehydrin (DHN), late embryogenesis abundance (LEA), cold responsive (COR) and responsive to abscisic acid (RBA), among others. Transcriptional activators, which activate cold tolerance structural genes, are triggered by low temperatures. In *Arabidopsis,* the transcription factor *ICE1* (Inducer of CBF Expression) is activated by the low temperature signaling pathway and subsequently activates the CBF (C-repeat-Binding Factor) protein family (Chinnusamy *et al.,* 2003). The CBF protein family is present in many plants, including *Arabidopsis* (Chinnusamy *et al.,* 2003), barley (Skinner *et al.,* 2005) and wheat (Jaglo *et al.,* 2001). Three transcriptional activators, *CBF1*, *CBF2* and *CBF3,* have been reported to activate cold tolerance genes. *CBF2* is constitutively expressed and suppresses *CBF1* and *CBF3* expression (Novillo *et al.,* 2004). Increased expression of *CBF1* and *CBF3* occurs early during cold acclimation. *ICE1* induces *CBF3* expression (Chinnusamy *et al.,* 2003) and causes an increase in *CBF1* expression. The high amount of *CBF1* and *CBF3* expression represses *CBF2* (Novillo *et al.,* 2004). *CBF2* represses *CBF1 and CBF3* activates the transcription of cold-regulated (COR) genes. Over time, the levels of the *CBF3* and *CBF1* genes decrease, allowing *CBF2* to be expressed. This slows the activation of cold acclimation genes and the cold tolerance response. Miller *et al*. (2006) recently reported that there are 13 different *CBF* genes in wheat, 11 of which are present in the same region of chromosome 5 as the *Fr-A2* locus. *Wcs120* and *Wcor410* are examples of cold-regulated COR genes (Kane *et al.,* 2005) that are activated by the CBF protein family. WCS120 is a 50 kDa dehydrin that accumulates in the cytoplasm and nucleus of cells. Along with *WCS120*, other COR genes responsible for cold tolerance include *WCS180, WCS200, WCS66* and *WCS40* (Vítámvás and Prášil, 2008); however, proteins that belong to *WCS120* showed higher transduction in winter cereals and were characterized as best in

cold tolerance (Vítámvás and Prášil, 2008). Induction of *Cor* genes by low-temperature stress is regulated by CBF and ABA-responsive element binding protein (AREB) (Kobayashi *et al.,* 2008). The activation of *CBF* gene expression is also ABA-dependent and ABA-independent (Zhou *et al.,* 2011). Very recently, the expression of the COR genes *WCS19* and *WCS120* was upregulated in wheat seedlings at 4 degrees upon the application of methyl jasmonate, which increased cold tolerance (Repkina *et al.,* 2021). A significant correlation between the level of induced frost tolerance and the accumulation of *WCS120* proteins was determined by immunoblot analysis probed with an anti-dehydrin antibody in cultivars grown at lower temperatures (9 and 4°C) (Vítámvás *et al.,* 2021). During freeze stress in wheat, in addition to *Wcs 120,* two other COR genes (*Wcor 15* and *WCOR 39*) were also expressed and their expression increased with the application of ABA (Zhang *et al.,* 2020). Pretreatment of seedlings with salicylic acid enhanced wheat freezing tolerance by upregulating the expression of both ABA-dependent (*ABI5* and *RAB17)* and ABA-independent cold-responsive genes (*CBF3*, *COR14*, *CS120)* (Wang *et al.*, 2018). At 3 and 11 weeks of cold treatment, the *cor14b*, CS120 and Wdhn13 genes tended to be more highly expressed in freezing-tolerant RILs than in freezing-susceptible RILs, suggesting that they are important for freezing tolerance (Kruse *et al.*, 2020). It has been established that in addition to cold, incident light also has a crucial role in the cold acclimation process. In barley, the expression of the *HvCBF14* gene and two well-characterized members of the C-repeat binding factor (CBF)-regulon *HvCOR14b* and *HvDHN5* increased at 5°C. Complementary far-red (FR) illumination induced the expression of *HvCBF14* and its target gene *HvCOR14b* at both temperatures. However, this supplementation did not significantly affect the expression of *HvDHN5* (Ahres *et al.*, 2020).

2.7 Inheritance pattern for cold stress tolerance

As already mentioned that cold tolerance is a crucial trait in wheat breeding, as it plays a vital role in ensuring stable and high wheat yields,

especially in regions with harsh winter climates. The ability of wheat plants to tolerate cold temperatures is determined by multiple genetic and environmental factors. The inheritance pattern of cold tolerance in wheat is complex and it involves both dominant and recessive genes, as well as multiple genes controlling the trait. To determine the inheritance pattern of cold tolerance in wheat, researchers have performed genetic crosses between plants with known cold tolerance levels, followed by phenotypic analysis of the resulting offspring. Limin and Fowler (1993) studied the inheritance of cold hardiness in bread wheat × synthetic hexaploid wheat crosses. Synthetic hexaploid wheat, produced by combining tetraploid wheat (AB genome) with *Triticum tauschii* (D genome), was crossed to modern hexaploid wheat (*Triticum aestivum* ABD genome) in an attempt to introduce new cold hardiness genes into the common hexaploid wheat gene pool. The cold hardiness of hybrids ranged from similar to parental means equal to the hardy parent, indicating that cold hardiness was controlled by both additive and dominant genes. As expected when dominant gene action is involved, differences between F_2 and parental means were smaller than comparable differences in the F_1. Frequency distributions of F_2-derived F_3 lines also suggested that dominant genes were involved in the control of cold hardiness in some crosses. Heritability estimates for cold hardiness ranged from 63 to 70 % indicating that selection for cold hardiness should be effective in populations arising from crosses between common and synthetic hexaploid wheat. However, in this study high selection pressure on the progeny of crosses that included the hardiest *T. aestivum, T. durum* and *T. tauschii* accessions as parents did not identify transgressive segregates for improved cold hardiness. In another study, Skinner *et al*. (2008) investigated the inheritance of freezing tolerance in F_2-derived F_4 populations from all possible crosses of winter wheat cultivars ‘Kestrel’, ‘Eltan’, ‘Tiber’, ‘Froid’ and germplasm line Oregon Feed Wheat (ORFW). When frozen to a temperature equal to the LT_{50} of the least freezing tolerant parent (ORFW), survival frequency distributions were skewed to greater survival in six of the 10 crosses, however, very few of the progeny from the four

crosses to ORFW survived. The inheritance of this freezing sensitivity was investigated with freezing of $F_{2:4}$ populations from the crosses of ORFW to 'Eltan' or 'Tiber' to the LT_{50} of the hardier parent. Very few of the $F_{2:4}$ populations survived as well as 'Eltan' or 'Tiber', indicating a small number of strongly dominant genetic factors in ORFW that conditioned freezing sensitivity. Molecular analysis indicated these factors were not spring-type vernalization alleles. Yet in another study, Asl *et al*. (2013) determined the mode of gene action and inheritance of cold tolerance genes (LT50) in winter × spring cross of bread wheat. In this study 'Mironovskaya' as tolerant parent (winter habit) was crossed with 'Pishtaz' as sensitive parent (spring habit) and F_1, F_2, BC_1 (back crossed to spring parent), BC_2 (back crossed to winter parent) and F2 generations were evaluated for freezing test. In this study it clarified that cold tolerance in wheat is controlled by additive, dominant and also epistatic genes. Overall, the number of genes related to cold tolerance in wheat was estimated between1 to 6. According to different methods, the broad sense (h^2BS) and narrow sense heritabilities were 0.89 and 0.61, respectively. The h^2BS shows that transmission of cold tolerance trait from tolerant to sensitive cultivars in breeding programs is possible. These studies provided valuable insights into the complex inheritance pattern of cold tolerance in wheat and highlight the need for continued research to develop effective breeding strategies for improving cold tolerance in this important crop.

Chapter- 3

MATERIAL AND METHODS

The present study entitled "**Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas"** was conducted during the year 2020-2023. The details of materials used and techniques adopted during the course of investigation are described in this chapter.

3.1 Evaluation of wheat germplasm for cold tolerance under field conditions

3.1.1 Location and experimental site

The screening of wheat core-set (4560 genotypes) for seedling cold tolerance was done under natural conditions in the field of the Division of Genetics and Plant Breeding, Faculty of Agriculture, SKUAST-Kashmir, Wadura, campus, Sopore, Kashmir, India. The experimental site is located between 33-37˚N latitude and 72-80 33-37˚E longitude and has altitude of 1584 m above mean sea level. On the other hand, the evaluation of diverse mini-core set which comprises of 350 genotypes for cold tolerance was done at Molecular Biology, Laboratory, Division of Genetics and Plant Breeding, Faculty of Agriculture, SKUAST-Kashmir, Wadura, campus, Sopore.

3.1.2 Planting/seed material

A core set of 4560 genotypes, which included the NBPGR core set, IIWBR core set, USDA core set, IC set, EC set, Mexican set, Iranian set, Indian released varieties set and Borlaug Institute for South Asia (BISA) genomic selection genotypes, was used for the evaluation of cold tolerance.

3.1.3 Experimental set up

All the 4560 genotypes were evaluated in the Augmented Block Design (ABD) at the research fields of the Division of Genetics and Plant Breeding,

Faculty of Agriculture, SKUAST-Kashmir Wadura, Sopore during the year 2020-21 and year 2021-22 with 4 checks (SKUA_52, SKUA_118, SKUA_4301, SKUA_1701) (Plate I). These checks were strategically chosen based on their known cold tolerance characteristics: SKUA_52 and SKUA_118, identified as cold-tolerant genotypes through our previous studies, served as benchmarks for superior cold resilience. In contrast, SKUA_4301 and SKUA_1701, identified as cold-susceptible genotypes, were included to provide a comparative context.

On the basis of field-based cold screening data of all 4560 genotypes, a mini-core set comprising of diverse 350 genotypes, which showed consistent performance over two years were selected. In October 2023, 350 genotypes were sown in an ABD design and then subjected to cold stress under natural conditions. Each genotype was sown in one row of 1m in length; 20 cm spacing between the rows and 10 cm spacing was kept between the plants. Plots were kept free from weeds, diseases, insects/pests throughout the cropping season. Standard agronomic practices were followed for normal crop growth during the crop cycle. The data was recorded on three random plants from each genotype.

3.1.4 Data recording

In order to study the magnitude of genetic variability and level of genetic divergence in the core set and mini-core set, the above material was sown in field in the month of October for two consecutive years to subject the germplasm to cold stress in the fourth coming winter i.e, from the month of November to March. The data on cold stress tolerance under natural conditions in the field was recorded using the following scale (0-4) (Zhao *et al.,* 2019) (Plate II).

0 = leaf has no frozen parts

1 = only tips of leaves frozen, with older leaves rarely or not frozen at all and the overall field is predominantly green.

2 = majority of leaf areas, especially of young leaves, are unfrozen and dead yellow leaves are laying on the ground.

Plate I: Overview of wheat germplasm including 4560 genotypes grown at Faculty of Agriculture, SKUAST-Kashmir, Sopore.

3 = majority of leaf areas are frozen and whole dead leaves constituting the bulk of intact leaves visible on the ground

4 = leaf frozen and dead, or whole plant dead.

The impact of low temperature on the membrane integrity of the whole plant of the whole mini-core set (350 genotypes) was determined by measuring the electrolyte leakage from damaged leaves using the protocol of (Nejadsadeghi *et al.*, 2014; Mir *et al.*, 2021). One hundred milligrams of fresh mass (FM) of leaf fragments were cut into two pieces and then deposited in glass tubes containing 10 ml of distilled water. The tubes were sealed and shaken at 250 rpm for 90 minutes. At 25℃, the electrical conductivity (μS^{-1}) of an extract containing released ions was measured (L1) using a digital Electrical Conductivity (EC) meter (Thermofisher ECtestr11+). In the second step, the tubes and their contents were placed in a boiling water bath for 10 minutes, followed by 30 minutes of stirring and their electrical conductivity was measured (L2). The electrolyte leakage index (ELI) (%) was calculated using the following formula:

$$ELI = L2/L1 * 100,$$

Where L1 is the electrolyte leakage of the cold stressed plants grown under natural conditions (field) and L2 is the same sample's electrical conductivity after boiling. The ELI represents the leakage of electrolytes from damaged plant tissues as the percentage of the leakage from tissues completely destroyed after boiling (100%).

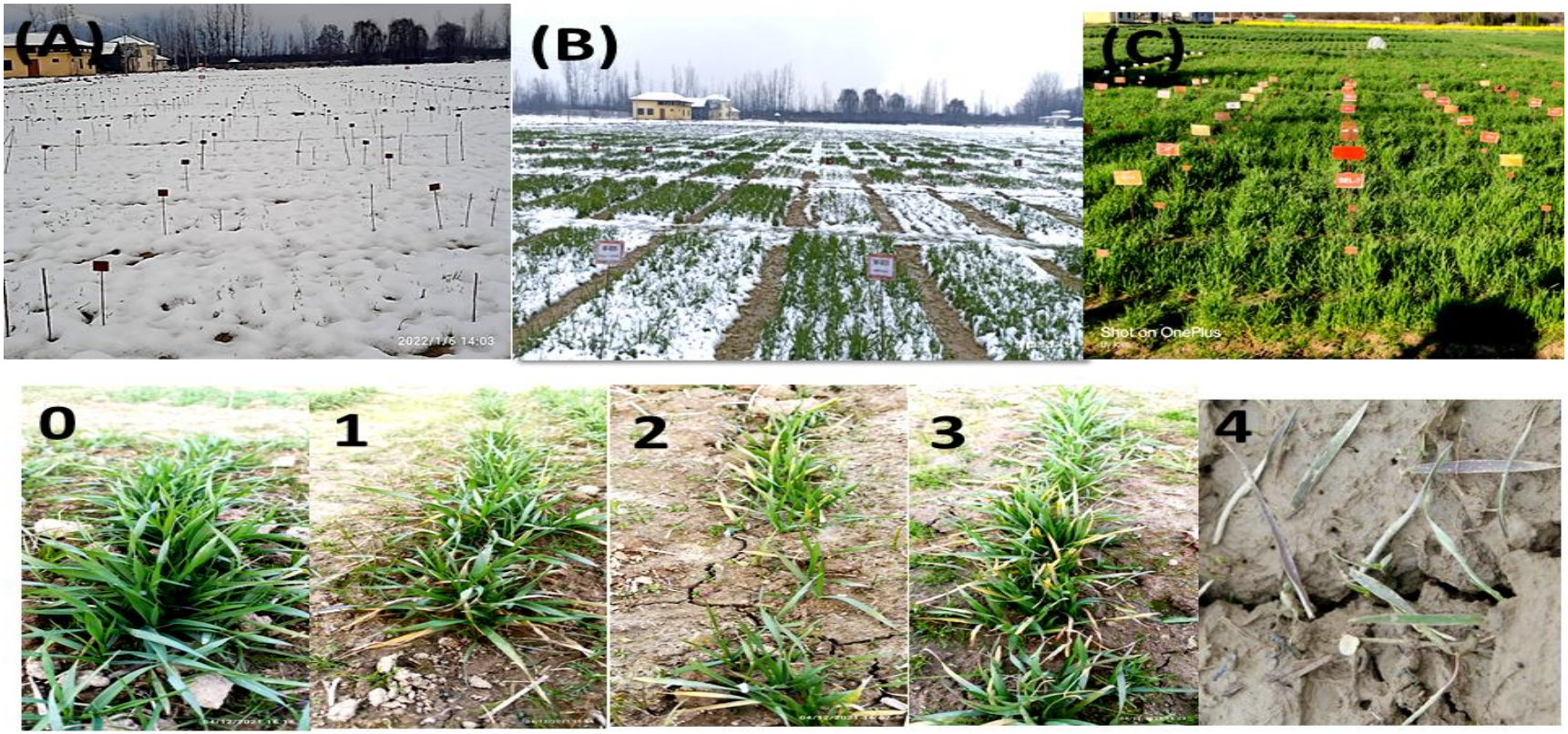


Plate II: **Overview of wheat field at Faculty of Agriculture (FoA), Wadura. The figure shows wheat germplasm (4560 wheat genotypes) under snow during winters subjected to cold/freezing stress (A and B) followed by its regrowth in summer (C). The figure also shows representative pics of the 0-4 scale (D) of (Zhao *et al.*, 2019) used during the present study for screening wheat germplasm for cold tolerance. In the scale, O=No cold injury; 1= only tips injured; 2 majority of old leaves damaged; 3 = majority of leaves dead and fallen on ground and 4= whole plant dead**

3.1.5 Statistical analysis

The cold screening data collected during the present study under in vivo conditions was analyzed using the R-software v.5.0. Frequency distribution analysis was performed to examine the distribution patterns and characteristics of the data. Furthermore, an augmented block design (ABD) analysis was conducted to assess the effect of cold stress on ELI levels. Subsequently, the mean values of ELI among the different cold tolerance groups were calculated using one-way ANOVA in the R software v.5.0 (https://www.r-project.org/). This statistical analysis allowed for the comparison of mean ELI values between the groups, providing insights into the variations in cold tolerance levels among the genotypes. Posthoc tests, such as Tukey's Honestly Significant Difference (HSD) test, were conducted to determine significant differences between specific groups.

3.2 Biochemical analysis of diverse wheat genotypes in response to cold stress under controlled condition

3.2.1 Location and experimental site

The evaluation of 50 diverse genotypes for various biochemicals induced by cold stress was done at Molecular Biology Laboratory of the Division of Genetics and Plant Breeding, Faculty of Agriculture, SKUAST-Kashmir, Wadura, campus, Sopore, Kashmir.

3.2.2 Planting material and experimental set up

Based on field screening and membrane stability assessed by electrolyte leakage, we selected 50 diverse genotypes across the cold screening scale. Seeds of these genotypes were sown in pots containing a mixture of soil, sand and farmyard manure. The plants were grown in a controlled growth chamber under specific conditions: a temperature of 25 ℃, an irradiance of 200 µmol m^2 s^{-1} from white light luminescent lamps, a 16/8 h light/dark photoperiod and 75% relative humidity for 14 days. Following this, the plants were exposed to an acclimation temperature of 4-5 ℃ for 14 days, maintaining the same photoperiod and

irradiance. Leaf samples were collected after 14 days under these acclimated conditions. Subsequently, the plants were transferred to a climatic chamber with a preliminary chilling temperature of 0℃. The temperature was gradually decreased to -5 ℃ at a rate of 0.5 ℃ per minute and the plants were incubated at this temperature for 24 hours. Non-acclimated plants were directly exposed to -5 ℃. The control group remained at 25°C under normal conditions. Physiological analyses were conducted on leaf samples harvested immediately after removing the plants from the cold exposure room. Measurements were taken from the middle parts of the first leaves of wheat seedlings in two replications (Nazari *et al.*, 2012).

In the course of conducting biochemical assays in this study, a variety of buffers and solutions were utilized to ensure precision and accuracy in measurement and analysis. Comprehensive details regarding the composition and preparation of these buffers and solutions are available in (Table 3.1 and Table 3.2). The adoption of standardized protocols was instrumental in maintaining consistency and reproducibility throughout the experimental procedures.

Table 3.1: List of the chemicals used in the biochemical assays during the current study

Chemical	Formula	Grade
Trichloroacetic acid (TCA)	C_2HCl_3	Analytical Grade
Thiobarbituric acid (TBA)	$C_{11}H_{10}N_2O_2S$	Analytical Grade
Potassium iodide	KI	Analytical Grade
Potassium phosphate buffer	K_2HPO_4/KH_2PO_4	Analytical Grade
Sulphosalicylic acid	$C_7H_6O_5S$	Analytical Grade
Ninhydrin	$C_9H_6O_4$	Analytical Grade
Acetic acid	CH_3COOH	Analytical Grade
Toluene	C_7H_8	Analytical Grade
Ascorbic acid	$C_6H_8O_6$	Analytical Grade
Hydrogen peroxide (H2O2)	H_2O_2	Analytical Grade
Guaiacol	$C_7H_8O_2$	Analytical Grade
EDTA (Ethylenediaminetetraacetic acid)	$C_{10}H_{16}N_2O_8$	Analytical Grade
Trisodium citrate	$C_6H_5Na_3O_7$	Analytical Grade
Methionine	$C_5H_{11}NO_2S$	Analytical Grade
NBT (Nitroblue tetrazolium)	$C_{40}H_{32}C_{l2}N_{10}O6$	Analytical Grade
Triton X-100	$C_{14}H_{22}O(C_2H_4O)n$	Analytical Grade
Riboflavin	$C_{17}H_{20}N_4O_6$	Analytical Grade

Table 3.2: List of buffers and solutions along with their method of preparation, used in the current study

TCA Extraction Buffer (1%)	Dissolve 1 g of Trichloroacetic acid (TCA) in 100 ml of water. Stir until fully dissolved.
TBA Solution (5% in 20% TCA)	Dissolve 5 g of Thiobarbituric acid (TBA) in 100 ml of 20% Trichloroacetic acid (TCA). Ensure complete dissolution.
0.1% TCA Solution	Dissolve 0.1 g of Trichloroacetic acid (TCA) in 100 ml of water. Stir until fully dissolved.
1 M Potassium Phosphate Buffer (pH 7.0)	Weigh and dissolve 136.1 g of Potassium dihydrogen phosphate (KH_2PO_4) and 174.2 g of Dipotassium hydrogen phosphate (K_2HPO_4) in distilled water. Mix in a 68:32 ratio to obtain a 1 M buffer at pH 7.0. Check and adjust the pH. Make the final volume to 100 ml with distilled water. Sterilize if required.
Ninhydrin Solution	Dissolve 1.25 g of ninhydrin in 30 ml of glacial acetic acid. Heat at 20-30°C, then add 20 ml of 6M orthophosphoric acid and store at 4°C for 24 hours.
0.5M EDTA Solution	Dissolve the 186.1 g Ethylenediaminetetraacetic acid (EDTA) in 1000 ml of distilled water. Adjust the pH of the solution to about 8-9 by adding small amounts of sodium hydroxide (NaOH).
3mM Ascorbic acid solution	Dissolve 0.0053 g of ascorbic acid in 1000 ml of distilled water.
96mM Guaicol (100 ml)	Dissolve 1.195 ml of guaicol in 200 ml of distilled water and finally make up the volume upto 100 ml.
75mM H_2O_2 (100 ml)	Dissolve 0.85 ml of 30% H_2O_2 in 100 ml of distilled water. Make the 3mM, 12mM from 75mM by dilution.
2.25 mM NBT (50 ml)	Dissolve 0.092 g of NBT in 50 ml distilled water. Stir until fully dissolved.
60 mM of Riboflavin	Dissolve 0.047 g of riboflavin in 25 ml of 0.1N NaoH, take 0.3 ml of it and dilute it with 25 ml of distilled water.
200 mM of Methionine	Dissolve 1.492 g of L-Methionine in 50 ml of distilled water.

3.2.3 Lipid peroxidation (MDA) analysis

Thiobarbituric acid (TBA) testing, which identifies MDA as a byproduct of lipid peroxidation, was used to measure lipid peroxidation in leaves (Heath *et al.*, 1968). Fresh mass (FM) leaflets (250 mg) were homogenized in 2ml of trichloroacetic acid (TCA) extraction buffer at 1% (w/v) before being centrifuged at 13,000 × g for 15 min. Then, 1 ml of the supernatant was added to 2ml of 5% (w/v) TBA in 20% (w/v) TCA. After 30 minutes of incubation in boiling water, the samples were placed in an ice bath to cease the process. The samples were then centrifuged at 10,000 × g for 10 min and a spectrophotometer was used to determine the absorbance of the supernatants at 532 and 600nm. OD600 values were subtracted from the MDA-TBA complex values at 532 nm. The MDA concentration was determined using the formula C = D/E× L, where L is the thickness of the layer of solution in the vessel (1 cm), E is the coefficient of molar extinction (1.56×10^5 cm^{-1} M^{-1}) and C is the concentration of MDA. µMol g^{-1} FM was used to express the amount of MDA.

3.2.4 Estimation of the hydrogen peroxide (H_2O_2) content

The amount of H_2O_2 was determined based on (Loreto and Velikova, 2001). FM (0.20 g) of leaf fragments was homogenized with 5 ml of 0.1% TCA in an ice bath after being pulverized in liquid nitrogen with a mortar and pestle. After centrifuging the homogenate at 12,000 × g for 15 min, 0.5 ml of the supernatant was added to 1 ml of 1 M potassium iodide and 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0). Using a spectrophotometer, the absorbance of the supernatant was determined at 390 nm. The amount of H_2O_2 was determined through comparison to a standard calibration curve that had previously been created using various H_2O_2 concentrations and was expressed in µmol g^{-1} FM.

3.2.5 Proline content

The proline content was measured according to Bates *et al.* (1973). Sulphosalicylic acid was used to homogenize samples (0.2 g leaflets), which were then filtered via filter paper. As soon as the acid ninhydrin and glacial acetic acid were added, the mixture was heated at 100°C for 1 hour in a water bath. After that, an ice bath prevented the reaction. Toluene was used to extract the mixture and at 520 nm, the absorbance of the toluene-aspirated fraction from the liquid phase was measured. Using a calibration curve, the concentration of proline was calculated and expressed as µmol g^{-1} FM.

3.2.6 Soluble protein content and antioxidant enzyme activity

According to Bradford (1976), the amount of total soluble protein was calculated and expressed in mg ml^{-1} protein. Enzyme extract for ascorbate peroxidase, Guaiacol peroxidase and Catalase was prepared by crushing 0.1g of fresh leaf tissue with 2ml of ice-cold 0.1M Potassium Phosphate Buffer (pH= 7.5 containing 0.5mM EDTA and 5% PvPP). The homogenate was centrifuged for 20 min at 15000 rpm at 4^0C and the supernatant was used as crude enzyme extract for enzyme activity assay.

3.2.6.1 APX activity

The reaction mixture contained: 1.5 ml potassium phosphate buffer, 0.5 ml ascorbic acid, 0.1 ml EDTA, 0.1 ml Hydrogen peroxide, 100 microliters of enzyme extract and 0.7 ml distilled water. The reaction was started with addition of 0.1 ml Hydrogen peroxide. The H_2O_2 dependent oxidation of Ascorbic acid is measured by decrease in absorbance at 290 nm for 3 minutes at the interval of 30 seconds in UV- visible spectrophotometer. The amount of ascorbate oxidized protein per minute per µMol of APX activity was expressed.

3.2.6.2 Catalase activity (CAT)

Catalase activity was measured immediately in fresh extract and the assay employed as described by Scebba *et al.* (1973). The reaction mixture contained

1.5 ml phosphate buffer, 0.5 ml H_2O_2, 200 µl enzyme extract and 1 ml distilled water. For the reaction to start H_2O_2 was added. For measurement of Catalase Activity, decline in absorbance at 240 nm was recorded for 3 minutes at the interval of 30 seconds. Assuming an extinction coefficient of 39.4 cm^{-1} mM^{-1}, CAT specific activity was expressed in µM of H_2O_2 decomposed min^{-1} mg^{-1} protein.

3.2.6.3 Guaiacol peroxidase activity (GPX)

The reaction mixture contains 1.5 ml potassium phosphate buffer, 0.5 ml guaiacol, 0.5 ml Hydrogen peroxide, 100 microliters of enzyme extract and 0.4 ml of distilled water. Addition of H_2O_2 started reaction. For measurement of guaiacol peroxidation, increase in absorbance at 480 nm was recorded for 3 minutes at the interval of 30 seconds. Assuming an extinction coefficient of 26.6 cm^{-1} mM^{-1}, GPX specific activity was expressed as µM of guaiacol oxidized min^{-1} mg^{-1} protein.

3.2.6.4 Superoxide dismutase (SOD) activity

SOD was determined according to the protocol of Beyer and Fridovich (1987). Phosphate buffer 100 mM (pH 7.8), EDTA 0.1 mM, methionine 12 mM, NBT 75 µM and triton ×100 0.025% (v/v) made up the reaction medium. The reaction medium was supplemented with 100 µl of crude enzyme extraction and 1 µl of riboflavin buffer. After 20 minutes of illumination at 25 ℃, turning off the lights put an end to the reaction. Blanks were non-illuminated solutions with minimal enzyme extraction. At 560 nm, the reaction was observed. The following equation was used to determine the activity: SOD (U ml^{-1}) = (P ×1,000)/(50 × mg protein) and P = (V - v/v) × 1,000, where V is the rate of reaction in the absence of the enzyme and v is the rate of reaction in its presence. P represents the percentage of prohibition. The specific activity of SOD was represented as U min^{-1} mg^{-1} protein for each sample and the activity of SOD was expressed in relation to the volume (ml) of enzyme extraction corresponding to a 50% prohibition rate.

3.2.6.5 Statistical analysis

Analysis of variance (ANOVA) was used to calculate the mean differences between cold stress levels and genotypes and their interaction at 0.05 and 0.01 significance levels in R-software. Pearson's correlation coefficient was assessed to find the linear relation between various biochemical parameters in R-software v.5.0. The differentiated genotypes were selected based on principal component analysis (PCA) and radar analysis. For this purpose, Origin Pro software was used. The statistically significant PCs were selected using eigenvalue standards as established by Kaiser (1960).

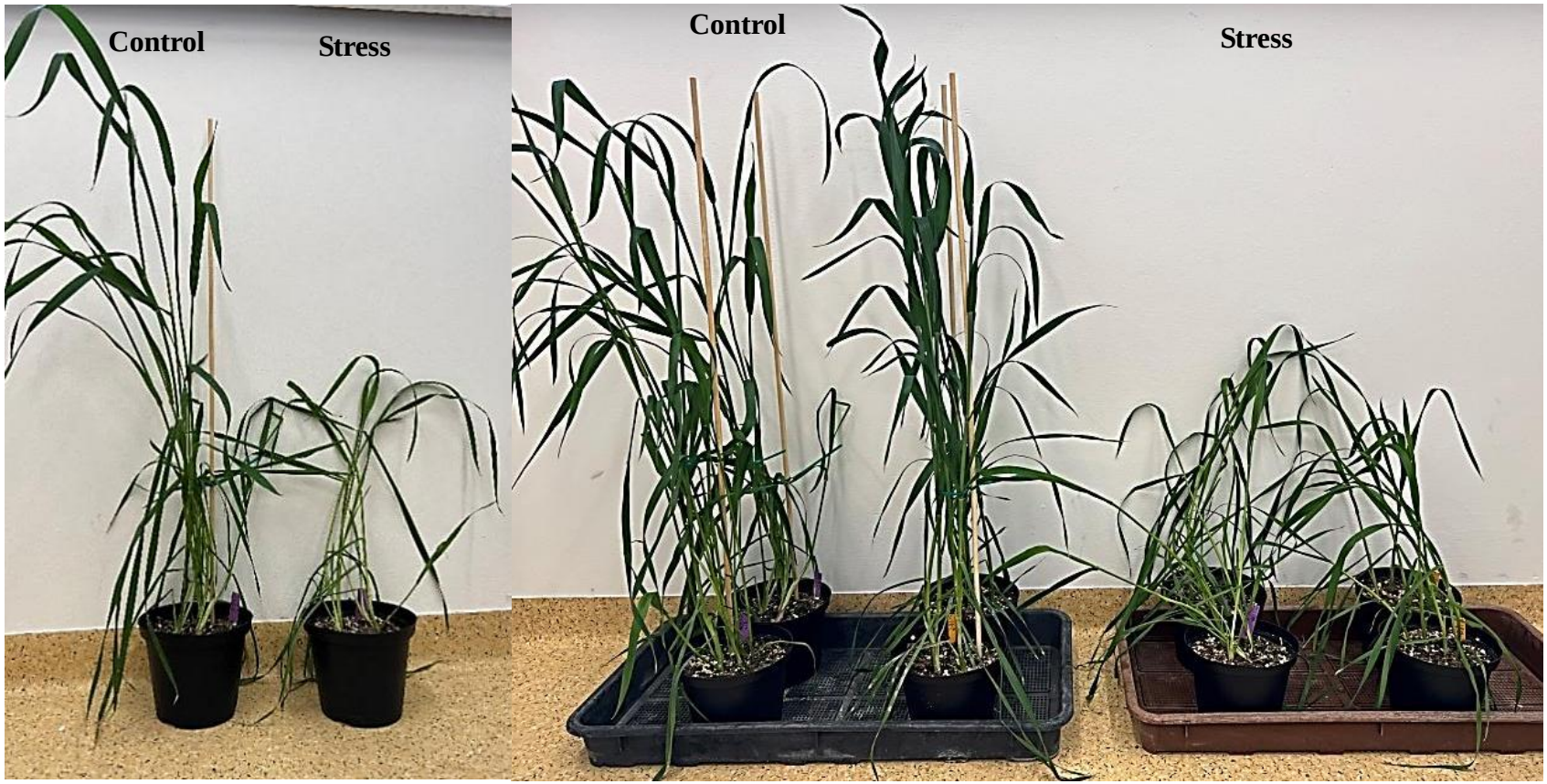
3.3 Comparative metabolome and lipidome profiling in contrasting wheat genotypes

3.3.1 Location and experimental site

The experiments were conducted at the National Institute of Plant Genome Research (NIPGR) in New Delhi. The research facility at NIPGR facilitated the study by providing the essential equipment, including gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). However, the plants used in this study were raised at Sher-e-Kashmir University of Agricultural Sciences and Technology (SKUAST), FoA, Wadura, Sopore, Kashmir, India. The controlled growth chamber facilities at SKUAST-K provided suitable environmental conditions for plant cultivation.

3.3.2 Planting material and experimental setup

Two genotypes, one cold tolerant (SKUA_52) and one susceptible (SKUA_4301) to cold stress, were selected based on the results of the field and biochemical screenings (Plate III). The plants were grown in individual pots under controlled growth conditions (25°C) at SKUAST-K, FoA, Wadura. The selected genotypes were replicated in two sets, each consisting of two biological replicates. One set was designated as the control group (25°C), while the other set was subjected to cold shock treatment at -5 °C after 14 days of cold acclimation at



(a) SKAU_52 (Tolerant) **(b) SKAU_4301 (Susceptible)**

Plate III: **Morphological comparison of (a) SKAU_52 (cold-tolerant) and (b) SKAU_4301 (cold-susceptible) wheat genotypes under normal conditions and after -5°C cold stress**

4°C. At the 3-leaf stage, the plants in the treatment group after acclimation phase were exposed to a controlled temperature of -5 °C for 24 hours to induce cold stress. The control group was maintained under normal growth conditions without any cold shock treatment. Following the cold shock treatment, leaves from both the control and stress groups were carefully harvested. The harvested leaves were immediately lyophilized to remove moisture content. Approximately 20 mg of lyophilized leaf tissue was used for defense phytohormones, flavonoids and untargeted analysis.

3.3.3 Methodology for analysis of defense hormones

The metabolomic profiling of defense hormones was performed for Jasmonic acid (JA), cis-12-oxo-phytodienoic acid (cis-OPDA), jasmonyl-isoleucine (JA-Ile), salicylic acid (SA) and abscisic acid (ABA) using liquid chromatography-mass spectrometry (LC-MS) using the following procedure:

1. Internal standard (IS) was prepared by mixing internal standard mix which contains known concentrations of stable isotope-labeled internal standards for each defense and methanol in 4:1 ratio.
2. An external solvent master mix was prepared by mixing internal standard (IS) and methanol in 4:1 ratio.
3. Lyophilized leaf tissue (25mg) was transferred to a clean and labeled microcentrifuge tube and 1 ml of external solvent master mix was added.
4. The samples were vortexed for 2 minutes to ensure proper mixing of the sample and extraction solvent.
5. The samples were shaken at 4°C for 30 minutes in tube rotator ensuring rotation end to end.
6. The samples were centrifuged at 12000×g at 4°C for 15 minutes.

7. 800µl of supernatant was transferred to a new 2ml eppendorf tube on ice.
8. The pellets were re-extracted by adding 500 µl methanol without IS. After vortexing for 2 minutes, the samples were shaken at 4°C for 10 minutes and centrifuged at 12000×g at 4°C for 20 minutes.
9. The supernatant was transferred and mixed with the old supernatant obtained in step 7.
10. The supernatant was dried in speed vaccum rotator in V-AL mode.
11. After allowing the samples to dry, 500 µl of methanol without IS was added. The samples were vortexed for 2 minutes.
12. The samples were centrifuged at 16000×g at 4°C for 5 minutes.
13. 400 µl of clear supernatant was transferred to HPLC vials.
14. The liquid chromatography (HPLC) system was set up coupled with a mass spectrometer for the analysis of defense hormones.
15. The mass spectrometer in multiple reaction monitoring (MRM) mode was set up, utilizing specific precursor-product ion transitions for each defense hormone and its corresponding internal standard.
16. LC-MS data for the samples was acquired, recording the peak areas or peak heights for the target analytes and their internal standards.

3.3.4 Methodology for flavonoid analysis

For the analysis of flavonoids, a similar LC-MS approach was employed. The lyophilized leaf tissue samples were extracted using appropriate solvents. The resulting extracts were analysed using LC-MS to determine the presence and levels of flavonoid compounds using the following procedure.

1. 25 mg of lyophilized tissue powder was taken.
2. 1000µl of 80% methanol: LCMS water extraction solvent was added.

3. Samples were vortexed for 10 minutes to ensure proper mixing of sample and extraction solvent.
4. The samples were sonicated for 20 minutes at room temperature in a sonicator.
5. The samples were centrifuged at 8000 rpm at 4˚C for 10 minutes.
6. The extract was filtered through 0.22 nm filter (Millipore) before separation and quantification of flavonoids using a LC-MS.
7. The liquid chromatography (HPLC) system was set up coupled with a mass spectrometer for the analysis of flavonoids.
8. An aliquot of the extracted supernatant (e.g., 5µL) was injected into the HPLC system for separation. A suitable reversed-phase column with appropriate mobile phase conditions was utilized to achieve optimal separation of the target flavonoids.
9. Mass spectrometer operating was used in the appropriate ionization mode (e.g., electrospray ionization, ESI) to ionize and detect the flavonoids.
10. The LC-MS data for the samples was acquired by recording the peak areas or peak heights for the target flavonoids.

3.3.5 Quantification and LC-MS data analysis for defense phytohormones and flavonoid

1. A calibration curve was generated by using a series of standard solutions containing known concentrations of each defense hormone. The calibration curve was obtained using the same extraction and analysis procedure as for the samples.
2. The concentration of each defense hormone in the samples was quantified by comparing the peak areas or peak heights of the analytes to the corresponding calibration curve.

3. The concentrations of the defense hormones to the internal standard concentrations was normalized to correct for any variations in extraction efficiency or instrument response.
4. Statistical analysis, such as t-tests was performed to determine significant differences in defense hormone levels between the control and stress groups in MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/MetaboAnalyst/).

3.3.6 Methodology for untargeted metabolomics analysis (GC-MS)

The untargeted metabolomics analysis was performed using gas chromatography-mass spectrometry (GC-MS). The lyophilized leaf tissue samples were subjected to derivatization, followed by GC-MS analysis to identify and quantify a wide range of metabolites using a following protocol:

1. An extraction buffer was prepared by mixing methanol and ribotol in 480:20 ratio.
2. 25 mg of lyophilized leaf tissue was taken and transferred to a clean and labeled 2ml eppendorf tube.
3. 500 µl of extraction solvent was added to the tube. The tubes were vortexed vigorously for a few seconds to ensure proper mixing of the sample and extraction solvent.
4. The samples were incubated in an incubator at 70˚C for 15 minutes.
5. 500µl of LC-grade water was added to each tube and then 250 µl of chloroform was added and mixed thoroughly followed by vortexing the samples for 2 minutes.
6. The samples were centrifuged at 2200g (rcf) for 10 minutes at 25˚C.
7. The supernatant was taken and dried in speed vaccum rotator (eppendorf concentrator plus) at 35˚C for 3 hours.

8. The dried fraction in 40µl of methoxaime hydrochloride pyrimidine was re-dissolved and incubated the samples for 90 minutes at 37°C.
9. 60µl of MSFTA was added to each sample and again incubated at 37°C for 30 minutes.
10. 100µl of derivatized samples was transferred in an insert containing GC-MS glass vial.
11. A gas chromatography-mass spectrometry (GC-MS) system was set up in full-scan mode to acquire the mass spectra of all compounds present in the sample.
12. The GC-MS data for the samples was acquired by recording the retention times and mass spectra of the metabolites.

3.3.6.1 Data processing and analysis of untargeted metabolites

1. The acquired GC-MS data was imported into appropriate data processing software, such as MetaboliteDetector.
2. Baseline correction, peak detection, deconvolution, alignment and normalization steps were performed to preprocess the data.
3. Metabolite identification was performed by comparing the obtained mass spectra with reference libraries, such as the NIST library.
4. Statistical analysis, such as t-test, multivariate analysis (e.g., principal component analysis, PCA), was used to identify metabolites showing significant differences between the control and stress groups in MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/MetaboAnalyst/).

3.3.7 Methodology for lipidomics analysis (GC-MS)

The lipidomics analysis was performed following the protocol of (Matyash *et al.,* 2008), using gas chromatography-mass spectrometry (GC-MS). The lyophilized leaf tissue samples were subjected to derivatization, followed by GC-

MS analysis to identify and quantify a wide range of lipids using a following protocol:

1. Lypohillised leaf tissue (~300 mg) was ground into a fine powder.
2. Grinding was done in a 5 mL polypropylene vial containing a tungsten bead.
3. A solution of cold methyl tert-butyl ether, methanol and water in a 6:3:1 ratio (by volume) was prepared.
4. 3 mL of this solvent mixture was added to the vial containing the leaf tissue.
5. The mixture was shaken for one hour at 4°C.
6. Subsequently, 750 µL of water was added to induce phase separation.
7. The vials were vortexed again and centrifuged for 15 minutes at 4°C.
8. Centrifugation resulted in phase separation, with the upper organic layer containing the lipids.
9. Gas Chromatography-Mass Spectrometry (GC-MS) was used for lipid profiling.

3.3.7.1 Data processing and analysis of untargeted metabolites

1. Analysis was conducted using a QP2010 SE GC–MS (Shimadzu, Kyoto, Japan).
2. An Rxi-5 MS capillary column (30 m × 0.25 mm with a 0.25 µm 5% diphenyl 95% dimethyl polysiloxane phase; Restek Corporation) was employed. Helium served as the carrier gas.
3. The GC injector temperature was maintained at 250°C.
4. The split ratio was set at 1:5 with a helium flow rate of 1.00 mL/min.
5. The initial GC column temperature was set to the boiling point of each

solvent for 1 min, then ramped to 300°C at 5°C/min with a 5 min hold at 300°C.

6. Mass spectrometry operated in the electron impact ionization mode at 70 eV and 0.1 kV detector voltage.
7. The ion source and MS interface temperatures were maintained at 200 and 250°C, respectively.
8. Mass spectra were obtained in the full scan mode, covering the mass range of 40–500 amu, scanned at a rate of 3,333 scans/s.
9. Peaks were identified and verified by comparison with a library of mass spectra (NIST).

3.4 Characterization and differential gene expression study of cold tolerance genes using qRT-PCR

3.4.1 Location and planting material

The two genotypes *viz,* SKUA_52 and SKUA_4301 selected for gene expression analysis were chosen based on their contrasting cold tolerance performance as determined through earlier field screening and biochemical screening. SKUA_52 demonstrated a high level of tolerance to cold stress, whereas the SKUA_4301, a susceptible genotype displayed a low level of tolerance to cold stress. These genotypes were identified from a pool of screened genotypes, consisting of diverse accessions, through rigorous evaluation of their cold tolerance traits and biochemical responses. The selection of these specific genotypes allowed for a focused investigation into the differential gene expression patterns associated with their contrasting cold tolerance phenotypes. The experiment of differential gene expression was conducted at the Chaudary Charan Singh Agricultural University (CCSAU), Hisar, Haryana.

3.4.2 Experimental set up

Seeds of the selected genotypes were planted in pots filled with a mixture of soil, sand and farmyard manure, following a completely randomized design (CRD). The experiment consisted of four biological replicates and within each replicate, there were three technical replicates per pot. The biological replicates were divided into two groups: the Control group and the Treated group. The plants were grown in a controlled growth chamber with specific conditions, including a temperature of 25 ℃, an irradiance of 200 µmol m^{-2} s^{-1} from white light luminescent lamps, a 16/8 h light/dark photoperiod and 75% relative humidity. This growth environment was maintained for 14 days. At the three-leaf stage, the seedlings in the Treated group were transferred to a low temperature acclimation chamber set at 4°C for a duration of 28 days, while the Control group was maintained at 20°C for the same period. After the acclimation period, the treated group was further exposed to a cold stress treatment at -5°C for 24 hours. Following the cold stress treatment, the crown tissues from 5 to 6 seedlings were pooled together and immediately frozen in liquid nitrogen for subsequent RNA extraction.

3.4.3 RNA extraction using maxwell RSC tissue RNA kit

RNA extraction was performed using the Maxwell RSC Tissue RNA Kit. Two biological replicates were processed independently for RNA extraction using the following protocol:

1. 100 mg of plant leaves was weighed.
2. The sample was ground to fine powder with liquid nitrogen in a pre-chilled mortar and pestle.
3. The sample was homogenized uniformly into 200 µl of chilled 1-thioglycerol/ homogenization solution.
4. The solution was transferred into 1.5 ml micro centrifuge tube.

5. 300 µl of lysis buffer was added to each tube and mixed well by vortexing for 15 seconds.
6. Centrifuged at 12000rpm for 2min
7. The tissue lysate was carefully added from MCT into well 1 of Maxwell RSC
8. The plunger was put in well 8 and 5 µl of DNase I was added in 4th well.
9. The cartridge was set up into deck tray and the elution tube (0.5ml) was put in front of each cartridge.
10. The deck tray was put into Maxwell RSC machine and the Maxwell R software on PC was started.
11. After completion of run, elution tube was collected and stored at -20°C till further use.

3.4.4 RNA quantification by nanodrop spectrophotometer

1. The RNA samples to be quantified were taken in ice cold bucket.
2. The Nanodrop system was switched on and the initialization process was allowed to complete.
3. The "ssRNA" tab was selected on the screen.
4. Both sides of the pedestal were cleaned with nuclease-free water and dried using a Kimwipe.
5. lµl of elution solution was loaded as a blank and the pedestal was closed.
6. Autoblanking was initiated and after completion, 1 µl of the RNA sample was loaded.
7. After placing the pedestal down, the instrument automatically measured the readings of the RNA samples.

8. A260/A280 ratios as well as the amount of RNA recovered (in ng/µl) were displayed on the screen.
9. The pedestals were cleaned with kimwipe and steps 7-8 were repeated for the quantification of remaining RNA samples.

3.4.5 Interpretation of nanodrop results

The A260/A280 ratio of 2.0-2.2 represented high quality of RNA. The A260/A230 ratio should be above 2.0. A lower ratio indicates the contaminations with wash solutions, phenols or proteins.

3.4.6 cDNA synthesis and its validation through qRT-PCR

3.4.6.1 Removal of genomic DNA from RNA samples

1. Prior to cDNA synthesis, the genomic DNA present in the extracted RNA was removed by treating with DNase I enzyme.
2. For each sample, 1µg of RNA was taken in a nuclease free PCR tube The volume was adjusted based on the RNA concentration.
3. 1 µl of 10X buffer containing $MgC1_2$ was added.
4. To the RNA, 1 µl DNase I enzyme was added
5. The volume was made up to 10 µl using nuclease free water (NFW).
6. The solution was incubated for 30 min at 37°C to facilitate DNase I digestion.
7. To inactivate the DNase, 1µl of 50 mM Ethylenediamine tetraacetic acid (EDTA) was added and incubated for 10 min at 65°C.

3.4.6.2 cDNA synthesis

cDNA synthesis was carried out using verso cDNA synthesis kit.

1. According to the instructions of the verso cDNA synthesis kit, following reagents were added into sterile, nuclease-free pcr tubes:

2. The ingredients were mixed gently and centrifuged at 3000 rpm for 1 minute.
3. The samples were incubated at 42°C for 30 minutes.
4. The reaction was terminated at 95°C for 2 minutes.
5. cDNA was used for gene expression through qRT- PCR.

Table 3.3: List of reagents and their concentrations used in cDNA synthesis during the current study

Reagent	Concentration
RNA tempelate	1µg
5X cDNA synthesis buffer	4 µl
Dntp Mix	2 µl
RNA primer	1 µl
RT enhancer	1 µl
Verso enzyme mix	1 µl
Nuclease-free water	Upto 20 µl

3.4.7 qRT PCR procedure

cDNA from the cold treated plants and from control plants were used in the real-time PCR experiment. The relative expression of genes was performed on the Applied Biosystems QuantStudio and 7 Flex Real-Time PCR system using Applied Biosystem`s SYBR Green PCR master mix. Actin genes were used as an internal control. The expression of target and endogenous gene (actin) was performed in triplicate for each sample. The reaction mixture containing following components was used for qRT- PCR (Table 3.4).

Table-3.4: List of reagents and their concentrations used in qRT- PCR during the current study

Reagent	Concentration
SYBR Green master mix	10 µl
Cdna	1 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Nuclease free water	8 µl
Total volume	20 µl

The primers used for each gene and the length of the fragment amplified are shown in (Table 3.5) (Guo *et al.,* 2019). The threshold was determined as the first cycle, above the background fluorescence that all samples were in the exponential amplification phase. This was manually set within the exponential phase of the amplification plots for all the samples (Adams, 2006). After the real-time PCR experiment was completed, a dissociation curve was conducted in order to determine if only one product was amplified. A single peak in the dissociation curve implied that only the gene of interest was amplified. If more than one peak was present then the fluorescence values for the gene of interest would not be accurate since SYBR Green I binds to all double stranded DNA.

Table-3.5: List of cold responsive genes used for qRT-PCR during the current study

Gene ID	Gene Name	Chromosome	Forward Primer (5'-3')	Reverse Primer (3'-5')
TaCBF2d	*Triticum aestivum*_C repeat Binding factor	2D	CTCGTACATGACGGTGTCGT	GAGCCATAGCCTCTGCTTCC
TaCBF11b	*Triticum aestivum*_C repeat Binding factor	11B	TACCTCCAGCGACTTGTCTG	CTAGTAGGTCCACAGCGACG
TaCBF16d	*Triticum aestivum*_C repeat Binding factor	16D	CGTCGATGACGGAACCTGTA	AACATGTCGCAGCCTAGTGT
TaICE1d	*Triticum aestivum*_Inducer of CBF expression	1D	AAGGAGGGTTGGAGTTGCAG	CGCCTCCGACGTGAGGTA
TaICE2b	*Triticum aestivum*_Inducer of CBF expression	2B	CTGCCTCCGACACCTACAAGC	TGACGACCCGACCTTCACGAA
TaCOR3d	*Triticum aestivum*_cold responsive gene	3D	CACCGCCAAATGAAGACCG	GCATCTTTGAATACACCGGACGA
TaCOR3b	*Triticum aestivum*_cold responsive gene	3B	GGATCATAGCCCTCTTTTCGTT	CTTTGCATACACTGGACGAAG

3.4.8 Relative quantification of target genes by comparative CT method

The average threshold cycle (Ct) comparative method was used to quantify the expression of the target genes (Livak and Schmittgen, 2001). The fold expression of these genes was compared to the calibrator sample. The control samples were used as calibrator. The ΔΔCT method was used to calculate the fold differences in the expression levels of target genes transcripts among different samples.

ΔCt (treated) = Avg Ct of target - Avg Ct of endogenous gene

ΔCt (control)= Avg Ct of target - Avg Ct of endogenous gene

ΔΔC-ACt (treated) - ΔCt (control)

Fold change in expression= $2^{-\Delta\Delta Ct}$

$2^{-\Delta\Delta Ct}$ represents the number of transcripts of the target gene as compared to the calibrator. when the value of 2-AAC> 1, the target gene expression was increased, but if the value of 2-4 <1, the expression of the target gene was decreased in comparison to the calibrator.

3.5 Study of the inheritance pattern for cold tolerance in wheat

3.5.1 Location and experimental setup

The genotypes viz, SKUA_52, SKUA_4301, SKAU_44 and SKAU_1422 selected for studying the inheritance pattern were chosen based on their contrasting cold tolerance performance as determined through earlier field screening and biochemical screening. SKUA_52 and SKAU_44 demonstrated a high level of tolerance to cold stress, whereas the SKUA_4301 and SKAU_1422, a susceptible genotype displayed a low level of tolerance to cold stress. Inheritance pattern was studied at experimental research field, Division of Genetics and Plant Breeding, Faculty of Agriculture, Wadura.

3.5.2 Methodology to study inheritance pattern

1. Spikes of the genotype SKUA_4301 and SKAU_1422 were emasculated carefully using forceps, followed by pollination from SKUA_52 and SKAU_44, respectively.
2. Tags or labels were used to mark each cross, providing essential information such as parental lines, date of pollination.
3. The fertilized flowers were allowed to develop into seeds within the bags.
4. The seed development process was monitored to ensure that the bags are well-ventilated to prevent fungal or bacterial infections.
5. Seeds once matured were harvested.

3.5.2.1 F_1 generation planting

F_1 seeds obtained from the crosses were sown in the greenhouse at Division of Genetics and Plant Breeding, Faculty of Agriculture, Wadura in the month of October during the year 2022. Phenotypic traits in the F1 generation, including cold stress tolerance were observed and recorded. This evaluation provided insight into the immediate outcomes of the hybridization.

3.5.2.2 F_2 generation field planting

Seeds from the F_1 generation were collected and planted in a field. The F_2 generation experienced natural environmental conditions, facilitating the assessment of cold stress tolerance under natural conditions scenarios. Phenotypic traits in the F_2 generation, including cold stress tolerance were observed and recorded. The cold tolerant plants were scored “0” and susceptible lines were scored “4”.

Chi Square Test was applied to test the goodness of fit for Mendelian ratios, after comparing observed and calculated/ expected values for each class separately for F_2 lines.

$$\chi^2 = \frac{\Sigma(O-E)^2}{E}$$

O = the frequencies of observed

E = the frequencies of observe

$\sum$ = the sum of

To accept or reject the null hypothesis total chi-square values were compared with tabulated values at appropriate degrees of freedom. Null hypothesis was, there are no significant differences between the observed and expected chi-square values, whereas the alternate hypothesis was, there are significant differences between the observed and expected chi-square values.

3.6 Methodology for transcriptome sequencing

3.6.1 Location and planting material

The two genotypes *viz*, SKUA_52 and SKUA_4301 selected for RNA ssequencing were chosen based on their contrasting cold tolerance performance as determined through earlier field screening and biochemical screening. SKUA_52 demonstrated a high level of tolerance to cold stress, whereas the SKUA_4301, a susceptible genotype displayed a low level of tolerance to cold stress. These genotypes were identified from a pool of screened genotypes, consisting of diverse accessions, through rigorous evaluation of their cold tolerance traits and biochemical responses. The selection of these specific genotypes allowed for a focused investigation into the differential gene expression patterns associated with their contrasting cold tolerance phenotypes. The experiment of RNA sequencing was outsourced and was done at NGB Diagnostics, New Dehli.

3.6.2 Experimental set up

Seeds of the selected genotypes were planted in pots filled with a mixture of soil, sand and farmyard manure, following a completely randomized design (CRD). The experiment consisted of four biological replicates and within each replicate, there were three technical replicates per pot. The biological replicates

were divided into two groups: the Control group and the Treated group. The plants were grown in a controlled growth chamber with specific conditions, including a temperature of 25 ℃, an irradiance of 200 µmol m^{-2} s^{-1} from white light luminescent lamps, a 16/8 h light/dark photoperiod and 75% relative humidity. This growth environment was maintained for 14 days. At the three-leaf stage, the seedlings in the Treated group were transferred to a low temperature acclimation chamber set at 4°C for a duration of 28 days, while the Control group was maintained at 20°C for the same period. After the acclimation period, the treated group was further exposed to a cold stress treatment at -5°C for 24 hours. Following the cold stress treatment, the crown tissues from 5 to 6 seedlings were pooled together and immediately frozen in liquid nitrogen for subsequent RNA extraction.

3.6.3 RNA extraction, Quality check and library preparation:

Transcriptome Sequencing of the samples SKAU_52 and SKAU_4301 under control conditions and cold stress conditions was done using NovaSeq 6000 2X(150) bp Chemistry at NGB DIAGNOSTICS, Noida, India. RNA was extracted from the samples in triplicates and was checked for quality using nanodrop 2500 (Thermo Fisher Scientific) and purity, integrity by Agilent 2100. The first strand cDNA was generated using random hexamer-directed reverse transcription, followed by synthesis of second strand cDNA. cDNA quality was checked using Qubit ds DNA estimation and agarose gel. 500ng gDNA and 200ng cDNA were pooled for each sample and taken forward for library preparation with an insert size of 300-400bp using NEBNext UltraII DNA library preparation kit for Illumina; Cat no: E7770 (New England Biolabs), according to manufacturers recommended protocol followed by sequencing was on S4 flowcell of NOVASEQ 6000 using 2x150bp paired-end chemistry. The samples were then demultiplexed and the indexed adapter sequences were trimmed using the CASAVA v1.8.2 software (Illumina Inc.).

3.6.4 Read statistics Read quality check–

FastQC (version 0.11.9, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) were used for Quality Check. of Base quality score distribution, Sequence quality score distribution, Average base content per read and GC distribution in the reads. Universal Illumina Adapters (AGATCGGAAGAGC) was removed using trim galore (version 0.6.7, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Sequences shorter than 20 bp were discarded. Other than adapter removal low-quality ends were trimmed from reads keeping phred score 20. The clean reads were mapped with reference genome of *Triticum aestivum* (version 2.1, https://wheat-urgi.versailles.inrae.fr/Seq-Repository/Assemblies) using hisat (version 2.2.1) with default parameters against reference (Kim *et al*., 2015). Cufflinks (http://cole-trapnell-lab.github.io/cufflinks, version 2.2.1) was used to assemble individual transcripts from RNA-seq reads that have been aligned to the genome using default parameters and quantifies their expression. Differential expression analysis of the new transcripts that were assembled and was performed using Cuffmerge at default parameters. Transcripts with a length greater than equal to 100 were used for further downstream analysis. Reads and the merged assembly were fed to Cuffdiff that calculated the expression levels and tested the statistical significance of observed changes at default parameters. DEGs at absolute (log2FC)>=2 and Pvalue <=0.05 were considered highly differential significant genes and Isoforms. Gene sequences were mapped against NR database using blastx (version 2.2.29+) annotation was done using uniprot database (https://www.uniprot.org/). Sequence based KEGG annotation was done using KAAS (https://www.genome.jp/kegg/kaas/). WEGO (Web Gene Ontology Annotation Plot. version 2.0, http://wego.genomics.org.cn/) was used for visualizing and plotting Gene Ontology of highly significant DEGs and DEIs. GO Enrichment analysis was done using topGO [version 2.34.0] an R package for gene set enrichment analysis. Sequences were also mapped against

Plant Transcription Factor Database (version 5.0, http://planttfdb.gao-lab.org/). Cold related genes were filtered using in house shell scripts.

3.7 Methodology for proteomics

3.7.1 Location and planting material

The two genotypes *viz*, SKUA_52 and SKUA_4301 selected for proteomice analysis were chosen based on their contrasting cold tolerance performance as determined through earlier field screening and biochemical screening. SKUA_52 demonstrated a high level of tolerance to cold stress, whereas the SKUA_4301, a susceptible genotype displayed a low level of tolerance to cold stress. These genotypes were identified from a pool of screened genotypes, consisting of diverse accessions, through rigorous evaluation of their cold tolerance traits and biochemical responses. The selection of these specific genotypes allowed for a focused investigation into the differential gene expression patterns associated with their contrasting cold tolerance phenotypes. The experiment of differential gene expression was done at Department of Ecogenomics and Systems Biology, University of Vienna, Vienna, Austria.

3.7.2 Experimental set up

Seeds of the selected genotypes were planted in pots filled with a mixture of soil, sand and farmyard manure, following a completely randomized design (CRD). The experiment consisted of four biological replicates and within each replicate, there were three technical replicates per pot. The biological replicates were divided into two groups: the Control group and the Treated group. The plants were grown in a controlled growth chamber with specific conditions, including a temperature of 25 ℃, an irradiance of 200 µmol m^{-2} s^{-1} from white light luminescent lamps, a 16/8 h light/dark photoperiod and 75% relative humidity. This growth environment was maintained for 14 days. At the three-leaf stage, the seedlings in the Treated group were transferred to a low temperature acclimation chamber set at 4°C for a duration of 28 days, while the Control group

was maintained at 20°C for the same period. After the acclimation period, the treated group was further exposed to a cold stress treatment at -5°C for 24 hours. Following the cold stress treatment, the crown tissues from 5 to 6 seedlings were pooled together and immediately frozen in liquid nitrogen for subsequent protein extraction.

3.7.3 Protein extraction

For proteomics analysis, leaf tissue was freeze-dried in liquid N2 and grounded to a fine poder using a motar and pestle. The proteins were extracted, pre-fractionated (40 µg of total protein were loaded onto the gel (1D SDS-PAGE), trypsin digested and desalted (using a C18 spec plate) according to a previously described method (Chaturvedi *et al*., 2013; Ghatak *et al*., 2021). Before mass spectrometric measurement, the tryptic peptide pellets were dissolved in 4% (v/v) acetonitrile and 0.1% (v/v) formic acid. One µg of each sample (three replicates for each cell types) was loaded on a C18 reverse-phase column (Thermo Scientific, EASY-Spray 500 mm, 2 µm particle size). Separation was achieved with a 90 min gradient from 98% solution A (0.1% formic acid in high purity water (MilliQ)) and 2% solution B (90% ACN and 0.1% formic acid) at 0 min to 40% solution B (90% ACN and 0.1% formic acid) at 90 min with a flow rate of 300 nL/min. nESI-MS/MS measurements were performed on Orbitrap QExactive (Thermo Fisher Scientific, Bremen, Germany) with the following settings: Full scan range 350–1800 m/z resolution 120 000 max. 20 MS2 scans (activation type CID), repeat count 1, repeat duration 30 s, exclusion list size 500, exclusion duration 30 s, charge state screening enabled with the rejection of unassigned and +1 charge states, minimum signal threshold 500.

3.7.4 Peptide and protein identification

Raw data were searched with the SEQUEST algorithm in Proteome Discoverer version 1.3 (Thermo, Germany), described previously (Ghatak *et al*., 2020). The raw data were also searched against the IWGSC (International Wheat

Genome Consortium Sequences) database containing an annotation of 106 914 genes. Peptides were matched against these databases plus decoys, considering a significant hit when the peptide confidence was high, which is equivalent to a false discovery rate (FDR) of 1% and the Xcorr threshold was established at 1 per charge (2 for +2 ions 3 for +3 ions, etc.). The variable modifications were set to acetylation of the N-terminus and methionine oxidation, with a mass tolerance of 10 ppm for the parent ion and 0.8 Da for the fragment ion. The number of missed and nonspecific cleavages permitted was two. There were no fixed modifications, as dynamic modifications were used. All the MS/MS spectra of the identified proteins and their meta-information from both databases were further uploaded to the PRIDE repository. The identified proteins were quantitated based on total ion count and normalized using the normalized spectral abundance factor (NSAF) strategy (Paoletti *et al.*, 2006). Statiscal significance of protiens in various comparisons was checked using t-test.

Chapter- 4

EXPERIMENTAL FINDINGS

The results of the present study entitled **"Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas"** have been presented under the following sub-headings**:**

4.1 Screening of wheat germplasm for cold tolerance at seedling stage under natural conditions in the field

4.2 Biochemical analysis of diverse wheat genotypes in response to cold stress under controlled condition

4.3 Comparative metabolome and lipidome profiling in contrasting wheat genotypes.

4.4 Characterization and differential gene expression study of cold tolerance genes using qRT-PCR.

4.5 Study of inheritance pattern of cold tolerance in bread wheat.

4.6 Transcriptomics to study differentially expressed transcripts/genes between two contrasting genotypes for cold tolerance.

4.7 Proteomics to study differentially expressing proteins in contrasting wheat genotypes for cold tolerance.

4.1 Screening of wheat germplasm for cold tolerance at seedling stage under natural conditions in the field

A total of 4,560 genotypes were screened for cold tolerance in the two environments, i.e, year 2020 and year 2021 at Faculty of Agriculture at Wadura, Kashmir, India. The cold weather conditions in the Kashmir valley prevailing during the winter season were found ideal for the screening of 4560 wheat genotypes for cold stress tolerance. During the year 2020–2021 (October–

February), the average high/day temperatures ranged from 5.8℃ to 18.23℃, while the range of average low/night temperature recorded was – 5.9℃ to 5.36℃. During the year 2021–2022 (October–February), the average high/day temperatures ranged from 6.2℃ to 21.45℃, while the range of average low/night temperature recorded was – 1.0℃ to 6.3℃. Furthermore, during the year 2022–2023 (October–February), the average high/day temperatures ranged from 8℃ to 22℃, while the range of average low/night temperature recorded was – 3℃ to 7℃ (Figure 4.1).

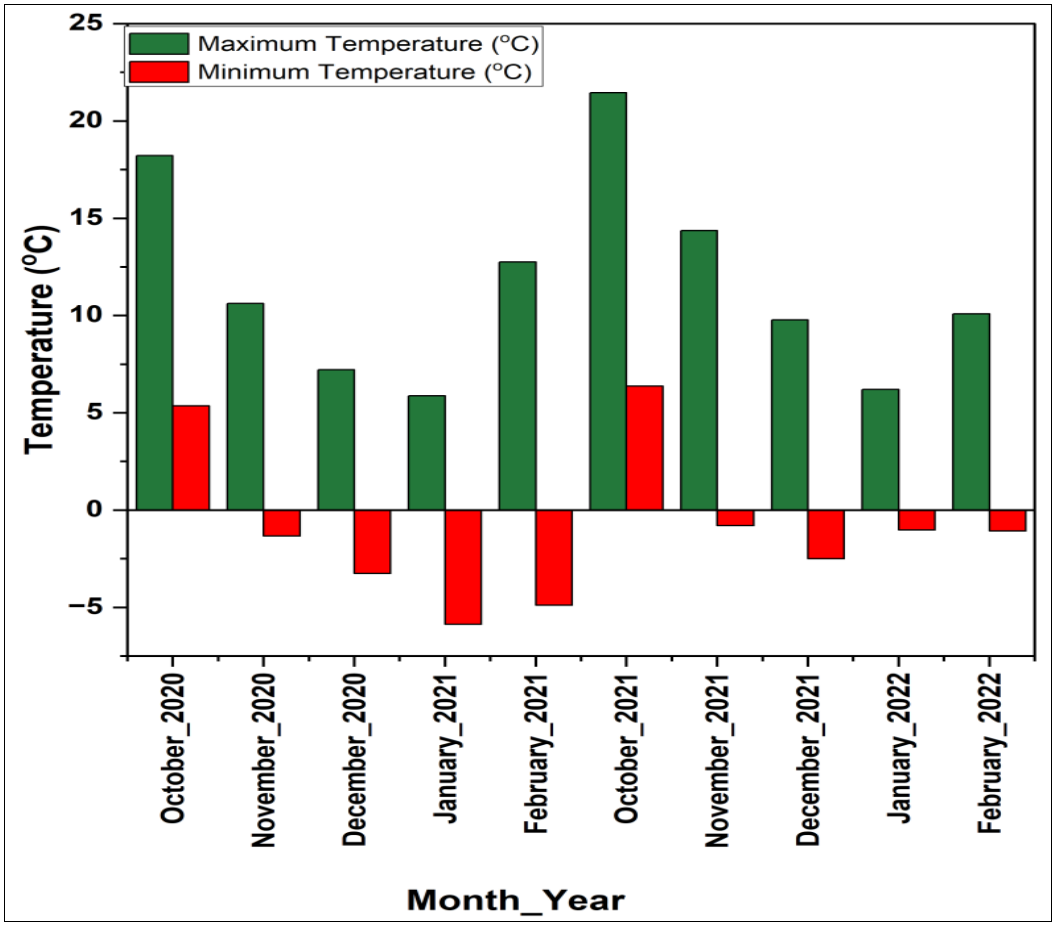


Fig: 4.1: **Bar graph showing variation in the temperature during the growing season of wheat (October_2020 to February_2021, October_2021 to February_2022, October_2022 to February_2022) at Faculty of Agriculture, SKUAST-K, Sopore.**

4.2 Screening for cold tolerance under natural conditions in the field

The frequency distribution of genotypes for cold stress tolerance for each year is shown in (Figure 4.2). The cold score for each genotype is presented in (Annexure-I). The frequency distribution of the results show that the majority of wheat genotypes screened in both years had a cold score of 2 (moderately resistant), with 1,164 genotypes in 2020 and 2,012 genotypes in 2021 falling into this category. A small number of genotypes had cold scores of 1 (cold tolerant), 3 (susceptible), or 4 (highly susceptible). During the year 2020, 311 genotypes had a cold score of 1, while in the year 2021, 1,043 genotypes had this score. Similarly, 1,812 genotypes in the year 2020 and 1,233 genotypes in the year 2021 had a cold score of 3, while 1,227 genotypes in the year 2020 and 166 genotypes in the year 2021 had a cold score of 4. Only a few genotypes, 46 genotypes in the year 2020 and 106 genotypes in the year 2021, were classified as highly cold tolerant (cold score of 0). Overall the results revealed that the majority of wheat genotypes screened in both years were moderately tolerant and had a cold score of 2. In contrast, a smaller number of genotypes were cold tolerant which had a cold score of 1 and an even smaller number were found highly cold tolerant which had a cold score of 0.

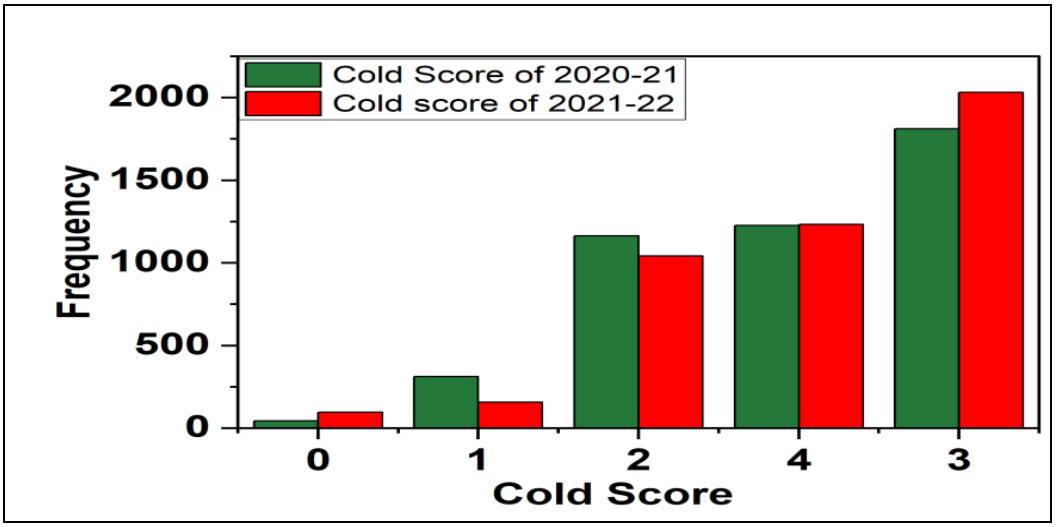


Fig: 4.2: Frequency distribution of 4560 wheat genotypes for cold stress tolerance during the year 2020- 2021 and year 2021-2022.

Based on the results of two-year field screening of wheat germplasm for cold stress tolerance, we selected a diverse set of 350 genotypes (including four checks) that showed consistent performance in both years of screening and were subsequently screened for membrane stability using electrical conductivity as a measure. The ELI values exhibited a wide range of variation among the genotypes, indicating the presence of substantial genetic variation for this trait in the germplasm studied (Figure 4.3).

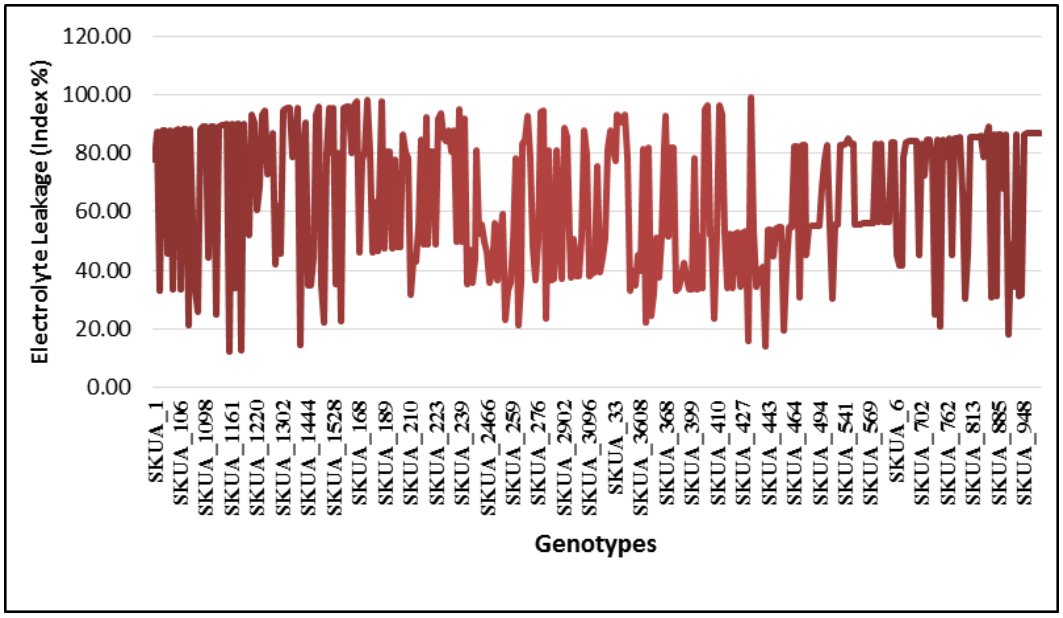


Fig. 4.3: Variation in cell membrane stability accessed by the measure of electrical conductivity (EC) in mini-core set (350 wheat genotypes during this study.

The analysis of variance (ANOVA) for the electrolyte leakage index (ELI) of 350 genotypes under field conditions showed that the variation among treatments (genotypes) was highly significant, while the variation among blocks (experimental units) was not significant. There were significant differences observed between the check genotypes and other treatments, as well as between the test and check genotypes (Table 4.1). The 350 wheat genotypes were grouped into five categories (HR, R, MR, S and HS) based on their cold scores in the field screening. Furthermore, we calculated the mean ELI values for each group to compare their membrane stability under cold stress conditions. The analysis of the

mean EC values for each group revealed significant differences ($p < 0.001$) between the groups, indicating variations in the membrane stability of the genotypes under cold stress. The mean EC values of the genotypes in the highly resistant (HR) group were the lowest (14.2 μS/cm), while the mean ELI values of the genotypes in the highly susceptible (HS) group were the highest (90.6 μS/cm).

Table-4.1: Analysis of variance (ANOVA) of cell membrane stability assessed by electrolyte leakage index in 350 genotypes in response to cold stress conditions prevailing in the field during the current study

Sources of variation	DF	Mean sum of squares
Block (ignoring treatments)	5	2547.08 *
Block (eliminating treatments)	5	4.7e-25 ns
Treatment (eliminating blocks)	349	675.15 *
Treatment (ignoring blocks)	349	711.53 *
Treatment: check	3	16762.06 *
Treatment: test vs. check	1	2682.54 *
Residuals	16	9.00E-02

DF= degrees of freedom; ***** = Significance at the P-value of 0.05, ns= non-significant

We further compared the mean values of EC among the different cold tolerance groups using one-way ANOVA, which revealed a significant difference between the groups (Figure 4.4). The box plots showed an increasing trend of ELI from the HR to HS groups, with the HR group having the lowest mean EC value (14.2 μS/cm) and the HS group having the highest mean EC value (90.6 μS/cm). Interestingly, we observed higher variability within each group in the more tolerant groups (HR, R and MR) compared to the more susceptible groups (S and HS)." Subsequently, we performed posthoc tests using the HR group as a control and found significant differences between the HR and R, HR and S and HR and HS groups (Figure 4.4).

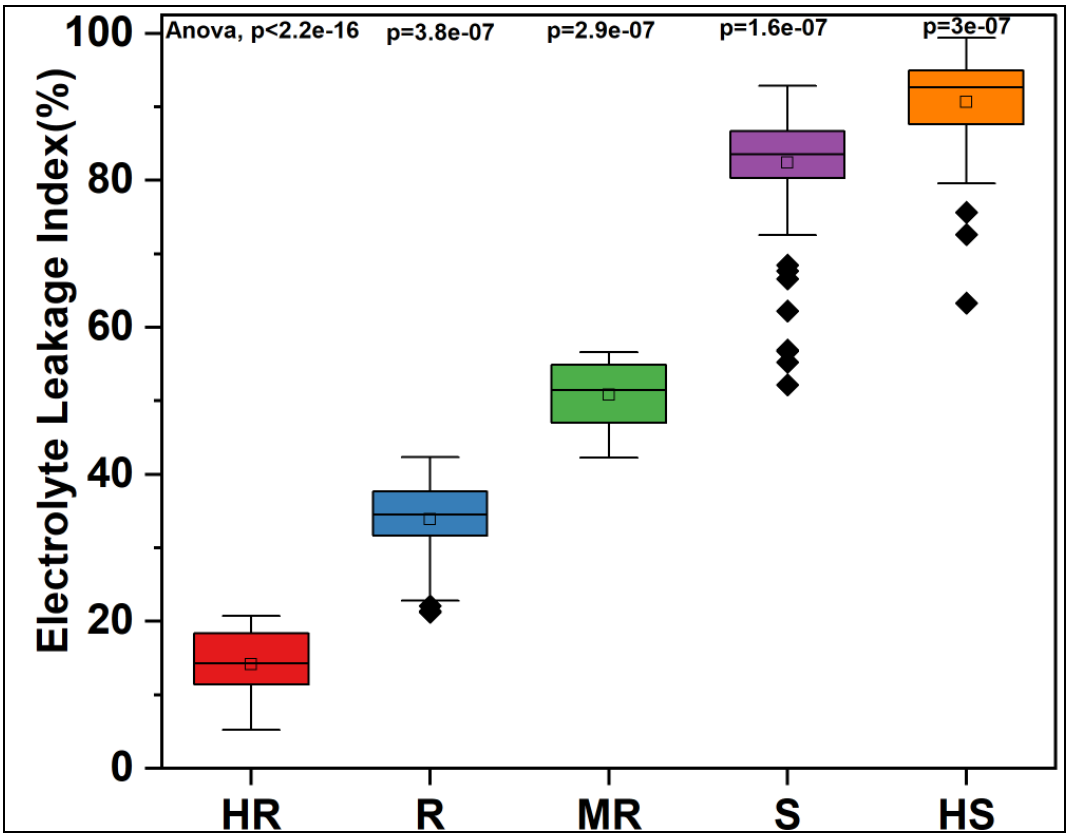
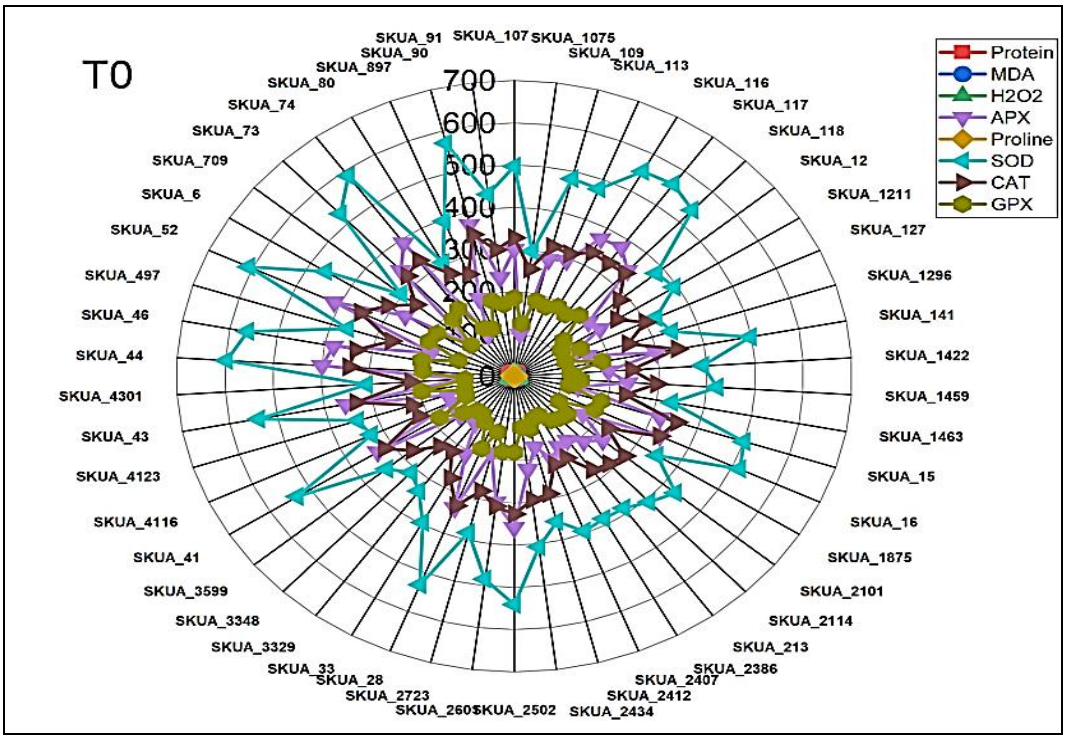


Fig: 4.4: **Box plots showing increasing trend of ELI (%) from HR (highly resistant) to HS (highly susceptible) genotypes. Box plots also show significant variability (P value<0.05) between highly resistant (HR), resistant(R), moderately genotypes (MR), susceptible genotypes(S) and highly susceptible genotypes(S)**

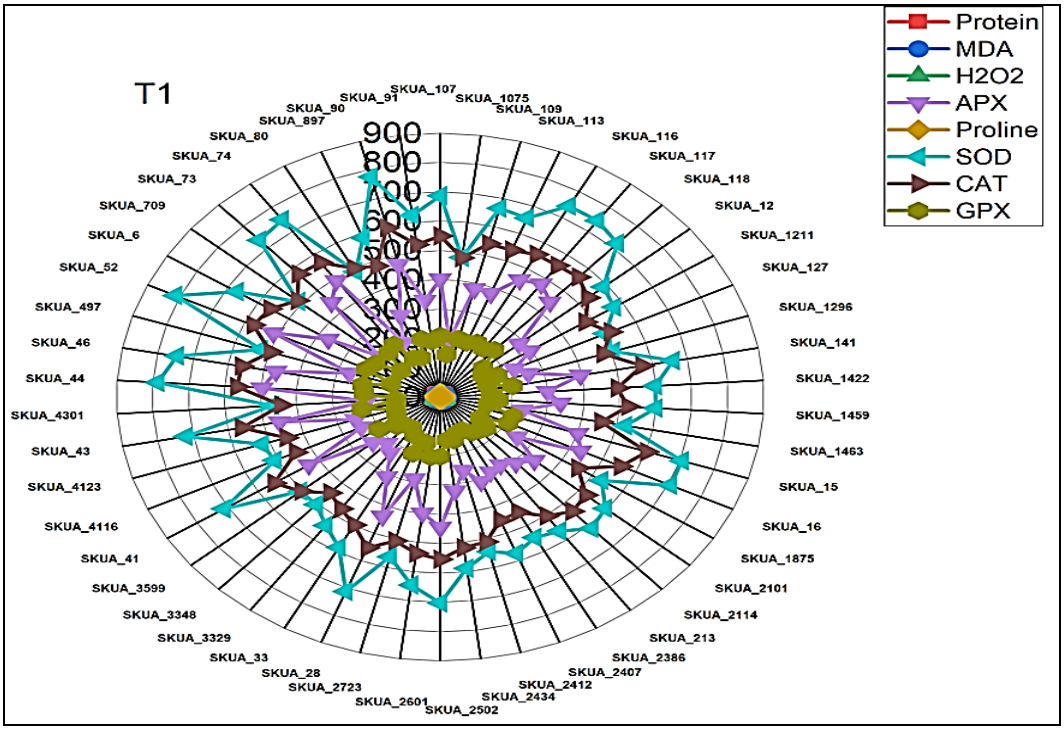
4.2.1 Biochemical analysis of diverse wheat genotypes in response to cold stress under controlled condition

Based on the results of cold screening in the field and membrane stability assessed by electrical conductivity assay, 50 diverse wheat genotypes were selected and analyzed for various stress-responsive biochemical parameters at different cold treatments viz, T0, T1, T2 and T3, where (T0=control (25℃), T1= acclimation phase at 4℃ for 14 days, T2=cold stress at -5℃ after acclimation and T3=cold stress at -5℃ without acclimation treatments, respectively). The biochemicals *viz,* malondialdehyde (MDA) levels, hydrogen peroxide (H_20_2) levels, protein content, antioxidant enzyme activities *viz*, ascorbate peroxidase

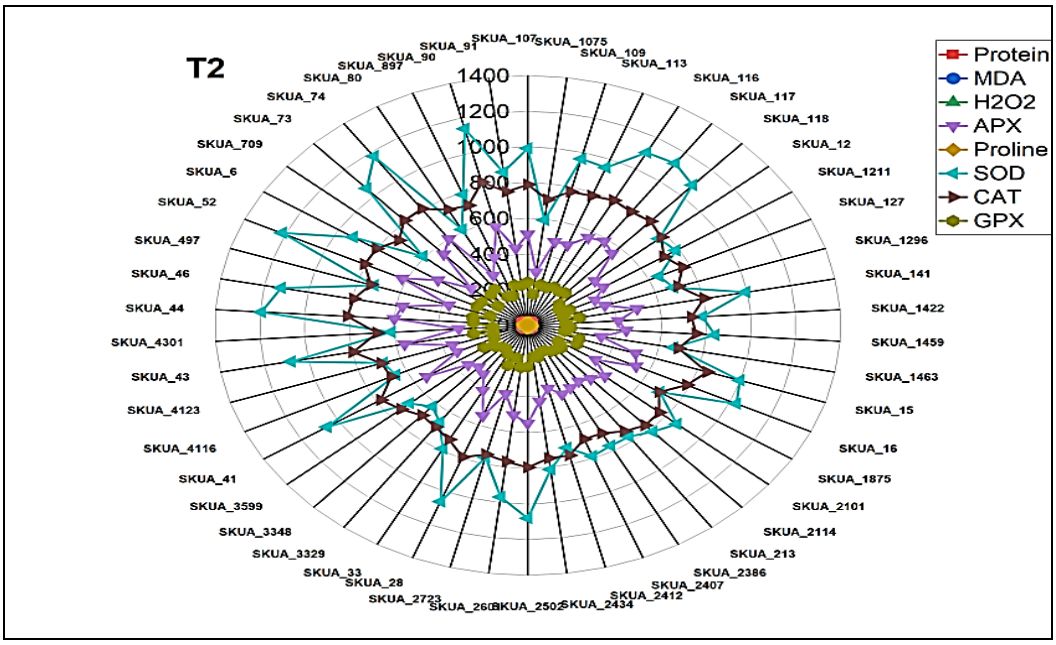
(APX) activity, superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPX) activity and osmoprotectant *viz*, proline content for which the genotypes were evaluated, depicted substantial variation as shown by radar graphs (Figure 4.5). Radar graphs showed that under control conditions (T0) relatively low levels of H_2O_2 and MDA; however a progressive increase in H_2O_2 and MDA was observed during acclimation phase (T1). In addition to this, antioxidants like APX, GPX, CAT and SOD, proline and protein also showed steep increase during acclimation phase. A cold stress followed by acclimation phase (T2) resulted in the sharp decline in H_2O_2 and MDA and heightened increase in antioxidants, proline and protein. Conversely, cold stress in the genotypes which were not acclimated prior (T3), resulted in the increase of MDA and H_2O_2 and decrease of antioxidants, proline and protein.


T0
Protein
MDA
H2O2
APX
Proline
SOD
CAT
GPX
700
600
500
400
SKUA_107
SKUA_1075
SKUA_109
SKUA_113
SKUA_116
SKUA_117
SKUA_118
SKUA_12
SKUA_1211
SKUA_127
SKUA_1296
SKUA_141
SKUA_1422
SKUA_1459
SKUA_1463
SKUA_15
SKUA_16
SKUA_1875
SKUA_2101
SKUA_2114
SKUA_213
SKUA_2386
SKUA_2407
SKUA_2412
SKUA_2434
SKUA_2502
SKUA_2601
SKUA_2723
SKUA_28
SKUA_33
SKUA_3329
SKUA_3348
SKUA_3599
SKUA_41
SKUA_4116
SKUA_4123
SKUA_43
SKUA_4301
SKUA_44
SKUA_46
SKUA_497
SKUA_52
SKUA_6
SKUA_709
SKUA_73
SKUA_74
SKUA_80
SKUA_897
SKUA_90
SKUA_91

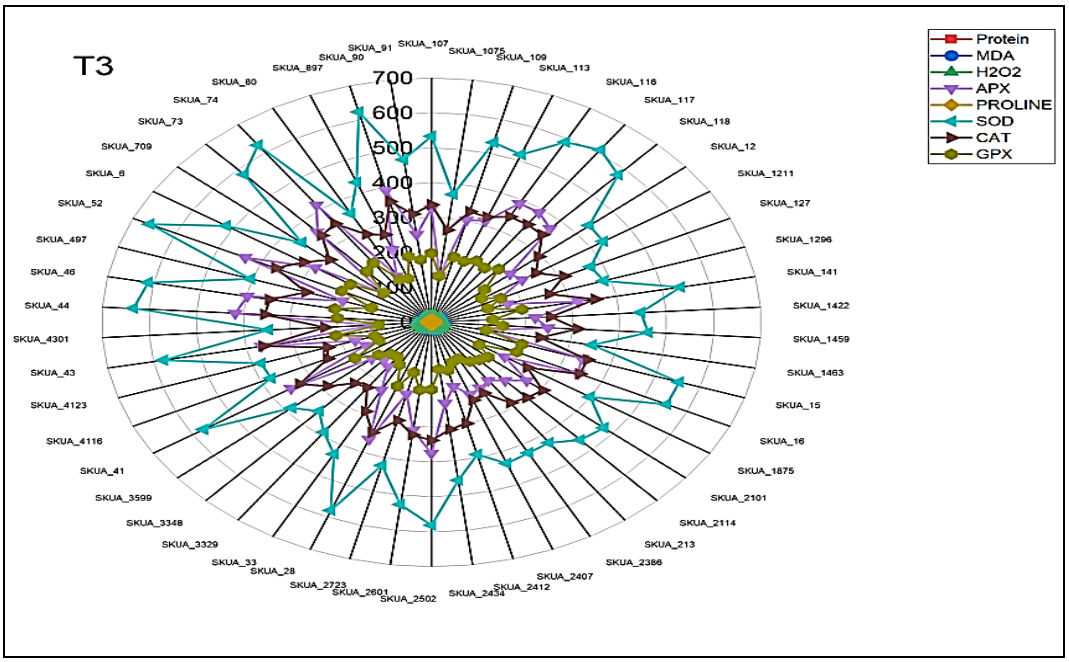
(a)


T1
Protein
MDA
H2O2
APX
Proline
SOD
CAT
GPX
900
800
700
600
500
400
SKUA_107
SKUA_1075
SKUA_109
SKUA_113
SKUA_116
SKUA_117
SKUA_118
SKUA_12
SKUA_1211
SKUA_127
SKUA_1296
SKUA_141
SKUA_1422
SKUA_1459
SKUA_1463
SKUA_15
SKUA_16
SKUA_1875
SKUA_2101
SKUA_2114
SKUA_213
SKUA_2386
SKUA_2407
SKUA_2412
SKUA_2434
SKUA_2502
SKUA_2601
SKUA_2723
SKUA_28
SKUA_33
SKUA_3329
SKUA_3348
SKUA_3599
SKUA_41
SKUA_4116
SKUA_4123
SKUA_43
SKUA_4301
SKUA_44
SKUA_46
SKUA_497
SKUA_52
SKUA_6
SKUA_709
SKUA_73
SKUA_74
SKUA_80
SKUA_897
SKUA_90
SKUA_91

(b)



(c)



(d)

Fig: 4.5: **Radar graphs showing variation in various biochemicals under (a) normal conditions (T0) (b) acclimation phase at 4℃ for 14 days (T1) (c) cold stress at -5℃ after acclimation (T2) and (d) cold stress at -5℃ without acclimation treatments (T3).**

4.2.2 Analysis of variance (ANOVA) of biochemical traits

The analysis of variance (ANOVA) of all biochemical results revealed significant differences in the genotypes for all analysed biochemical parameters (Table 4.2). Additionally, the effects of various cold treatments on the biochemical profiles of the genotypes were highly significant. Furthermore, the interaction between genotype and treatment demonstrated significant effects on all biochemical parameters except for H202 and GPX ($p > 0.05$). Post-hoc LSD (least significant difference) analysis was performed to determine specific differences among genotypes and among the treatments. The LSD values for each parameter, representing the minimum significant difference required for pairwise comparisons between genotypes and between the treatments, are provided in (Table 4.3).

One-way analysis of variance (ANOVA) was conducted to assess the significance of differences in the mean values of various biochemical parameters among different cold treatments. The results of the one-way ANOVA indicate that there are significant differences in the mean values of biochemical parameters among the different treatments (Figure 4.6). The highest mean values for protein, oxidative stress, hydrogen peroxide accumulation and antioxidant activity were generally observed in the cold stress after acclimation treatment. The box plots revealed interesting insights into the variations among the different cold treatments. In the control group (T0), the median values for protein, MDA, H_2O_2, APX, proline, SOD, CAT and GPX were 7.13, 4.06, 5.79, 244, 3.22, 434, 290 and 150, respectively. These values suggest a certain baseline level of biochemical activity under normal conditions. Upon acclimation (T1), we observed an increase in protein, MDA and APX levels, indicating a response to the acclimation process. The mean values for these parameters were 10.5, 12.1 and 355, respectively. Additionally, the proline, SOD, CAT and GPX levels showed a notable increase compared to the control group, with mean values of 7.59, 618, 519 and 173, respectively.

Table-4.2: Analysis of variance (ANOVA) of various biochemical traits for 50 diverse wheat genotypes recorded under normal and different cold stress conditions in this study

SOV	DF	Protein	MDA	H_2O_2	APX	Proline	SOD	CAT	GPX
Genotype	49	23*	84.9*	43*	69424*	9.8*	108472*	14738*	9294*
Treatment	3	3974*	2859.9*	4740*	839339*	1460*	3805111*	4830630*	53005*
Genotype*Treatment	147	1*	5.2*	7ns	37*	0.6*	4391*	77*	4ns
Error	200	0.05	0.05	6	25	0.05	4	3	3

DF= degrees of freedom; Sign P-value code: 0.05 '*'; MDA= malondialdehyde; H_2O_2 = hydrogen peroxide; SOD= superoxide dismutase; CAT= catalase; GPX= glutathione peroxidase.

Table-4.3: Mean values of biochemical parameters (APX, CAT, GPX, SOD, MDA, H2O2, proline and protein) measured across different wheat genotypes

Genotype	Protein	MDA	H_2O_2	APX	Proline	SOD	CAT	GPX
SKUA_107	14.143 ^{b}	6.365 ^{y}	8.27 ^{nop}	384.8 ^{k}	7.0375 ^{jkl}	677.1 ^{m}	500.7 ^{j}	208.6 ^{de}
SKUA_1075	10.2775 ^{uv}	11.955 ^{h}	11.335 ^{efghijk}	178.645 ^{F}	6.6075 ^{no}	429.79 ^{O}	427.75 ^{z}	145.15 ^{s}
SKUA_109	12.6745 ^{jkl}	5.745 ^{z}	8.5525 ^{mnop}	363.645 ^{m}	7.3575 ^{hi}	661.04 ^{o}	489.1 ^{m}	204.5 ^{gh}
SKUA_113	12.6085 ^{klm}	7.2325 ^{v}	9.5375 ^{ijklmno}	367.745 ^{m}	9.0025 ^{b}	650.49 ^{q}	488.55 ^{m}	197.925 ^{j}
SKUA_116	14.0405 ^{b}	8.73 ^{q}	9.035 ^{klmnop}	444.675 ^{e}	9.0275 ^{b}	748.35 ^{f}	508.05 ^{h}	211.425 ^{bc}
SKUA_117	13.3345 ^{fgh}	8.63 ^{qr}	8.255 ^{op}	456.52 ^{c}	8.2775 ^{cd}	759.79 ^{e}	504.75 ^{i}	203.45 ^{hi}
SKUA_118	13.556 ^{de}	6.1825 ^{y}	8.50875 ^{mnop}	427.87 ^{g}	7.1825 ^{ijk}	729.24 ^{g}	510.1 ^{g}	219 ^{a}
SKUA_12	10.7225 ^{rs}	11.795 ^{hi}	12.8275 ^{bcdefgh}	273.18 ^{t}	6.98 ^{kl}	542.11 ^{A}	471.05 ^{p}	155.675 ^{p}
SKUA_1211	10.0975 ^{vwx}	14.845 ^{e}	11.69125 ^{defghij}	283.745 ^{s}	6.2025 st	545.49 ^{z}	428.1 ^{z}	136.475 ^{v}
SKUA_127	13.285 ^{ghi}	7.095 ^{vw}	9.8 ^{ijklmno}	215.895 ^{B}	6.3175 ^{pqrs}	463.54 ^{K}	474.7 ^{o}	175.075 ^{l}
SKUA_1296	9.3875 ^{y}	10.67 ^{l}	11.33 ^{efghijk}	238.305 ^{x}	5.82 ^{wx}	479.86 ^{H}	423.35 ^{A}	135.3 ^{vw}
SKUA_141	12.425 ^{mn}	7.1325 ^{vw}	7.935 ^{op}	378.43 ^{l}	7.35 ^{i}	672.36 ^{n}	518.35 ^{d}	206 ^{fg}
SKUA_1422	9.06 ^{z}	13.955 ^{f}	13.035 ^{bcdefg}	280.305 ^{s}	5.16 ^{z}	554.61 ^{x}	430.7 ^{y}	139.85 ^{u}
SKUA_1459	11.6125 ^{p}	10.6825 ^{l}	9.59875 ^{ijklmno}	315.37 ^{p}	6.0825 ^{tuv}	580.49 ^{u}	470.6 ^{p}	162.3 ^{n}
SKUA_1463	8.165 ^{B}	17.23 ^{a}	14.3175 ^{abc}	213.425 ^{B}	5.9175 ^{vw}	472.1 ^{I}	405.1 ^{B}	136.5 ^{v}
SKUA_15	12.684 ^{jkl}	8.28 ^{s}	8.095 ^{op}	391.18 ^{j}	6.95 ^{lm}	689.61 ^{l}	541.7 ^{a}	211.325 ^{bc}
SKUA_16	13.1405 ^{hi}	6.355 ^{y}	8.5025 ^{mnop}	411.3 ^{i}	7.9375 ^{e}	702.1 ^{k}	505.1 ^{i}	209.8 ^{cd}
SKUA_1875	10.435 ^{tu}	14.82 ^{e}	14.8425 ^{ab}	240.895 ^{wx}	7.2475 ^{ij}	494.79 ^{F}	405.05 ^{B}	131.425 ^{yz}

Contd….

Table 4.3: contd…

Genotype	Protein	MDA	H_2O_2	APX	Proline	SOD	CAT	GPX
SKUA_2101	11.5975 ^{p}	10.53 ^{lm}	11.1675 ^{fghijkl}	321.805 ^{o}	8.48 ^{c}	592.36 ^{t}	473.05 ^{o}	166.2 ^{m}
SKUA_2114	11.5275 ^{p}	10.1825 ^{no}	8.34875 ^{nop}	289.93 ^{r}	6.53 ^{op}	575.86 ^{v}	470.75 ^{p}	157.4 ^{op}
SKUA_213	10.5275 st	10.645 ^{l}	10.23 ^{ijklmno}	266.495 ^{u}	6.0925 ^{tuv}	536.74 ^{B}	448.85 ^{v}	149.25 ^{r}
SKUA_2386	9.335 ^{y}	15.47 ^{c}	13.725 ^{abcde}	273.805 ^{t}	5.61 ^{xy}	532.36 ^{C}	397.6 ^{E}	127.75 ^{A}
SKUA_2407	10.05 ^{wx}	15.28 ^{cd}	13.7875 ^{abcde}	289.37 ^{r}	5.7425 ^{wxy}	547.99 ^{y}	400.25 ^{CD}	130.95 ^{z}
SKUA_2412	12.8225 ^{jk}	9.4675 ^{p}	9.5125 ^{ijklmno}	245.675 ^{w}	6.5875 ^{no}	499.6 ^{E}	461.6 ^{r}	152.975 ^{q}
SKUA_2434	12.275 ^{n}	10.105 ^{o}	10.37875 ^{hijklmno}	301.52 ^{q}	6.5175 ^{opq}	566.04 ^{w}	468.35 ^{q}	146.05 ^{s}
SKUA_2502	12.8525 ^{j}	5.8325 ^{z}	7.9725 ^{op}	431.68 ^{fg}	6.75 ^{mn}	726.11 ^{h}	503.6 ^{i}	202.225 ^{i}
SKUA_2601	13.515 ^{def}	8.505 ^{r}	10.1075 ^{ijklmno}	379.305 ^{l}	7.17 ^{ijk}	656.11 ^{p}	485.75 ^{n}	204.7 ^{gh}
SKUA_2723	12.56 ^{lm}	10.3825 ^{mn}	10.925 ^{ghijklm}	270.87 ^{tu}	6.5725 ^{no}	532.99 ^{C}	456.2 ^{s}	158.1 ^{o}
SKUA_28	13.087 ^{i}	6.72 ^{x}	8.3 ^{nop}	419.02 ^{h}	8.8575 ^{b}	721.04 ^{j}	504.05 ^{i}	206.95 ^{ef}
SKUA_33	11.025 ^{q}	12.5275 ^{g}	12.84125 ^{bcdefgh}	289.3 ^{r}	6.2275 ^{rst}	549.6 ^{y}	448.75 ^{v}	149.45 ^{r}
SKUA_3329	10.2275 ^{uvw}	14.145 ^{f}	13.465 ^{abcdef}	216.93 ^{AB}	5.12 ^{z}	484.61 ^{G}	406.35 ^{B}	131 ^{yz}
SKUA_3348	11.635 ^{p}	14.04 ^{f}	14.145 ^{abcd}	203.675 ^{C}	5.6475 ^{xy}	447.1 ^{L}	401.7 ^{C}	134.075 ^{wx}
SKUA_3599	8.7525 ^{A}	11.745 ^{hi}	11.3725 ^{efghijk}	224.43 ^{yz}	6.15 ^{stu}	479.86 ^{H}	450.1 ^{uv}	155.75 ^{p}
SKUA_41	12.7185 ^{jkl}	7.5925 ^{u}	8.6475 ^{mnop}	413.495 ^{i}	6.9825 ^{kl}	720.49 ^{j}	494.25 ^{l}	202.65 ^{i}
SKUA_4116	9.95 ^{x}	16.37 ^{b}	14.8275 ^{ab}	221.52 ^{zA}	5.2475 ^{z}	466.04 ^{J}	393.1 ^{F}	132.8 ^{xy}
SKUA_4123	10.7225 ^{rs}	15.2325 ^{d}	15.9525 ^{a}	228.87 ^{y}	6.9425 ^{lm}	480.49 ^{H}	398.55 ^{DE}	128.45 ^{A}
SKUA_43	13.8025 ^{c}	8.1575 ^{s}	7.915 ^{op}	434.37 ^{f}	7.1025 ^{jkl}	729.24 ^{g}	510.25 ^{g}	218.125 ^{a}

Contd….

Table 4.3: contd…

Genotype	Protein	MDA	H_2O_2	APX	Proline	SOD	CAT	GPX
SKUA_4301	8.74 ^{A}	15.33 ^{cd}	13.7275 ^{abcde}	187.43 ^{E}	5.59 ^{y}	433.61 ^{N}	390.7 ^{G}	122.35 ^{B}
SKUA_44	13.3875 ^{efg}	7.5025 ^{u}	8.345 ^{nop}	478.425 ^{b}	7.6975 ^{fg}	803.35 ^{b}	518.6 ^{d}	210.975 ^{bc}
SKUA_46	13.7925 ^{c}	5.2325 ^{A}	8.00875 ^{op}	453.895 ^{cd}	7.6775 ^{fg}	761.04 ^{e}	509.85 ^{gh}	218.55 ^{a}
SKUA_497	11.61 ^{p}	11.63 ^{ij}	10.75875 ^{ghijklmn}	253.68 ^{v}	6.44 ^{opqr}	503.61 ^{D}	445.7 ^{w}	142.325 ^{t}
SKUA_52	15.65 ^{a}	7.145 ^{vw}	6.54875 ^{p}	496.305 ^{a}	10.61 ^{a}	824.61 ^{a}	524.75 ^{c}	219.6 ^{a}
SKUA_6	13.66 ^{cd}	8.67 ^{qr}	10.045 ^{ijklmno}	351.305 ^{n}	6.3 ^{qrst}	643.36 ^{r}	498.6 ^{k}	211.75 ^{b}
SKUA_709	10.85 ^{qr}	12.4825 ^{g}	10.085 ^{ijklmno}	199.805 ^{CD}	5.96 ^{uvw}	445.86 ^{L}	452.1 ^{t}	139.05 ^{u}
SKUA_73	12.8465 ^{j}	7.245 ^{v}	9.24 ^{jklmno}	421.805 ^{h}	7.37 ^{hi}	723.36 ^{i}	512.35 ^{f}	208.3 ^{de}
SKUA_74	12.675 ^{jkl}	7.8925 ^{t}	8.70125 ^{lmnop}	473.805 ^{b}	8.16 ^{d}	782.36 ^{c}	514.7 ^{e}	219.85 ^{a}
SKUA_80	11.9725 ^{o}	11.295 ^{k}	12.01125 ^{cdefghi}	195.02 ^{D}	6.4575 ^{opq}	441.04 ^{M}	451.6 ^{tu}	151.5 ^{q}
SKUA_897	10.6725 ^{rs}	11.43 ^{jk}	10.86 ^{ghijklm}	282.8 ^{s}	7.8675 ^{ef}	549.6 ^{y}	433.05 ^{x}	144.95 ^{s}
SKUA_90	12.2815 ^{n}	6.9325 ^{wx}	8.24125 ^{op}	449.93 ^{d}	7.57 ^{gh}	775.86 ^{d}	532.1 ^{b}	203.75 ^{hi}
SKUA_91	13.7275 ^{cd}	9.615 ^{p}	9.21625 ^{jklmno}	314.425 ^{p}	6.9375 ^{lm}	598.35 ^{s}	473.35 ^{o}	191.75 ^{k}

Superscripts (a, b, c….z) following the mean values indicate significant differences between genotypes, with distinct superscripts signifying statistical significance. Genotypes sharing the same superscript are not significantly different from each other, while differing superscripts denote significant variations.

During cold stress after acclimation (T2), the biochemical parameters exhibited further changes. The mean values for protein, H202 and SOD increased to 21.1, 6.13 and 868, respectively. However, the MDA and proline levels decreased compared to T1, with mean values of 8.27 and 12, respectively. The CAT and GPX levels also showed an increase, with median values of 756 and 204, respectively. In contrast, cold stress without acclimation (T3) led to different patterns in the biochemical parameters. The mean values for MDA and H202 significantly increased to 16.6 and 20.5, respectively, indicating oxidative stress. Protein and proline levels decreased compared to T2, with median values of 8.65 and 4.86, respectively. The SOD, CAT and GPX levels exhibited slight variations, with median values of 478, 302 and 163, respectively.

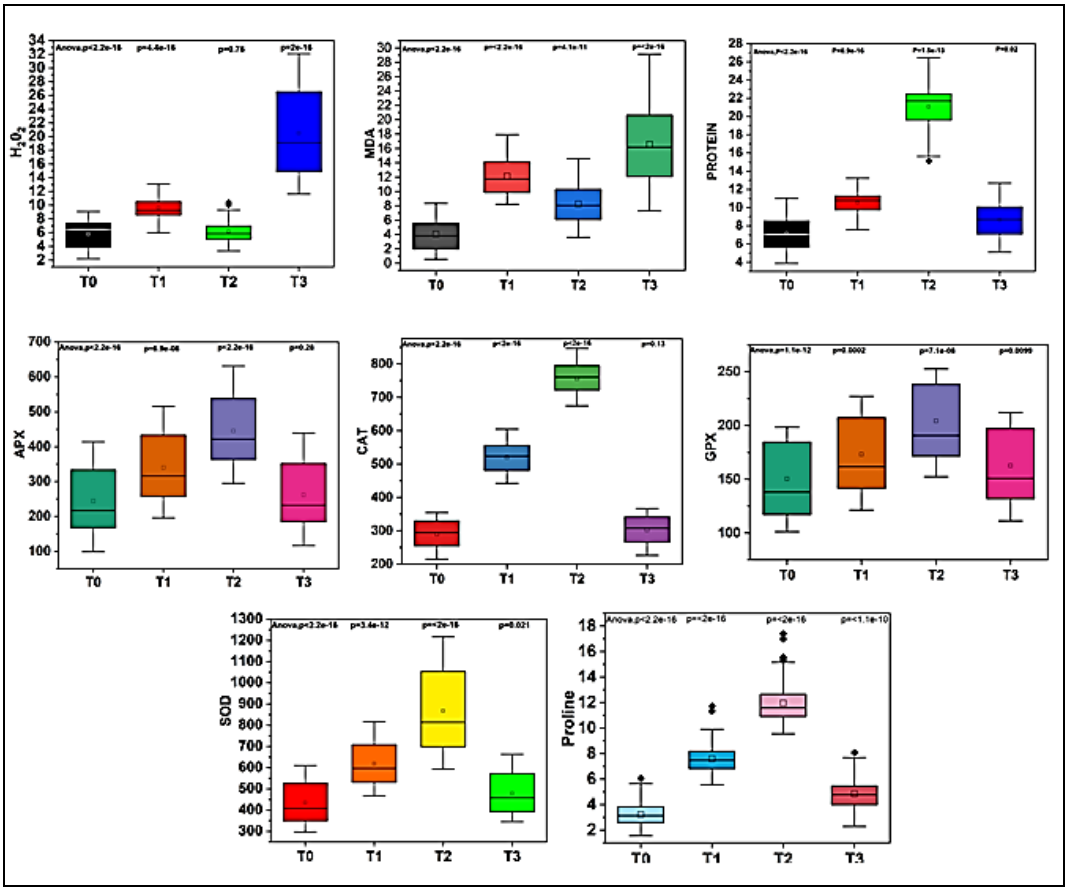


Fig: 4.6: **Box plots revealing significant differences in the mean values of various biochemicals MDA, H202, APX, GPX, SOD, CAT, proline and protein under the different cold treatments.**

4.2.3 Correlation analysis among different biochemical traits

The correlation analysis revealed significant associations among the various biochemical parameters studied (Table 4.4). The correlation results highlighted the interconnectedness of the antioxidant system in response to the different cold treatments and control conditions in this study (Figure 4.7). Protein levels exhibited strong negative correlations with MDA levels and H_2O_2 content. H_2O_2 levels showed positive correlations with MDA and negative correlations with antioxidant enzymes APX, GPX, SOD and CAT. APX levels demonstrated positive correlations with protein content. Proline levels exhibited positive correlations with APX, SOD and CAT. SOD and CAT levels showed strong positive correlations with protein levels. GPX levels displayed positive correlations with protein content.

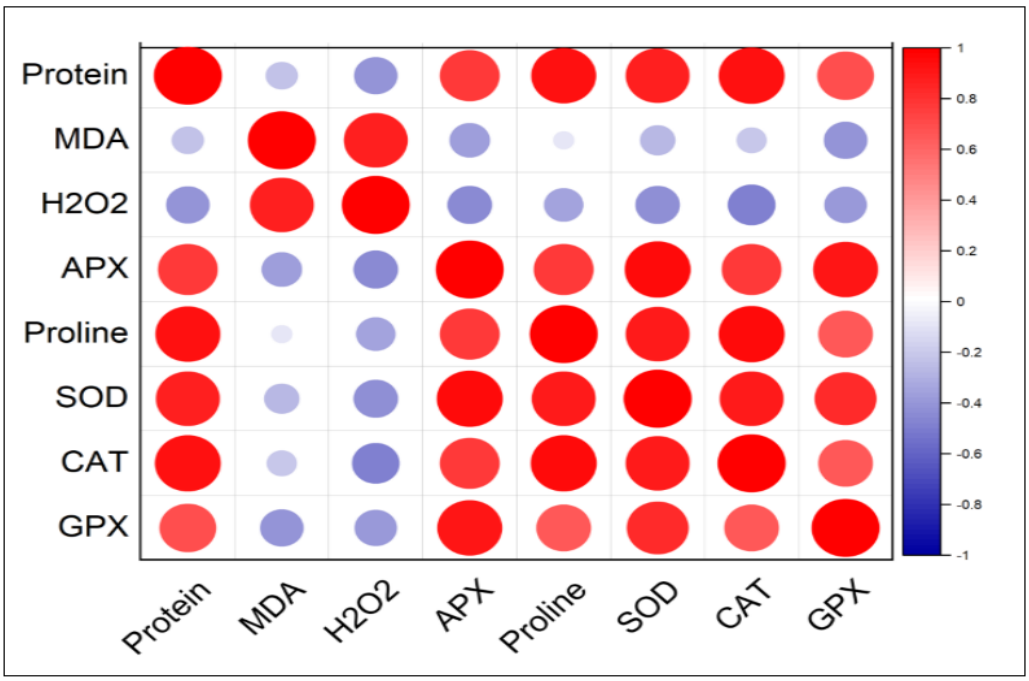


Fig. 4.7: Correlation network analysis showing biochemical interactions underlying cold stress tolerance in wheat in this study. The size of each circle is proportional to its degree of correlation with other parameters; larger circles indicate stronger correlations. The color intensity of circles corresponds to the magnitude of correlation, with deeper shades indicating stronger correlation

Table- 4.4: Correlation matrix among biochemical traits under normal conditions (T0), acclimation phase at 4℃ for 14 days (T1), cold stress at -5℃ after acclimation (T2) and cold stress at -5℃ without acclimation treatments (T3)

Traits	Level	Protein	MDA	H_2O_2	APX	Proline	SOD	CAT
MDA	T0	-0.88*						
	T1	-0.72*						
	T2	-0.73*						
	T3	-0.83*						
H_2O_2	T0	-0.86*	0.81*					
	T1	-0.69*	0.85*					
	T2	-0.57*	0.78*					
	T3	-0.79*	0.90*					
APX	T0	0.86*	-0.81*	-0.89*				
	T1	0.55*	-0.77*	-0.83*				
	T2	0.56*	-0.74*	-0.90*				
	T3	0.86*	-0.74*	-0.73*				
Proline	T0	0.86*	-0.83*	-0.85*	0.83*			
	T1	0.57*	-0.62*	-0.60*	0.72*			
	T2	0.45*	-0.42*	-0.16ns	0.55*			
	T3	0.66*	-0.58*	-0.54*	0.65*			

Contd….

Table 4.4: contd…

Traits	Level	Protein	MDA	H_2O_2	APX	Proline	SOD	CAT
SOD	T0	0.86*	-0.81*	-0.90*	0.99*	0.84*		
	T1	0.57*	-0.77*	-0.67*	0.98*	0.73*		
	T2	0.55*	-0.75*	-0.58*	0.99*	0.55*		
	T3	0.85*	-0.74*	-0.73*	0.99*	0.63*		
CAT	T0	0.90*	-0.95*	-0.86*	0.87*	0.89*	0.86*	
	T1	0.65*	-0.90*	-0.82*	0.83*	0.65*	0.85*	
	T2	0.67*	-0.86*	-0.73*	0.82*	0.47*	0.83*	
	T3	0.89*	-0.90*	-0.91*	0.86*	0.62*	0.87*	
GPX	T0	0.92*	-0.92*	-0.87*	0.89*	0.86*	0.90*	0.94719*
	T1	0.70*	-0.90*	-0.75*	0.90*	0.70*	0.90*	0.91*
	T2	0.70*	-0.88*	-0.67*	0.89*	0.49*	0.90*	0.91*
	T3	0.91*	-0.87*	-0.82*	0.90*	0.60877*	0.90*	0.94*

P-value= 0.01'*'; MDA= malondialdehyde; H_2O_2 = hydrogen peroxide; SOD=superoxide dismutase; CAT= catalase; GPX= glutathione peroxidase

4.2.4 Principal component analysis

Principal component analysis (PCA) was performed to investigate the relationships between genotypes and biochemical traits in our study. We generated biplots based on the PCA results to visually explore the relationships between genotypes and traits in the reduced-dimensional space and to select the parents for breeding programs (Figure 4.8).

In the comprehensive analysis of biochemical traits through PCA biplots, it was discerned that certain key traits played pivotal roles in contributing to the maximum variance within the dataset. Specifically, under control (T0), the biochemical traits MDA, APX and SOD prominently stood out, as reflected by their exceptionally long vectors in the PCA biplot. Moving forward in the stress timeline, Protein took precedence, exhibiting the lengthiest vector in T1, indicating its significant contribution to the observed variance. Notably, as the stress progressed to T2 and T3, Proline emerged as a dominant contributor, characterized by the longest vectors in these respective time points.

We obtained a total of eight eigenvalues, representing the principal components, with varying degrees of contribution to the overall variance (Table 4.5). Out of 8 principal components of eigenvalues, the first three PCs found with eigenvalues larger than 1 under normal and stress conditions were selected. The other three PCs data were considered non-significant and were not useable for further analysis due to eigenvalues less than 1. The first three PCs showed 95.96% of the total variation under normal conditions (T0). Under cold acclimation (T1), the first three PCs accounted for 92.73% of the total variation. Meanwhile, under cold stress after acclimation (T2), the first three PCs showed 91.96% of the total variation and under cold stress without acclimation, the first three PCs showed 95.93% of the total variation. The 1st PCs accounted for 85.60% of the variance at T0, 75.79% of the variance at T1, 72.96% of the variance at T2 and 82.93% of the variance at T3 (Table 4.5).

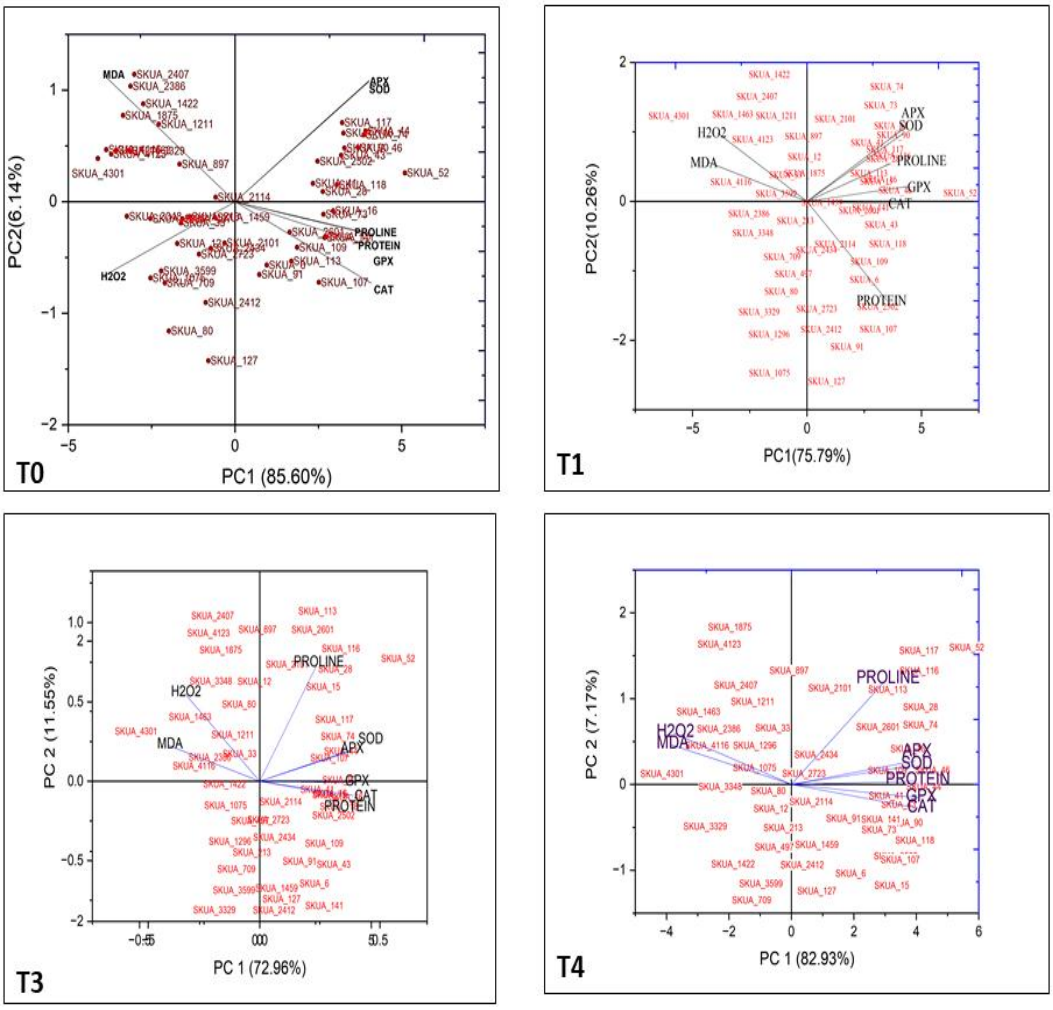


Fig: 4.8: **Biplots between the genotypes and various studied biochemical traits under normal conditions (T0), acclimation phase at 4℃ for 14 days (T1), cold stress at -5℃ after acclimation (T2) and cold stress at -5℃ without acclimation treatments (T3).**

Table- 4.5: Eigenvalues, variability and cumulative of wheat seedling traits normal conditions (T0), acclimation phase at 4℃ for 14 days (T1), cold stress at -5℃ after acclimation (T2) and cold stress at -5℃ without acclimation treatments (T3)

	Treatment	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Eigen Vector	T0	4.17	2.33	1.18	0.13	0.11	0.05	0.03	0.00
	T1	3.30	2.66	1.45	0.30	0.14	0.09	0.05	0.01
	T2	3.84	1.92	1.60	0.30	0.17	0.12	0.05	0.00
	T3	4.63	1.57	1.44	0.16	0.09	0.07	0.03	0.00
Percentage of Variance (%)	T0	85.60%	6.14%	4.22%	1.68%	1.48%	0.56%	0.27%	0.05%
	T1	75.79%	10.27%	6.67%	3.70%	1.78%	1.06%	0.59%	0.14%
	T2	72.96%	11.55%	7.45%	3.76%	2.14%	1.45%	0.66%	0.03%
	T3	82.93%	7.17%	5.49%	1.96%	1.19%	0.83%	0.39%	0.04%
Cumulative (%)	T0	85.60%	91.74%	95.96%	97.64%	99.12%	99.68%	99.95%	100.00%
	T1	75.79%	86.06%	92.73%	96.43%	98.21%	99.27%	99.86%	100.00%
	T2	72.96%	84.51%	91.96%	95.72%	97.86%	99.31%	99.97%	100.00%
	T3	82.93%	90.10%	95.59%	97.55%	98.73%	99.57%	99.96%	100.00%

4.2.5 Comparison between cold tolerant and susceptible genotypes

To identify cold-tolerant lines among the genotypes studied, several key criteria were considered based on the field cold screening data, ELI and biochemical data. First, genotypes exhibiting lower cold score values (0) and low EC values were regarded as having improved cold tolerance. Additionally, the selection process favored genotypes demonstrating low MDA value, low H_20_2 content and higher activities of important antioxidant enzymes, including APX, SOD, CAT and GPX, as these enzymes play a crucial role in mitigating oxidative damage. Last, genotypes characterized by increased accumulation of proline, a compatible solute with cryoprotective properties, were given preference. For cold-susceptible genotypes, certain characteristics were used to distinguish them from cold-tolerant lines. These included higher cold score values, indicating increased susceptibility to cold stress and elevated EC values, indicating compromised membrane stability under cold conditions. Furthermore, cold-susceptible genotypes exhibited higher levels of MDA and H_2O_2, indicating higher oxidative damage. The activities of antioxidant enzymes, such as APX, SOD, CAT and GPX, were comparatively lower in cold-susceptible genotypes, suggesting a reduced capacity to counteract oxidative stress. By employing these criteria, the study aimed to identify the most 5 promising cold-tolerant lines and 5 cold-susceptible genotypes under different (Table 4.6 and Table 4.7**)**. Furthermore, the comparison between cold-tolerant and cold-susceptible genotypes based on the data obtained in this study revealed significant differences between cold-tolerant and cold-susceptible genotypes in response to cold stress conditions. Overall, cold-tolerant genotypes exhibited higher levels of antioxidants, such as SOD CAT, APX, GPX, proline, while as, in cold susceptible genotypes the opposite trend was observed. The box plots revealed significant differences between the tolerant and susceptible genotypes under different cold treatments. For Treatment T1 and T2, the tolerant genotype exhibited higher protein levels, proline and antioxidants enzymatic activity as compared to the susceptible genotypes. This

indicates genotype-dependent variations in these biochemical expression in response to different treatments. Furthermore, the susceptible genotype showed increased levels of malondialdehyde (MDA), H_2O_2, as compared to tolerant genotypes, in all the treatments indicating higher oxidative stress, compared to the tolerant genotypes. These findings suggest that the susceptible genotype may be more susceptible to oxidative damage under the given experimental conditions.

Table-4.6: List of cold tolerant genotypes selected on the basis of various biochemical parameters recorded under normal conditions (T0), acclimation phase at 4℃ for 14 days (T1), cold stress at -5℃ after acclimation (T2) and cold stress at -5℃ without acclimation treatments (T3)

Treatment	Genotypes	Protein	Genotypes	MDA	Genotypes	H_2O_2	Genotypes	APX	Genotypes	Proline	Genotypes	SOD	Genotypes	CAT	Genotypes	GPX
T0	SKUA_52	10.82	SKUA_52	0.69	SKUA_46	2.24	SKUA_52	412.18	SKUA_52	5.85	SKUA_52	608.18	SKUA_52	358.00	SKUA_52	202.20
	SKUA_44	9.77	SKUA_107	1.44	SKUA_44	2.47	SKUA_44	399.68	SKUA_74	4.81	SKUA_44	597.68	SKUA_43	353.00	SKUA_118	197.10
	SKUA_116	9.74	SKUA_41	1.52	SKUA_52	2.52	SKUA_74	389.68	SKUA_16	4.61	SKUA_74	585.68	SKUA_90	346.35	SKUA_74	196.00
	SKUA_46	9.47	SKUA_44	1.55	SKUA_43	2.52	SKUA_46	380.90	SKUA_117	4.51	SKUA_90	569.18	SKUA_141	343.60	SKUA_46	195.60
	SKUA_43	9.43	SKUA_73	1.58	SKUA_74	2.60	SKUA_117	377.90	SKUA_90	4.49	SKUA_46	562.90	SKUA_44	343.10	SKUA_43	194.00
T1	SKUA_52	13.03	SKUA_52	8.32	SKUA_52	6.18	SKUA_52	514.18	SKUA_52	11.52	SKUA_52	814.54	SKUA_52	603.20	SKUA_52	226.00
	SKUA_127	12.33	SKUA_118	8.45	SKUA_46	7.52	SKUA_44	496.68	SKUA_116	9.69	SKUA_44	788.03	SKUA_90	596.35	SKUA_44	221.00
	SKUA_6	12.28	SKUA_16	8.65	SKUA_109	8.01	SKUA_74	491.68	SKUA_74	9.63	SKUA_90	775.54	SKUA_73	575.60	SKUA_74	220.40
	SKUA_91	12.23	SKUA_109	8.73	SKUA_107	8.27	SKUA_117	476.90	SKUA_28	9.41	SKUA_74	747.04	SKUA_46	575.00	SKUA_118	220.30
	SKUA_107	12.00	SKUA_90	8.96	SKUA_141	8.34	SKUA_90	468.18	SKUA_2101	9.14	SKUA_46	744.69	SKUA_141	571.60	SKUA_46	220.20
T2	SKUA_52	26.26	SKUA_52	3.64	SKUA_52	3.51	SKUA_52	621.68	SKUA_52	17.19	SKUA_52	1214.54	SKUA_52	845.20	SKUA_74	255.40
	SKUA_6	24.61	SKUA_109	4.11	SKUA_109	3.92	SKUA_44	599.68	SKUA_116	15.36	SKUA_44	1194.03	SKUA_90	828.35	SKUA_52	250.20
	SKUA_127	24.51	SKUA_46	4.40	SKUA_141	4.20	SKUA_74	599.18	SKUA_113	15.30	SKUA_74	1170.04	SKUA_44	809.10	SKUA_118	249.30
	SKUA_91	24.36	SKUA_118	4.78	SKUA_91	4.21	SKUA_117	582.40	SKUA_74	15.08	SKUA_90	1136.54	SKUA_73	807.60	SKUA_46	249.20
	SKUA_107	24.16	SKUA_16	4.81	SKUA_46	4.31	SKUA_90	571.18	SKUA_44	14.81	SKUA_46	1124.69	SKUA_74	807.00	SKUA_43	247.00
T3	SKUA_52	12.49	SKUA_52	7.33	SKUA_52	13.99	SKUA_52	437.18	SKUA_52	7.88	SKUA_52	661.18	SKUA_52	365.20	SKUA_52	210.50
	SKUA_43	11.27	SKUA_109	7.97	SKUA_43	14.29	SKUA_44	417.68	SKUA_46	7.20	SKUA_44	633.68	SKUA_43	364.00	SKUA_74	210.40
	SKUA_116	11.06	SKUA_46	8.85	SKUA_46	14.35	SKUA_74	414.68	SKUA_113	7.12	SKUA_74	626.68	SKUA_90	357.35	SKUA_118	209.30
	SKUA_44	11.01	SKUA_118	9.61	SKUA_16	14.41	SKUA_46	397.90	SKUA_116	7.04	SKUA_90	622.18	SKUA_44	355.10	SKUA_46	209.20
	SKUA_117	10.86	SKUA_16	9.87	SKUA_107	15.09	SKUA_90	389.18	SKUA_117	6.62	SKUA_46	611.90	SKUA_141	354.60	SKUA_43	206.20

Table-4.7: **List of Cold susceptible genotypes selected on the basis of various biochemical parameters recorded under under normal conditions (T0), acclimation phase at 4℃ for 14 days (T1), cold stress at -5℃ after acclimation (T2) and cold stress at -5℃ without acclimation treatments (T3).**

Treatment	Genotypes	Protein	Genotypes	MDA	Genotypes	H202	Genotypes	APX	Genotypes	Proline	Genotypes	SOD	Genotypes	CAT	Genotypes	GPX
T0	SKUA_4301	4.12	SKUA_1463	7.74	SKUA_33	8.06	SKUA_3348	130.68	SKUA_2386	2.15	SKUA_3348	312.68	SKUA_1463	222.55	SKUA_4116	109.35
	SKUA_1422	4.40	SKUA_4123	7.82	SKUA_1463	8.09	SKUA_709	121.18	SKUA_1875	2.13	SKUA_80	306.90	SKUA_3348	221.10	SKUA_1463	108.00
	SKUA_1463	4.55	SKUA_4116	7.87	SKUA_3348	8.20	SKUA_80	110.90	SKUA_3329	2.07	SKUA_4301	306.68	SKUA_3329	220.60	SKUA_4123	106.55
	SKUA_3599	4.59	SKUA_1875	8.06	SKUA_4116	8.60	SKUA_4301	108.68	SKUA_1422	2.06	SKUA_1463	305.18	SKUA_4116	219.35	SKUA_2386	105.10
	SKUA_2386	4.60	SKUA_4301	8.09	SKUA_4301	8.87	SKUA_1463	99.90	SKUA_1463	2.05	SKUA_1075	297.90	SKUA_4301	215.95	SKUA_4301	101.95
T1	SKUA_1296	8.69	SKUA_1211	16.06	SKUA_2407	11.84	SKUA_709	220.18	SKUA_2386	6.27	SKUA_3599	505.04	SKUA_2407	452.00	SKUA_1875	132.05
	SKUA_1422	8.64	SKUA_1463	16.16	SKUA_1463	12.09	SKUA_3348	215.68	SKUA_4301	6.25	SKUA_3348	503.03	SKUA_1463	449.55	SKUA_3329	130.80
	SKUA_3599	8.28	SKUA_2386	16.57	SKUA_3348	12.20	SKUA_1463	212.90	SKUA_1422	6.07	SKUA_1463	488.69	SKUA_3348	446.20	SKUA_4123	129.75
	SKUA_1463	8.24	SKUA_4116	17.43	SKUA_4116	12.60	SKUA_4301	205.68	SKUA_3329	6.03	SKUA_1075	479.69	SKUA_2386	443.10	SKUA_2386	128.30
	SKUA_4301	7.81	SKUA_4301	17.65	SKUA_4130	12.87	SKUA_1075	196.90	SKUA_1463	5.80	SKUA_4301	468.04	SKUA_4301	429.00	SKUA_4301	122.15
T2	SKUA_1296	17.48	SKUA_4116	12.44	SKUA_2407	8.63	SKUA_709	325.68	SKUA_3348	10.44	SKUA_3348	624.03	SKUA_41	689.10	SKUA_2412	161.60
	SKUA_1422	17.13	SKUA_2407	12.50	SKUA_3348	8.98	SKUA_3348	320.68	SKUA_1422	10.39	SKUA_1463	612.69	SKUA_2407	687.35	SKUA_3348	160.80
	SKUA_3599	16.38	SKUA_2386	12.75	SKUA_1463	9.09	SKUA_1463	320.40	SKUA_3348	10.32	SKUA_4301	612.04	SKUA_2412	684.00	SKUA_2407	159.30
	SKUA_4301	16.31	SKUA_4301	13.31	SKUA_4301	10.20	SKUA_4301	308.68	SKUA_1463	10.08	SKUA_709	608.54	SKUA_4301	675.95	SKUA_4116	158.75
	SKUA_1463	15.37	SKUA_1463	14.31	SKUA_52	13.99	SKUA_1075	299.90	SKUA_4301	9.99	SKUA_1075	594.69	SKUA_1463	675.35	SKUA_4301	153.15
T3	SKUA_1211	6.22	SKUA_4123	25.02	SKUA_1463	29.09	SKUA_3348	147.68	SKUA_4116	3.59	SKUA_709	358.18	SKUA_1463	234.35	SKUA_2407	121.60
	SKUA_1422	6.07	SKUA_2407	25.10	SKUA_4116	29.60	SKUA_1075	135.90	SKUA_1422	3.57	SKUA_80	355.90	SKUA_4123	232.55	SKUA_1875	120.05
	SKUA_3599	5.83	SKUA_2386	25.55	SKUA_4123	29.75	SKUA_709	132.18	SKUA_709	3.23	SKUA_3348	348.68	SKUA_3329	231.60	SKUA_1463	118.75
	SKUA_4301	5.79	SKUA_1463	26.87	SKUA_1875	30.76	SKUA_4301	126.68	SKUA_4301	2.43	SKUA_1463	347.68	SKUA_2386	231.10	SKUA_2386	118.30
	SKUA_1463	5.36	SKUA_4301	28.87	SKUA_4301	31.87	SKUA_1463	117.90	SKUA_1463	2.39	SKUA_4301	346.90	SKUA_4301	226.95	SKUA_4301	112.15

4.3 Comparative metabolome and lipidome profiling in contrasting wheat genotypes

Based on the results of cold screening in the field and biochemical screening, we selected two contrasting genotypes, SKUA_52 and SKUA_4301, for further metabolomic and lipidomic analysis. SKUA_52 exhibited cold tolerance, while SKUA_4301 was susceptible to cold stress. Metabolomic and lipidomic analyses were performed to investigate the metabolic and lipid profiles of these genotypes under control conditions (25℃) and under cold stress at -5℃ after acclimation for 14 days at 4℃.

4.3.1 Metabolomics results

In the metabolomics analysis, we focused on studying the defense hormone profiles of Salicylic Acid (SA), Jasmonic Acid (JA), Abscisic Acid (ABA), JA-Isoleucine (JA-ILE) and cis-Octadecanoid-derivative (cis-OPDA) and flavonoid content of the selected genotypes using liquid chromatography-mass spectrometry (LC-MS) and untargeted metabolomic analysis using gas chromatography-mass spectrometry (GC-MS) between contrasting genotypes viz, SKUA_52 (cold tolerant) and SKUA_4301 (cold susceptible) under control conditions (25℃) and under cold stress at -5℃ after acclimation for 14 days at 4℃. The results of metabolomic analysis are presented under the following headings:

4.3.1.1 Defense harmone analysis

The analysis of defense harmones revealed variations in hormone levels among the cold tolerant and cold susceptible genotypes and across the control (25℃) and -5℃ after acclimation for 14 days at 4℃ (Figure 4.9).

Among the studied defense hormones, Salicylic Acid (SA) exhibited notable differences between the genotypes. Under cold stress, SKUA_52 demonstrated significantly higher SA concentration (748.90 ng/g) compared to SKUA_4301 (208.13 ng/g). Similarly, Jasmonic Acid (JA) levels followed a

similar trend, with SKUA_52 (355.11 ng/g) surpassing SKUA_4301 (207.97 ng/g) under cold stress. Likewise, Abscisic Acid (ABA) concentrations also reflected the divergent responses, with SKUA_52 (145.01 ng/g) showing a markedly higher level than SKUA_4301 (33.80 ng/g) under cold stress.

Further, the analysis revealed that JA-Isoleucine (JA-ILE) and cis-Octadecanoid-derivative (cis-OPDA) exhibited similar trends. SKUA_52 displayed elevated concentrations of JA-ILE (330.59 ng/g) and cis-OPDA (96.13 ng/g) under cold stress, outperforming SKUA_4301 (JA-ILE: 115.27 ng/g, cis-OPDA: 29.90 ng/g).

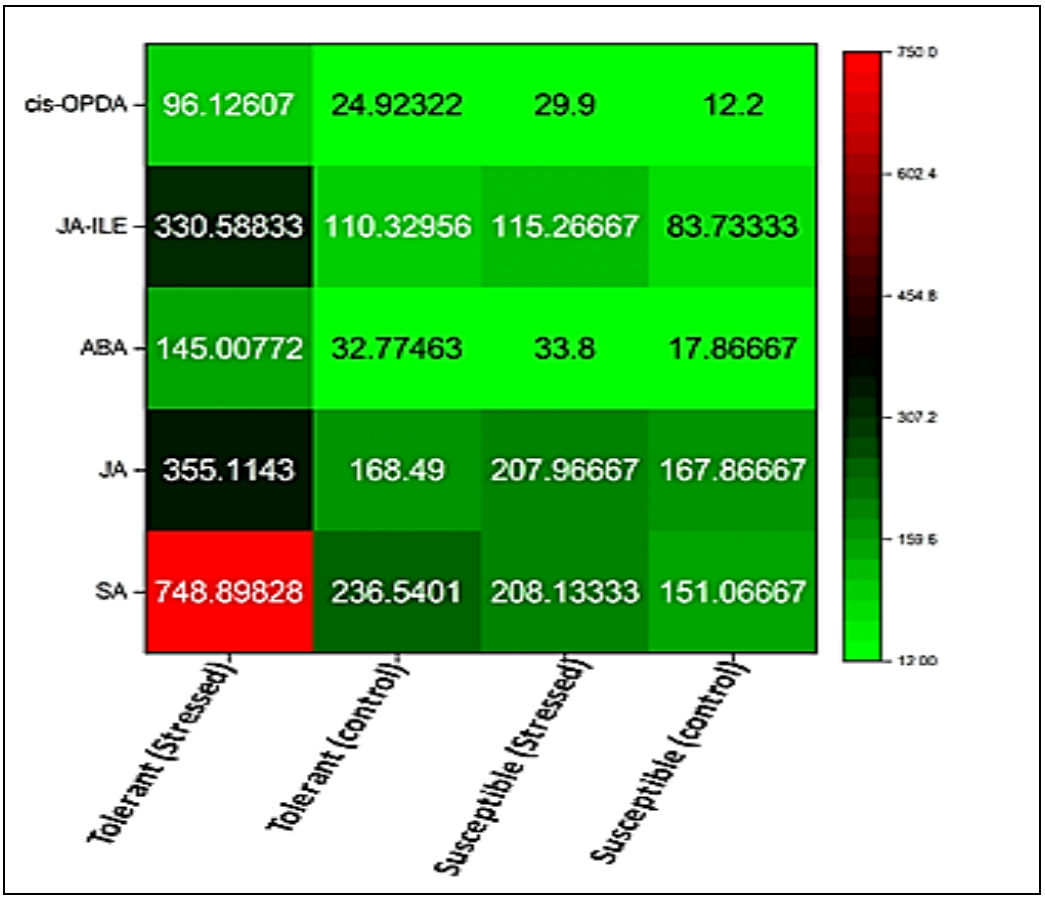


Fig. 4.9: **Heat map representing defense hormone concentrations in cold-tolerant (SKUA_52) and cold-susceptible (SKUA_4301) wheat genotypes under cold stress (-5℃) and control conditions (25℃) and. Color intensity reflects hormone levels, with warmer colors indicating higher concentrations.**

The comparative analysis of defence hormones was also conducted to investigate the variations and relationships between these hormone levels within and between the contrasting genotypes under control and cold stress conditions. The study included four specific comparisons: SKUA_52 (Stressed) vs SKUA_52 (Control), SKUA_52 (Stressed) vs SKUA_4301 (Stressed), SKUA_4301 (Stressed) vs SKUA_4301 (Control) and SKUA_52 (Control) vs SKUA_4301 (Control). The results of the comparative analysis of defence hormone levels revealed significant differences in fold changes (FC) between different genotypes and treatments (Figure 4.10). Specifically, when comparing the genotype SKUA_52 (Stressed) to SKUA_52 (Control), the fold changes for SA, JA, ABA, JA-ILE and cis-OPDA was 3.16, 2.10, 4.42, 2.99 and 3.85, respectively. Similarly, when comparing SKUA_4301 (Stressed) to SKUA_4301 (Control) the fold changes for SA, JA, ABA, JA-ILE and cis-OPDA was 1.37, 1.23, 1.89, 1.37 and 2.45, respectively. Comparing SKUA_52 (Stressed) to SKUA_4301 (Stressed) revealed fold changes of 3.59, 1.70, 4.29, 2.86 and 3.214 for SA, JA, ABA, JA-ILE and cis-OPDA, respectively. Lastly, comparing SKUA_52 (Control) to SKUA_4301 (Control) showed fold changes of 1.56, 1.00, 1.83, 1.31 and 2.04 for SA, JA, ABA, JA-ILE and cis-OPDA, respectively. These fold changes indicate significant differences in defense hormone levels between the control conditions of SKUA_52 and SKUA_4301 genotypes (Figure 4.10).

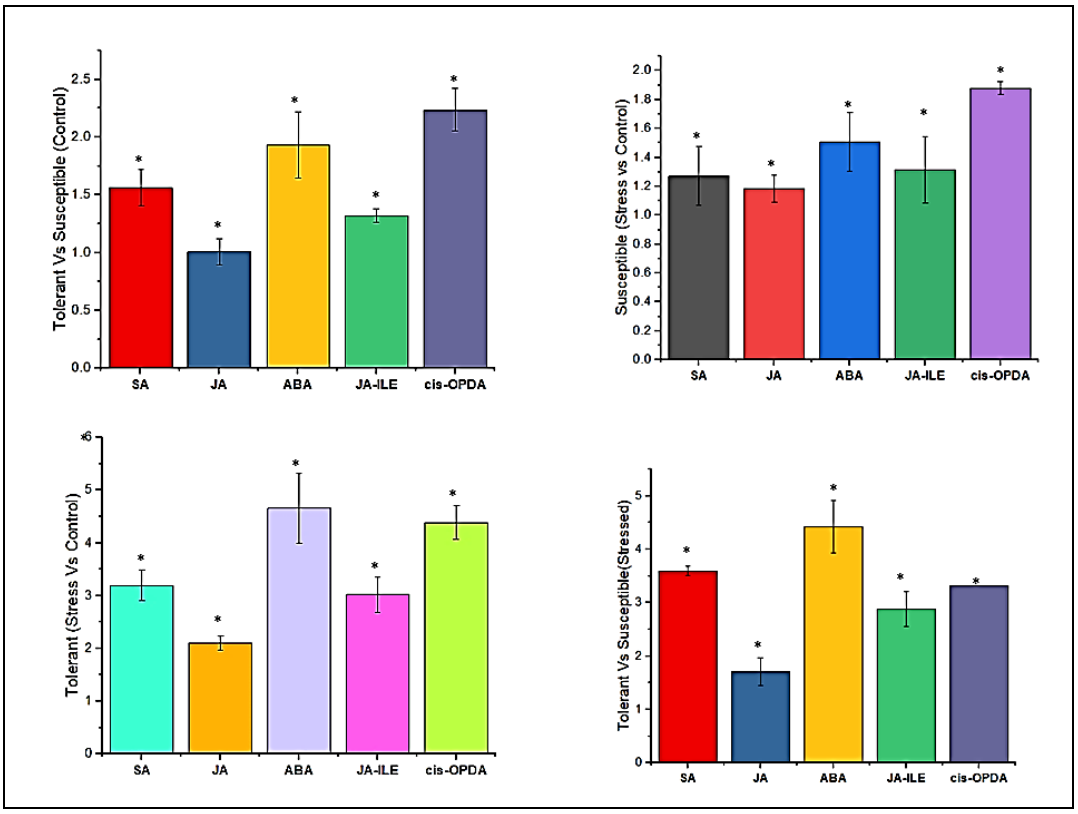


Fig. 4.10: Bar plots depicting fold changes in defense hormone concentrations between cold-tolerant (SKUA_52) and cold-susceptible (SKUA_4301) wheat genotypes under cold stress (-5℃) and control conditions (25℃)

4.3.2 Flavonoid analysis

The LC-MS analysis of flavonoids was performed to investigate the concentrations and differential expression of flavonoids, including Naringenin, Rutin, Quercitin, Petunidin, Peonidin and Malvidin, between contrasting genotypes viz, SKUA_52 and SKUA_4301 under control conditions (25℃) and under cold stress at -5℃ after acclimation for 14 days at 4℃. Among the investigated flavonoids, Naringenin, Rutin, Quercitin, Petunidin, Peonidin,and Malvidin, showed differential accumulation patterns across genotypes and treatment**s.** Notably, the cold-tolerant genotype SKUA_52 under cold stress consistently displayed higher levels of flavonoids compared to the cold-susceptible genotype (4301_Stressed and 4301_Control). Specifically, Naringenin

exhibited a higher level in SKUA_52_Stressed (0.15 ng/mg) compared to both SKUA_4301_stressed (0.025 ng/mg) and 4301_control (0.018 ng/mg). Rutin also showed a higher level in SKUA_52_Stressed (0.072 ng/mg) compared to SKUA_4301_Stressed (0.024 ng/mg) and SKUA_4301_Control (0.032 ng/mg). Similarly, Quercitin exhibited higher levels in SKUA_52_Stressed (0.251 ng/mg) compared to both SKUA_4301_Stressed (0.028 ng/mg) and SKUA_4301_stressed (0.019 ng/mg). Petunidin, Peonidin and Malvidin also showed higher levels in SKUA_52_Stressed compared to the SKUA_4301_Stressed (Figure 4.11).

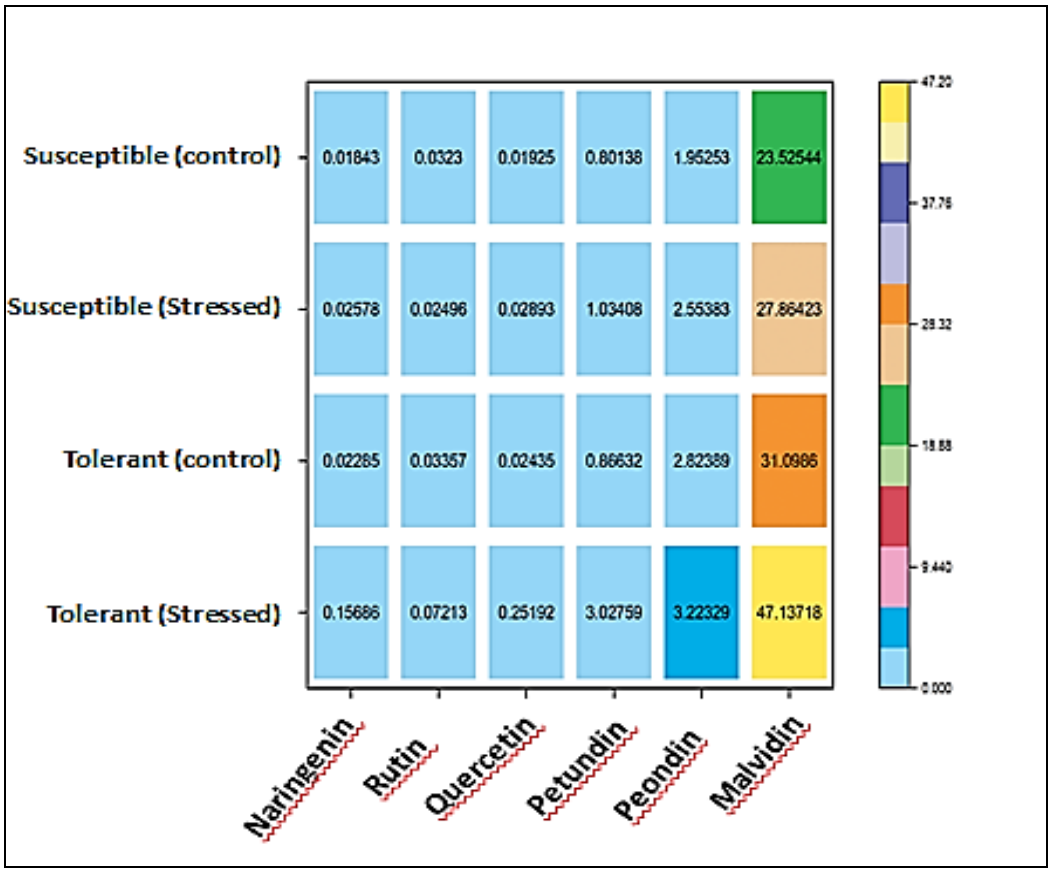


Fig: 4.11: **Heat map representing flavonoid concentrations in cold-tolerant (SKUA_52) and cold-susceptible (SKUA_4301) wheat genotypes under cold stress (-5℃) and control conditions (25℃). Color intensity reflects hormone levels, with warmer colors indicating higher concentrations.**

Furthermore, we conducted comparative flavonoid analysis using fold change and determined the significance based on a p-value threshold of 0.05. Importantly, we observed significant up-regulation or down-regulation in all the comparisons conducted (Figure 4.12).

The analysis of “Naringenin” in the comparison of SKUA_52 (Stressed) vs SKUA_52 (Control), revealed a significant fold change of 2.7. Additionally, when comparing SKUA_52 (Stressed) vs SKUA_4301 (Stressed), a fold change of 2.5 was observed. In the comparison of SKUA_4301 (Stressed) vs SKUA_4301 (Control), the fold change was 0.4, indicating a moderate change. Lastly, the comparison of SKUA_52 (Control) vs SKUA_4301 (Control) showed a fold change of 0.2926 (Figure 4.12).

The analysis of “Rutin” in the comparison of SKUA_52 (Stressed) vs SKUA_52 (Control) exhibited a fold change of 1.1. Similarly, in the comparison of SKUA_52 (Stressed) vs SKUA_4301 (Stressed), fold change of 1.5233 indicated a significant difference between the genotypes. However, in the comparison of SKUA_4301 (Stressed) vs SKUA_4301 (Control), a negative fold change of -0.3446 suggested a decrease in the stressed genotype compared to the control. The comparison of SKUA_52 (Control) vs SKUA_4301 (Control) showed a slight upregulation with a fold change of 0.04 (Figure 4.12).

Similarly, “Quercitin” in the comparison of SKUA_52 (Stressed) vs SKUA_52 (Control) demonstrated a remarkable fold change of 3.37. Similarly, in the comparison of SKUA_52 (Stressed) vs SKUA_4301 (Stressed), a fold change of 3.12 was observed, indicating a significant difference between the two genotypes. In the comparison of SKUA_4301 (Stressed) vs SKUA_4301 (Control), the fold change was 0.58. The comparison of SKUA_52 (Control) vs SKUA_4301 (Control) showed a fold change of 0.35, indicating a slight difference between the control genotypes (Figure 4.12).

Another flavonoid “Petunidin” in the comparison of SKUA_52 (Stressed) vs SKUA_52 (Control) displayed a moderate fold change of 1.80. In the comparison of SKUA_52 (Stressed) vs SKUA_4301 (Stressed), a fold change of 1.54 was observed. The comparison of SKUA_4301 (Stressed) vs SKUA_4301 (Control) exhibited a log2 fold change of 0.3613. The comparison of SKUA_52 (Control) vs SKUA_4301 (Control) showed a slight upregulation with a log2 fold change of 0.1278 (Figure 4.12).

The “Peonidin” in the comparison of SKUA_52 (stressed) vs SKUA_52 (Control), a relatively low fold change of 0.17 was observed, indicating a slight upregulation in the cold-stressed genotype. Similarly, in the comparison of SKUA_52 (Stressed) vs SKUA_4301 (Stressed), a fold change of 0.35 was observed. The comparison of SKUA_4301 (Stressed) vs SKUA_4301 (Control) exhibited a fold change of 0.37. Lastly, the comparison of SKUA_52 (Control) vs SKUA_4301 (Control) showed a fold change of 0.5141 (Figure 4.12).

Lastly the flavonoid “Malvidin” in the comparison of SKUA_52 (Stressed) vs SKUA_52 (Control) displayed a fold change of 0.92. In the comparison of SKUA_52 (Stressed) vs SKUA_4301 (Stressed), a fold change of 0.75 was observed. The comparison of SKUA_4301 (Stressed) vs SKUA_4301 (Control) exhibited a fold change of 0.2 was observed. The comparison of SKUA_52 (Control) vs SKUA_4301 (Control) showed a fold change of 0.37 (Figure 4.12).

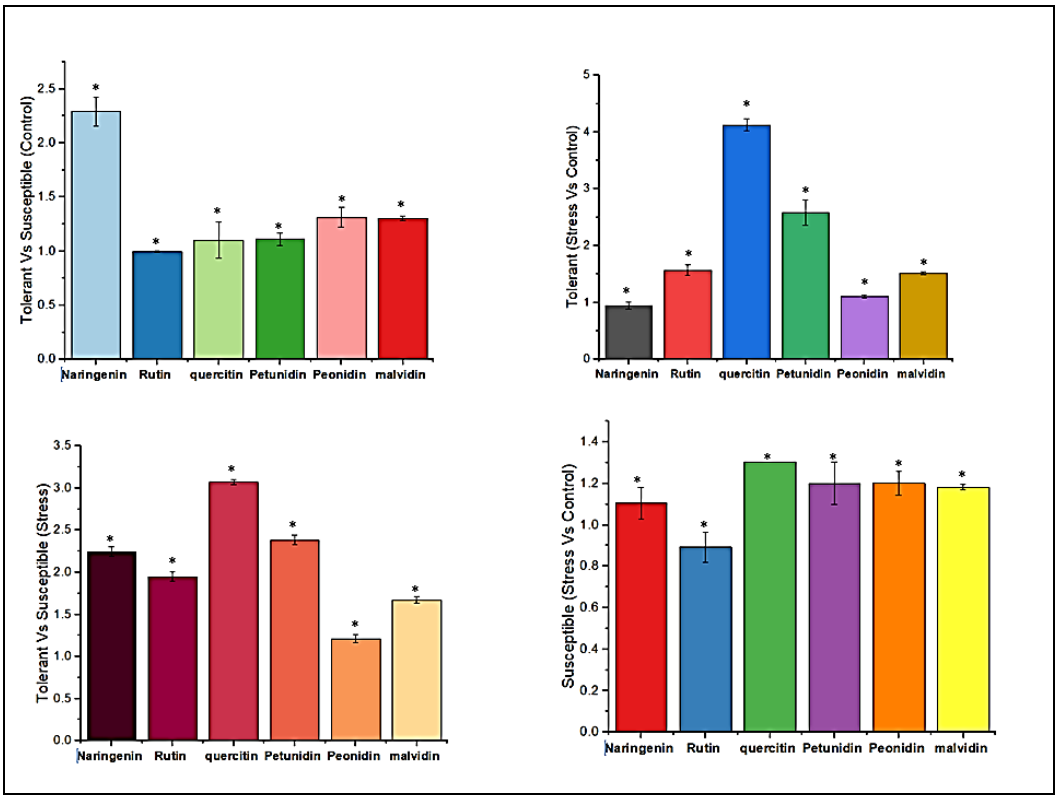


Fig: 4.12: Bar plots depicting fold changes in flavonoid concentrations between cold-tolerant (SKUA_52) and cold-susceptible (SKUA_4301) wheat genotypes under cold stress (-5℃) and control conditions (25℃).

4.3.3 Untargeted analysis of metabolites by GC-MS

In this study, metabolomics analysis using GC-MS was conducted to investigate the metabolic differences between two genotypes, SKUA-52 (cold-tolerant) and SKUA-4301 (cold-susceptible), under control conditions and after exposure to cold stress. Through this comprehensive analysis a total of 190 metabolites were identified, which belong to different compound classes (Figure 4.13). Among the compound classes identified, carbohydrates emerged as the most abundant, with a total of 43.9% compounds. Amino acids and their derivatives, comprising 7.3% compounds, were also prominently featured. The analysis further revealed the presence of 11% organic oxygen compounds, encompassing alcohols, ketones, ethers and other molecules with diverse roles in biological processes. Additionally, 8.1% compounds belonging to the class of

flavonoids were also identified. Also, 7.3% Nucleotide and its derivative metabolites, were also detected. Furthermore, several other classes of metabolites were identified. These included organic acids and derivatives, phenolamines, fatty acids and conjugates and organic acid derivatives. While less represented, classes such as alcohols, vitamins, indole and its derivatives and others were also identified.

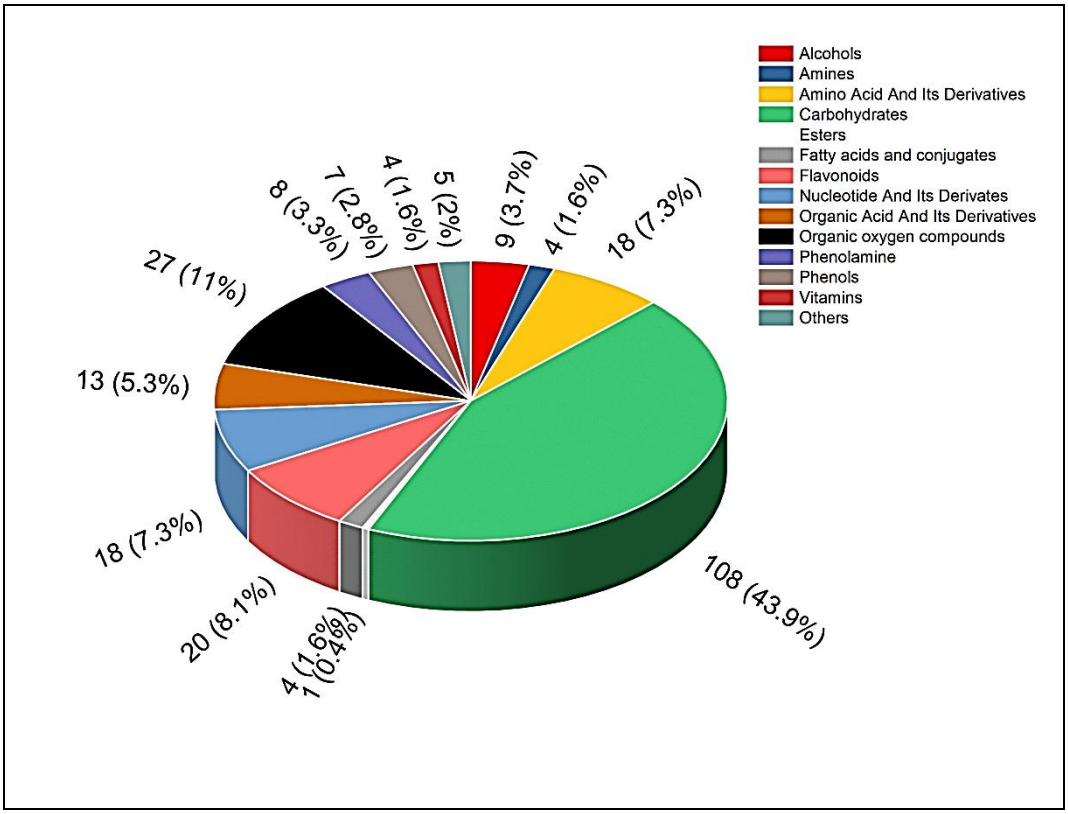


Fig. 4.13: Distribution of 190 identified metabolites across diverse compound classes in a comprehensive analysis by GC-MS.

Furthermore, to explore the specific changes in metabolite profiles under cold stress conditions and to identify genotype-specific variations in metabolite responses, the untargeted metabolite analysis in our study focused on investigating the impact of cold stress treatment on the regulation of metabolites and comparing the responses between different genotypes. These comparisons aimed to identify metabolites that exhibited differential expression in response to the treatments, using a significance threshold of p-value < 0.05.

Comparing the cold-tolerant genotype SKUA_52 under cold stress with its control conditions, a comprehensive untargeted metabolite analysis revealed significant differential expression of 66 metabolites (Annexure II). This indicates that the cold stress treatment had a considerable impact on the metabolomic profile of SKUA_52. Among these metabolites, 43 showed up-regulation, indicating an increase in their abundance, while 23 showed down-regulation. The differentially expressed metabolites spanned various compound classes, including carbohydrates, amino acids, flavonoids, nucleotides and organic compounds. Notably, sucrose exhibited the highest log2 fold change (+0.9), followed by glyceraldehyde and fructose (+0.8). Oxyproline, an amino acid, showed a significant increase of 2-fold under cold stress. Flavonoids, such as Naringenin 7-O-glucoside and Chrysoeriol, were also found to be up-regulated by 1.5-fold. Additionally, nucleotides, organic compounds and phenolamines exhibited both up-regulation and down-regulation in response to cold stress in SKUA_52. These findings highlight the dynamic metabolic adaptations occurring in SKUA_52 under cold stress conditions, with specific metabolites playing key roles in response to the stressor (Figure 4.14).

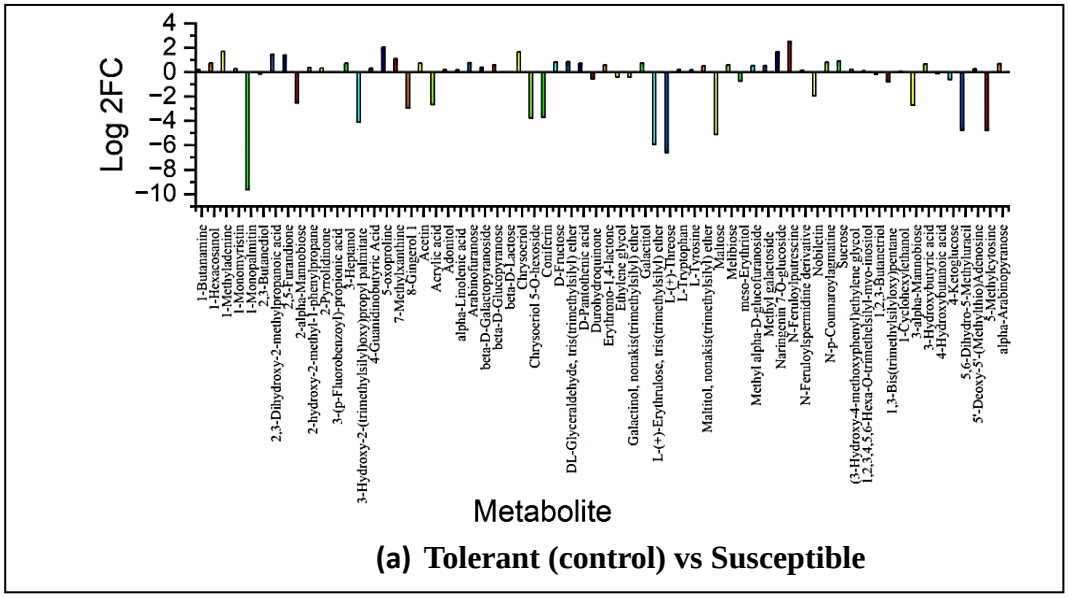
Similarly, Comparing the cold-tolerant genotype SKUA_52 with the cold-susceptible genotype SKUA_4301 under cold stress conditions, a total of 43 metabolites exhibited significant up-regulation, while 6 metabolites showed down-regulation (Annexure III). The up-regulated metabolites encompassed various compound classes, including carbohydrates, fatty acids and conjugates, flavonoids, phenolamines, nucleotides, organic compounds, amino acids and vitamins. Notably, sucrose showed the highest up-regulation with a log2 fold change of 4.5, followed by beta-D-Allopyranose and monolaurin with log2 fold changes of 3.7 and 3.5, respectively. Tricin 5-O-hexosyl-O-hexoside, N', N'', N'''-DiFeruloyl,Sinapoylspermidine and spermidine derivative were also significantly up-regulated by 3.2, 3.2 and 3.1 log2 fold changes, respectively. In contrast, several metabolites exhibited down-regulation, including talose, 5,6-Dihydro-5-

Methyluracil, diosmetin, L-(+)-Erythrulose and L-Rhamnose, with log2 fold changes ranging from -4.6 to -6.0. These findings highlight the distinct metabolic responses between the cold-tolerant and cold-susceptible genotypes under cold stress conditions, with specific metabolites playing crucial roles in adaptation to the stressor (Figure 4.14).

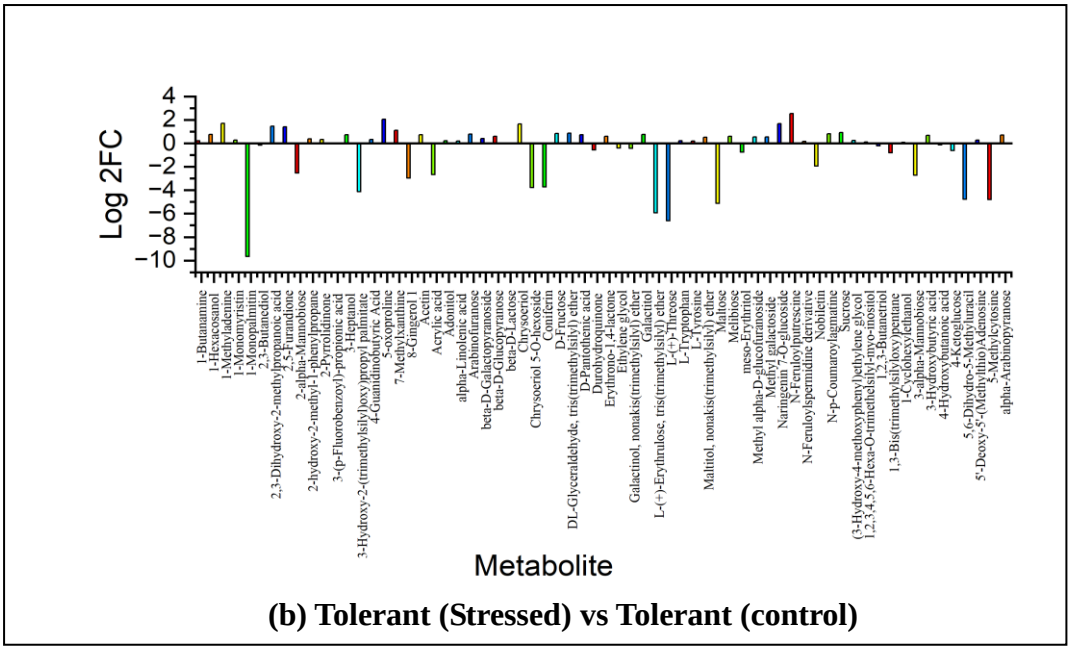
Comparing the stressed samples of the cold-susceptible genotype SKUA_4301 to the control samples, a total of 12 metabolites displayed significant up-regulation, while 29 metabolites showed down-regulation (Annexure IV). The up-regulated metabolites belonged to diverse classes, including carbohydrates, vitamins, nucleotides, amino acids and its derivatives, flavonoids, phenols and organic compounds. Notably, D-Fructose and Vitamin B2 exhibited the highest up-regulation with log2 fold changes of 2.6098 and 2.5615, respectively. Other up-regulated metabolites included Melibiose, N-Acetylglucosamine 1-phosphate, 5-Methylcytosine, L-Rhamnose and N-Acetylaspartate, with log2 fold changes ranging from 1.1 to 0.7. Conversely, numerous metabolites showed down-regulation, including D-Trehalose, Galactopyranose, D-Glucopyranose, Chrysoeriol and alpha-D-Lactose, with log2 fold changes ranging from -0.20 to -0.79. These findings highlight the distinct metabolic responses of the cold-susceptible genotype SKUA_4301 under cold stress conditions and reveal specific metabolites that may play crucial roles in its adaptation to cold stress.

Comparing the control samples of the cold-tolerant genotype SKUA_52 to the control samples of the cold-susceptible genotype SKUA_4301, a total of 60 metabolites showed significant differential expression (Annexure V). Out of these, 41 metabolites were up-regulated and 19 metabolites were down-regulated. The up-regulated metabolites spanned various classes, including carbohydrates, nucleotides and its derivatives, vitamins, phenols, amino acids and its derivatives, organic acids and derivatives, flavonoids and others. Notable up-regulated metabolites included D-Fructose, 5-Methylcytosine, N-Acetylglucosamine 1-phosphate, Melibiose and D-Lactose, with varying log2 fold changes.

Additionally, several down-regulated metabolites were observed, such as Chrysoeriol, 1-Hexacosanol, 3-Heptanol and beta-Sitosterol, also with varying log2 fold changes (Figure 4.14).



(a) Tolerant (control) vs Susceptible



(b) Tolerant (Stressed) vs Tolerant (control)

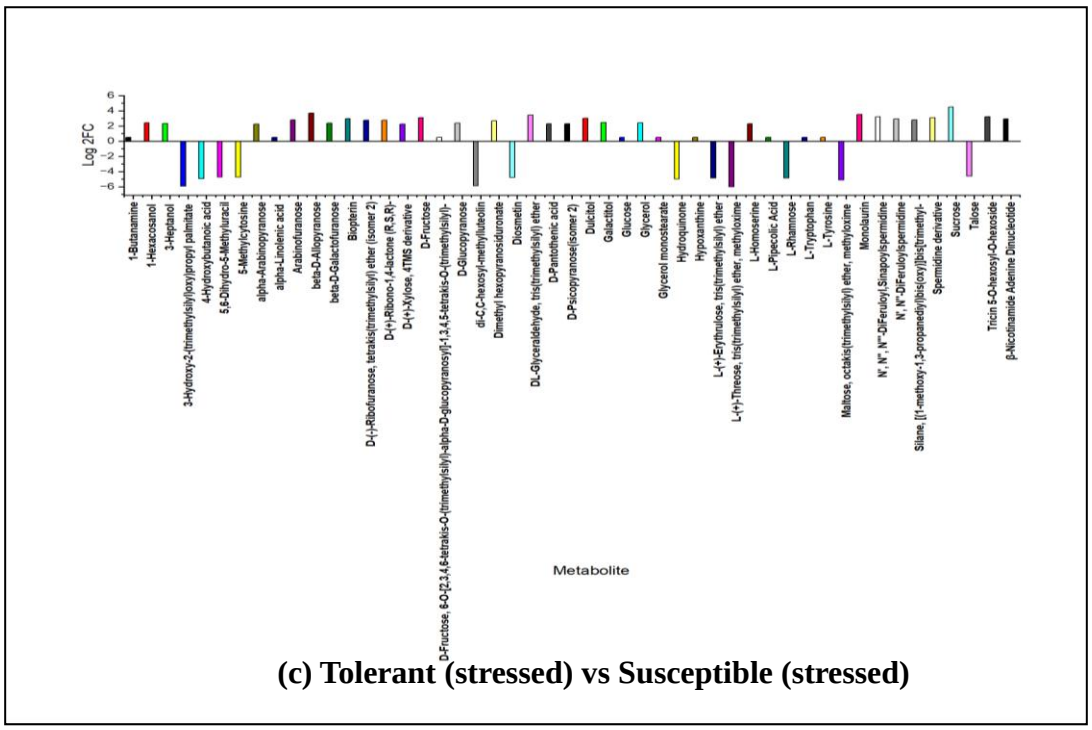


Fig. 4.14: **Bar plots depicting log_2 fold changes in metabolite profiles of (a) tolerant (control) vs susceptible (control) (b) tolerant (stressed) vs tolerant (control) (c) tolerant (stressed) vs susceptible (stressed) (d) susceptible (stressed) vs susceptible (control).**

4.4 Lipidomics in response to cold stress

In this comprehensive study, we employed lipidomics analysis by GC-MS to investigate the lipid dynamics and changes in lipid profiles in response to cold stress in two contrasting genotypes, SKUA_52 and SKUA_4301. The genotypes were selected based on their divergent cold tolerance characteristics, with SKUA_52 known for its high cold tolerance and SKUA_4301 considered more susceptible to cold stress. Based on the analysis of lipidomics data using GC-MS, a total of 90 lipids were identified. Among the lipid identified, we observed 34 lipids belonging to Phosphatidylcholines (PC), 24 lipids categorized as Phosphatidylethanolamines (PE) and 8 lipids falling under the class of Phosphatidylglycerols (PG). Additionally, our analysis unveiled 7 lipids classified as Lysophosphatidylcholines (LPC), 7 lipids identified as Phosphatidylinositols (PI), 4 lipids recognized as Digalactosyldiacylglycerols (DGDG), 3 lipids attributed to Monogalactosyldiacylglycerols (MGDG), 2 lipids belonging to Sulfoquinovosyldiacylglycerols (SQDG) and finally, 1 lipid classified as Lysophosphatidylglycerol (LPG) (Figure 4.15).

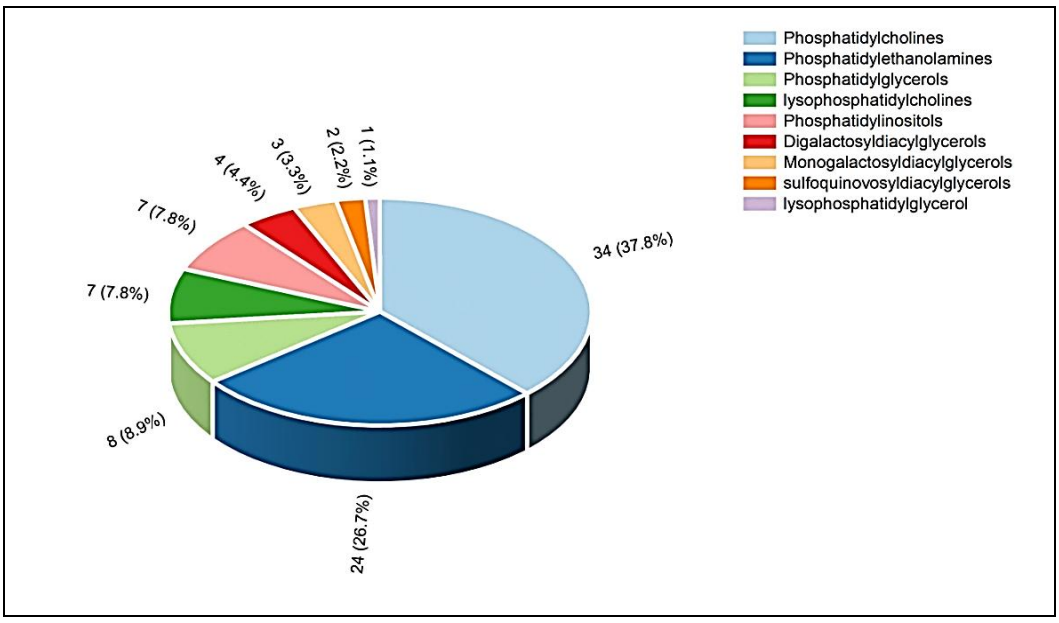


Fig. 4.15: Distribution of lipids identified in cold tolerant and susceptible genotype using GC-MS.

Comparative lipidomic analysis was done to identify differentially expressed lipid between the two genotypes under that exhibited significant changes in response to the treatments, employing a p-value threshold of <0.05. The comparative lipidomic analysis across the two different genotypes unveiled substantial variations in the expression patterns of lipids.

The lipidomic analysis comparing SKUA-52 (Control) and SKUA-4301 (Control) revealed significant differences in the expression levels of specific lipids (Annexure VI). Among the identified lipids, LPC (24:4) showed Log_2 Fold Change of 0.45), in SKUA-52 (Control) compared to SKUA-4301 (Control). Similarly, PC(32:1) exhibited Log2 Fold Change of 1.42 in SKUA-52 (Control). On the other hand, PC(33:4), PC(35:3), PC(36:6), PC(37:5), PG(34:4) and PI(36:2) showed Log2 Fold Change of -3.1, -1.0, -1.4, -0.07, -0.04, -1.86, respectively) in SKUA-52 (Control) compared to SKUA-4301 (Control).

The comparative analysis between SKUA-52 (Stressed) and SKUA-52 (Control) revealed differential expression of lipids. Among these lipids, several saturated lipids were identified, which exhibited down-regulation in response to the cold stress, as indicated by their negative log2 fold change values (Annexure VII). Specifically, LPC(16:0), PC(34:0), LPG(16:0), LPC(15:0) and PE(34:0) showed significant down-regulation, with log2 fold change values of -2.6, -2.1, -1.1, -0.2 and -0.1, respectively in SKUA-52 (Stressed). In addition to the down-regulated saturated lipids, two unsaturated lipids were also identified as being down-regulated in response to the cold stress in SKUA-52 (Stressed) compared to SKUA-52 (Control). These unsaturated lipids, PC(35:3) and PC(34:1), exhibited negative log2 fold change values of -0.08 and -0.01, respectively. In contrast to the down-regulated unsaturated lipids, several unsaturated lipids were found to be up-regulated in SKUA-52 (Stressed) compared to SKUA-52 (Control). These up-regulated unsaturated lipids include DGDG(34:3), DGDG(36:6), PC(35:2), PC(32:1), PE(32:2), PC(36:3), PC(35:1), PC(33:4), LPC(18:3), PC(36:6),

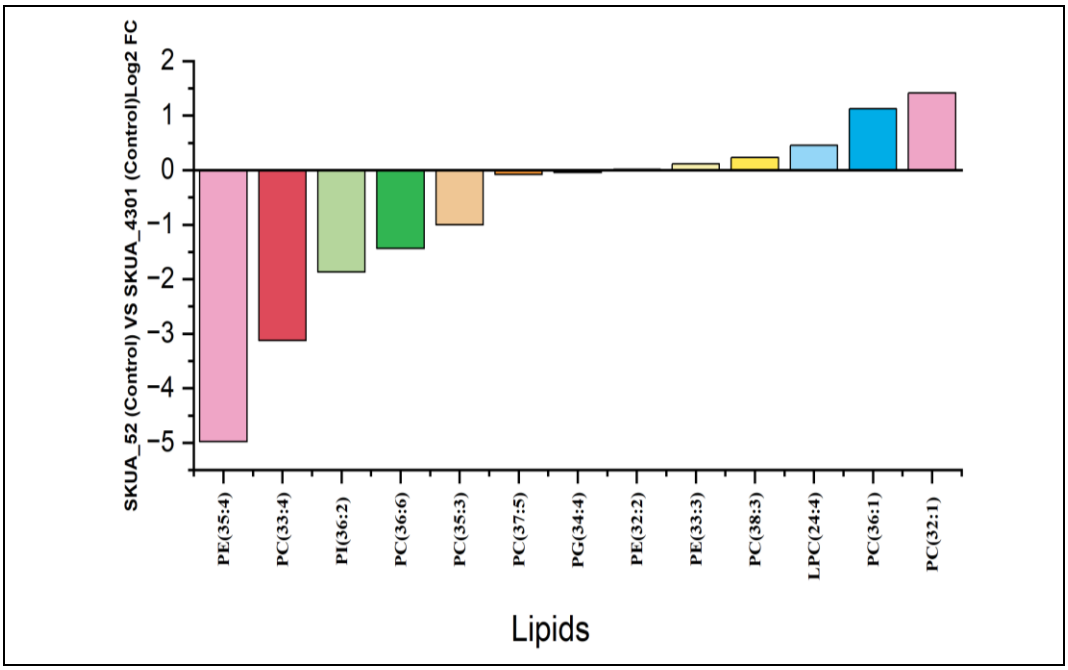
PC(34:4), PC(36:4), PC(38:2), PC(33:3), PC(32:2) and PC(33:2). These lipids exhibited positive log2 fold change values ranging from 0. 68 to 2.09.

The comparative analysis between the susceptible genotype SKUA-4301 under cold stress (Stressed) and SKUA-4301 without cold stress (Control) revealed differential expression of 37 lipids, all of which are unsaturated lipids (Annexure VIII). Among these lipids, 28 lipids exhibited down-regulation in response to cold stress. They include PC(38:6), PE(35:4), PC(38:4), SQDG(38:4), PC(39:4), DGDG(38:6), PC(35:3), PC(33:3), PG(36:7), PI(36:4), PC(37:5) and PC(39:6). On the other hand, 9 lipids demonstrated up-regulation in the susceptible genotype under cold stress. These include DGDG(36:3), PC(36:6), MGDG(36:6), PE(38:4), PC(32:0), PC(35:5), PE(36:6), PC(36:3) and PG(36:5) (Figure 4.16).

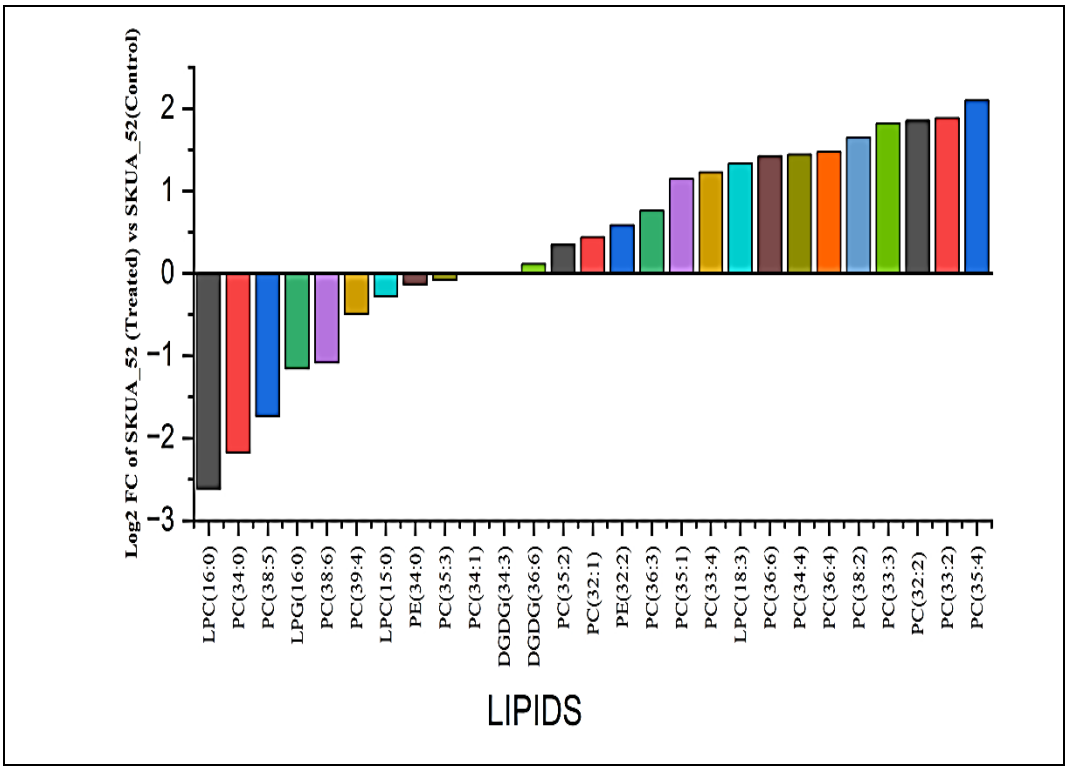
In the comparative analysis between the cold-tolerant genotype SKUA-52 after cold shock (Stressed) and the cold-susceptible genotype SKUA-4301 under cold stress (Stressed), a total of 33 differentially expressed lipids were identified (Annexure IX). These lipids exhibited significant changes in their expression levels, indicating their potential involvement in the response to cold stress. Among the differentially expressed lipids, 12 unsaturated lipids and two saturated lipids were found to be down-regulated in SKUA-52 (Stressed) compared to SKUA-4301 (Stressed). The down-regulated unsaturated lipids included PC(38:6), PE(35:4), PC(38:4), SQDG(38:4), PC(39:4), DGDG(38:6), PC(35:3), PC(33:3), PG(36:7), PI(36:4), PC(37:5) and PE(38:3). These lipids exhibited log2 fold change values ranging from -4.8 to -0.03, indicating a substantial decrease in their expression levels in SKUA-52 (Stressed) compared to SKUA-4301 (Stressed). Additionally, two saturated lipids, PC(36:0) and LPC(17:0), were also down-regulated in SKUA-52 (Stressed). These lipids showed log2 fold change values of -1.322 and -1.221, respectively, indicating a significant decrease in their expression levels compared to SKUA-4301 (Stressed) (Figure 4.16).

Conversely, 19 lipids demonstrated up-regulation in SKUA-52 (Stressed) compared to SKUA-4301 (Stressed). These up-regulated lipids included polyunsaturated lipid viz, DGDG(36:3), PC(36:6), MGDG(36:6), PE(38:4), PC(32:0), PC(35:5), PE(36:6), PC(36:3), DGDG(36:6), PE(40:4), PC(38:3), PE(34:2), PC(34:2), PC(34:3), DGDG(34:3), PC(35:4), PC(38:5), PG(34:4) and PG(36:6). These lipids exhibited log2 fold change values ranging from 0.019 to 2.582, indicating a substantial increase in their expression levels in SKUA-52(Stressed) compared to SKUA-4301 (Stressed).

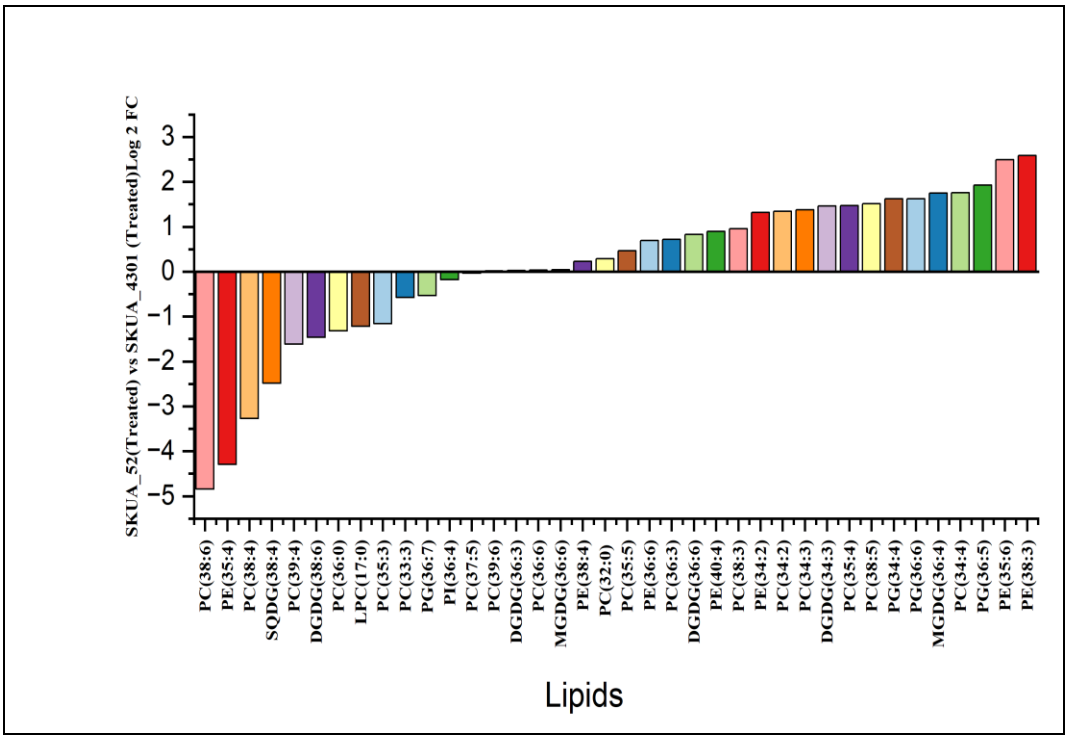
In the comparative analysis between the susceptible genotype SKUA 4301 under stress and without stress conditions, several lipids exhibited differential expression, as indicated by their log2 fold changes. These fold changes provide insights into the magnitude of the differences in lipid abundance under the respective conditions. Among the saturated lipids, PC (32:0) showed a down-regulation with a log_2 fold change of -1.4, suggesting a significant decrease in its abundance under stress conditions. On the other hand, PC (36:0) displayed a slight up-regulation with a log_2 fold change of 0.03, indicating a minor increase in its abundance. In the case of unsaturated lipids, PC (35:4), PE(40:4), PG(34:4), LPC(15:0) and LPC(16:0) exhibited different fold changes. PC (35:4) showed a small up-regulation with a log_2 fold change of 0.014228075, indicating a slight increase in its abundance under stress conditions. Similarly, PE(40:4), PG(34:4), LPC(15:0) and LPC(16:0) also displayed up-regulation with log_2 fold changes of 0.08, 0.07, 0.57 and 0.91, respectively, suggesting noticeable increases in their abundances. Additionally, other unsaturated lipids such as LPC(18:3), PC(32:1), PC(34:1), PC(35:1), PC(35:2), PE(33:1), PE(33:3), LPC(18:1), LPC(24:4), PC(36:1), PE(32:1), PE(33:2), PE(34:1), PE(38:1), PE(38:2), PE(38:6), PI(34:2), PI(36:2) and PI(36:5) exhibited various fold changes. These changes range from down-regulation to up-regulation, indicating diverse responses in their abundances under stress conditions. Notably, PI (34:2) showed a significant down-regulation with a log_2 fold change of -3.31, suggesting a substantial decrease in its abundance under stress conditions (Figure 4.16).


SKUA_52 (Control) VS SKUA_4301 (Control)Log2 FC
2
1
0
−1
−2
−3
−4
−5
PE(35:4)
PC(33:4)
PI(36:2)
PC(36:6)
PC(35:3)
PC(37:5)
PG(34:4)
PE(32:2)
PE(33:3)
PC(38:3)
LPC(24:4)
PC(36:1)
PC(32:1)
Lipids

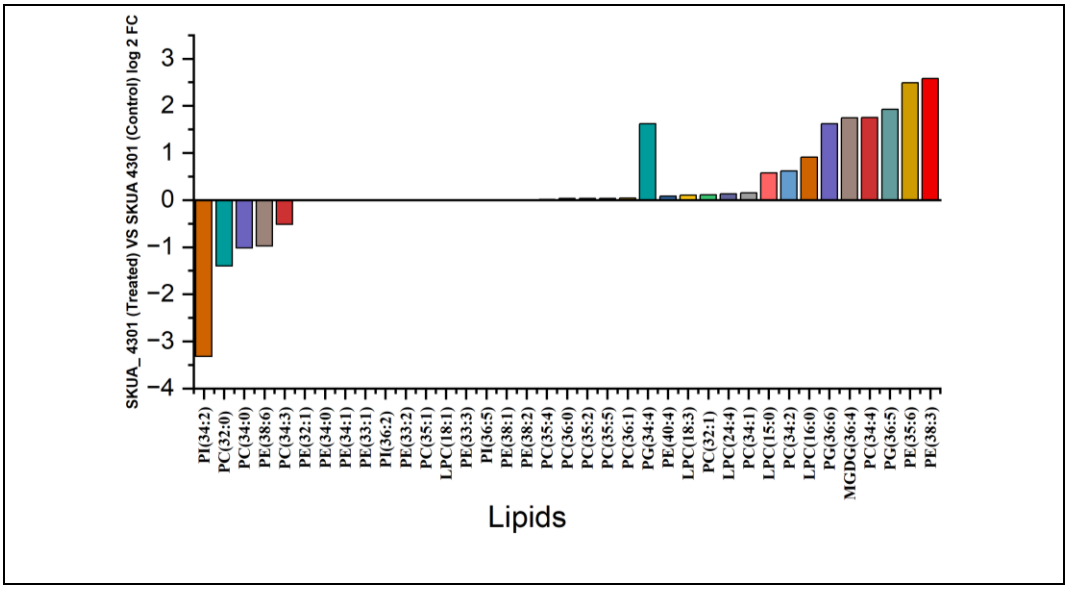
(a)


Log2 FC of SKUA_52 (Treated) vs SKUA_52(Control)
2
1
0
−1
−2
−3
LPC(16:0)
PC(34:0)
PC(38:5)
LPG(16:0)
PC(38:6)
PC(39:4)
LPC(15:0)
PE(34:0)
PC(35:3)
PC(34:1)
DGDG(34:3)
DGDG(36:6)
PC(35:2)
PC(32:1)
PE(32:2)
PC(36:3)
PC(35:1)
PC(33:4)
LPC(18:3)
PC(36:6)
PC(34:4)
PC(36:4)
PC(38:2)
PC(33:3)
PC(32:2)
PC(33:2)
PC(35:4)
LIPIDS

(b)



(c)



(d)

Fig: 4.16: **Bar plots depicting comparative log2 fold changes in significant lipids under control and cold stress conditions in (a) tolerant (control) vs susceptible (control) (b) tolerant (stressed) vs tolerant (control) (c) tolerant (stressed) vs susceptible (stressed) (d) susceptible (stressed) vs susceptible (control).**

4.5 Characterization and differential gene expression study of cold tolerance genes using qRT-PCR.

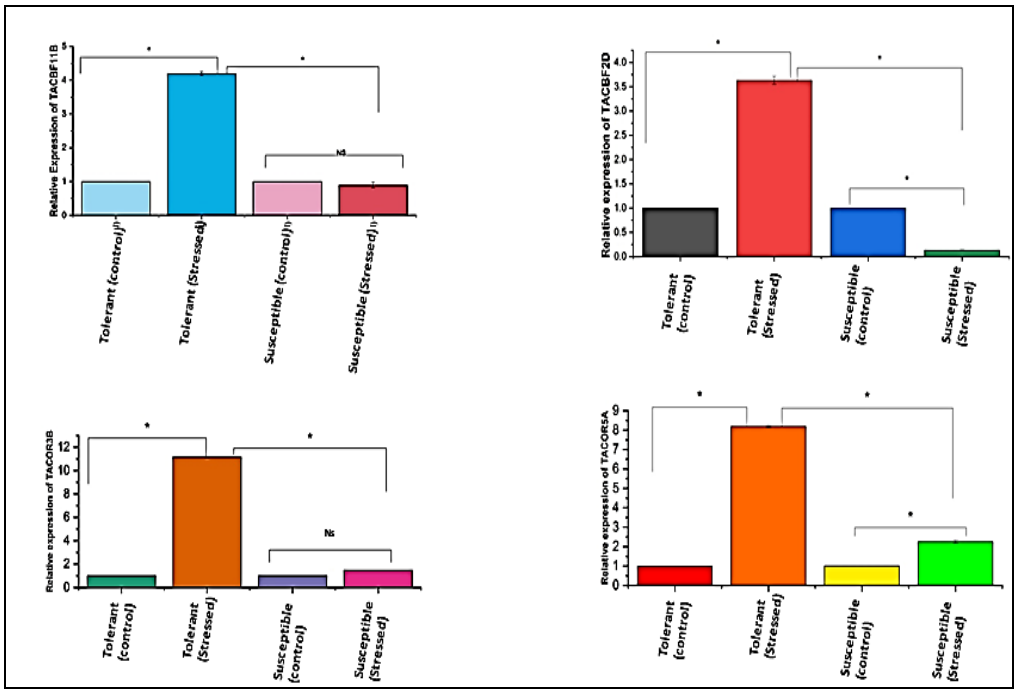
In this study, we aimed to characterize and investigate the differential expression of cold tolerance-related genes in two wheat genotypes, SKUA_52 (cold-tolerant) and SKUA_4301 (cold-susceptible), under cold stress conditions. A total of seven cold tolerance genes, including TaICE1D, TaICE2B, TaCOR5a, TaCOR3d, TaCBF11b, TaCBF2d and TaCOR3b, were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Relative gene expression levels were quantified using the ΔΔCT method and statistical significance was determined by paired t-tests at a significance level of 0.05. The qRT-PCR results for the expression levels of key genes involved in stress response revealed significant differences between the cold-tolerant genotype, SKUA_52 and the cold-susceptible genotype, SKUA_4301, under stressed conditions and their respective controls (Figure 4.17).

In SKUA_52 under cold stress, TaICE1D exhibited a substantial upregulation, indicating a 3.997-fold change compared to its control. Conversely, SKUA_4301 displayed a reduced expression of TaICE1D under stress (0.482-fold change) in comparison to its control. In addition to this, TaICE2B in SKUA_52 exhibited a significant upregulation (1.748-fold change) under stress, while in SKUA_4301, a milder upregulation (0.981-fold change) was observed.

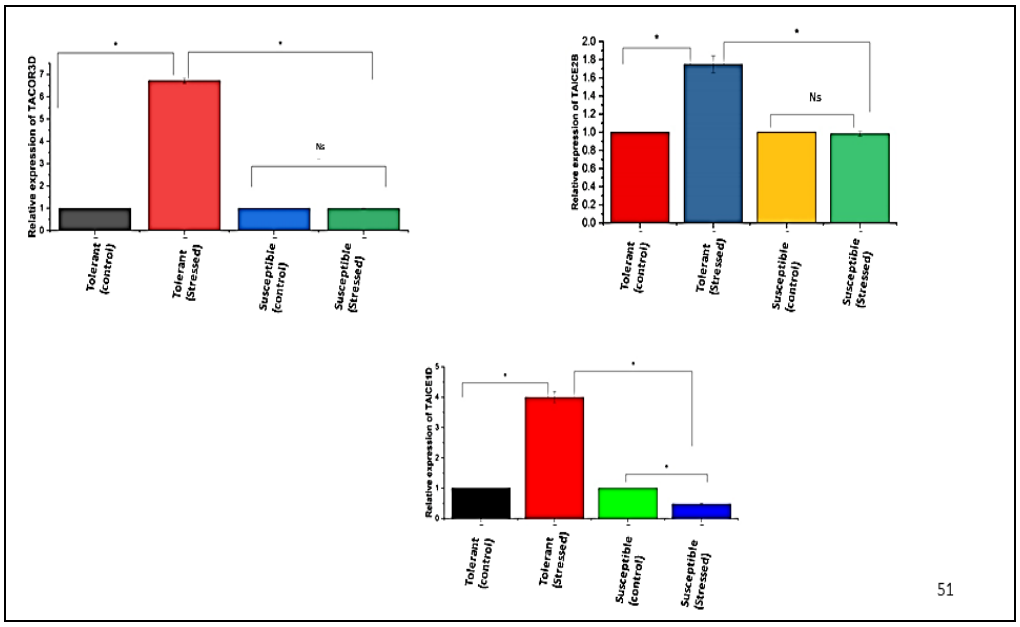
The cold-tolerant genotype, SKUA_52, demonstrated remarkable upregulation of TaCOR5a under cold stress (8.182-fold change). In SKUA_4301, although TaCOR5a was also upregulated (2.245-fold change) under stress, the extent was comparatively lower than in SKUA_52. Additionally, TaCOR3d exhibited a significant upregulation of 6.720-fold change in SKUA_52 under stress, while SKUA_4301 showed a moderate upregulation of TaCOR3d (0.989-fold change) under the same conditions. Similarly, SKUA_52 displayed a substantial upregulation of TaCOR3b under stress (11.157-fold change), whereas

SKUA_4301 exhibited a milder upregulation (1.486-fold change) of TaCOR3b under stress.

Regarding TaCBF11b, SKUA_52 demonstrated increased expression under stress (4.198-fold change), while SKUA_4301 exhibited a significant downregulation (0.001-fold change) of TaCBF11b. Furthermore, TaCBF2d was found upregulated in both genotypes under stress, with SKUA_52 showing a higher fold change (3.630) compared to SKUA_4301 (0.132).



(a)



(b)

Fig. 4.17: **Differential expression of cold tolerance-related genes for (a) *TaCBF11b, TaCBF2d, TaCOR3b, TaCOR5a (b) TaICE1D, TaICE2B, TaCOR3d* in response to cold stress in wheat genotypes SKUA_52 (cold-tolerant) and SKUA_4301 (cold-susceptible) analysed using qRT-PCR.**

4.6 Inheritance pattern of cold stress

The outcomes of hybridization experiments aimed at assessing the inheritance pattern of cold stress tolerance in F2 generation of contrasting wheat genotypes are detailed in (Table 4.8). In the cross-involving SKAU_52 and SKAU_4301, the observed distribution revealed 145 individuals with tolerance to cold stress and 63 susceptible individuals, totalling 208 progeny, which closely approximates the expected 3:1 ratio (156 resistant and 52 susceptible). The calculated chi-square (χ^2) value was found to be 3.096, which is below than the tabulated value of 3.84 at a 0.05 level of significance with 1 degree of freedom. Although, there is slight deviation in the calculated and tabulated (χ^2) value, but the observed variance is within an acceptable range and there is no strong evidence to reject the null hypothesis. Similarly, in the cross between SKAU_44 and SKAU_1422, 139 individuals displayed cold tolerance, 59 exhibited cold susceptibility, summing up to 198 progeny. The observed ratio aligns well with the expected 3:1 ratio (148 resistant and 49 susceptible) resulted in a chi-square (χ^2) value of 2.52, which is less than the tabulated value of 3.84.

Table-4.8: Inheritance pattern of cold tolerance in crosses SKAU_52 × SKAU_4301 and SKAU_44 × SKAU_1422 during the current study

Cross	F_1	F_2 population				
		Resistant (observed)	Susceptible (observed)	Total	Expected ratio	Chi-square (χ^2)
SKAU_52 x SKAU_4301	Resistant	145	63	208	3:1 (156 resistant and 52 susceptible)	3.096
SKAU_44 x SKAU_1422	Resistant	139	59	198	3:1 (148 resistant and 49 susceptible)	2.52

4.7 Transcriptomics to study differentially expressed transcripts/genes between two contrasting genotypes for cold tolerance.

4.7.1 Global analysis of transcriptome data:

Twelve libraries, representing four samples with three replications each, were sequenced, generating approximately 19.73 GB of data. Quality control (QC) statistics for RNA sequencing indicated consistent and reliable measurements. Notably, "SKAU-4301-Control" and "SKAU_52-Control" displayed 17,598,958 and 16,224,928 reads, respectively, both within a sequence length range of 20-150 base pairs and a GC content of 55%. Similarly, "SKAU_4301_stressed" and "SKAU_52_stressed" exhibited comparable read counts of 15,239,812 and 16,714,285, with a slightly increased GC content of 57%. The proportion of clean reads mapped to the wheat reference genome ranged from 93.68% to 93.89%.

Furthermore, 7302 differentially expressed genes (DEGs) were identified. In SKAU 4301, 3,916 DEGs were identified, with 859 upregulated and 3,057 downregulated genes. SKAU 52 exhibited 3,386 DEGs under cold stress, with 2,652 upregulated and 734 downregulated genes. Comparison between SKAU 4301 and SKAU 52 demonstrated a higher number of DEGs in SKAU 4301. Notably, SKAU 52 showed a significantly greater number of upregulated genes under cold stress compared to the cold-susceptible SKAU 4301. Among these DEGs, 846 were common to both genotypes, with 2540 specifically differentially expressed in SKAU 52 and 3070 in SKAU 4301 compared to their controls. Furthermore, inherent differences were observed between the two genotypes under control conditions, with 3938 common DEGs, including 314 up-regulated in SKAU-52 and 3623 downregulated. The top 30 DEGs are presented in Figure 4.1.8.

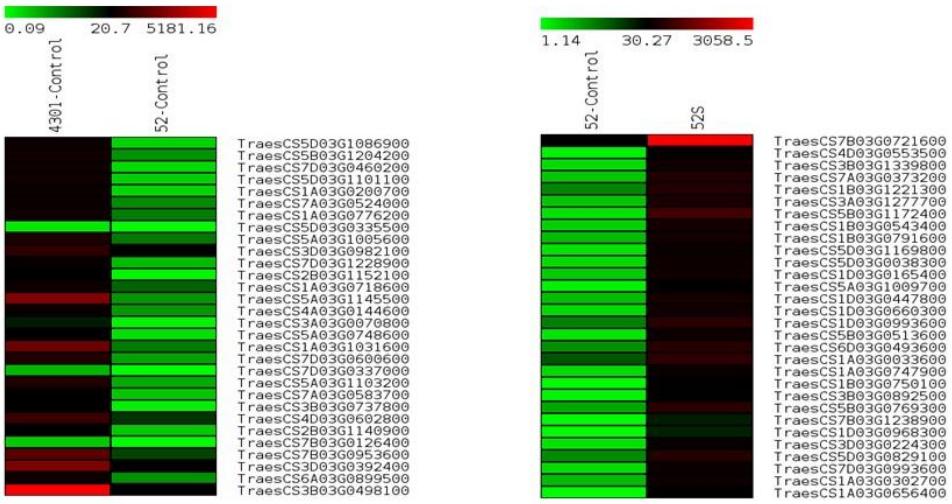


Fig. 4.18: Heat map depicting the expression profiles of the top 30 differentially expressed genes in cold-tolerant genotype SKAU_52 compared to its control and cold-susceptible genotype SKAU_4301 compared to its control. The color gradient represents the level of gene expression, with red indicating upregulation and green indicating downregulation.

4.7.2 Transcription factors

In our analysis of the cold-tolerant genotype SKAU 52, we identified a total of 1,656 differentially expressed transcription factors (TFs) under cold stress conditions. The NAC family exhibited the highest frequency with 158 members and other prominent TF families included ERF, bHLH, C2H2 and MYB-related families. Families like WRKY, HSF and bZIP were also active participants in the genotype's response to cold stress. In contrast, SKAU 4301 showed 1,833 differentially expressed TFs, with bHLH, ERF and NAC families being notably abundant. Additionally, C2H2, MYB-related, WRKY and B3 families played significant roles in the transcriptional response to cold stress. Common differentially expressed genes (DEGs) shared between genotypes highlighted the central roles of ERF and NAC families, along with the active participation of C2H2, bHLH and MYB-related families.

4.7.3 Gene annotation

Gene ontology analysis illuminated distinctive molecular responses between SKAU 52 and the SKAU 4301 under cold stress compared to their controls at 25°C. Under control conditions, both genotypes share common features, with membrane-related functions dominating the cellular component category. However, under cold stress, SKAU 52 exhibits significant upregulation in genes associated with protein-containing complexes, cell parts and membranes, indicating structural adaptations. Molecular function categories in SKAU 52 show enhanced enzymatic and binding responses, along with upregulated antioxidant and transcription regulator activities. Conversely, SKAU 4301 displays distinct changes under cold stress, featuring alterations in cellular structure and intensified enzymatic and binding responses (Figure 4.19).

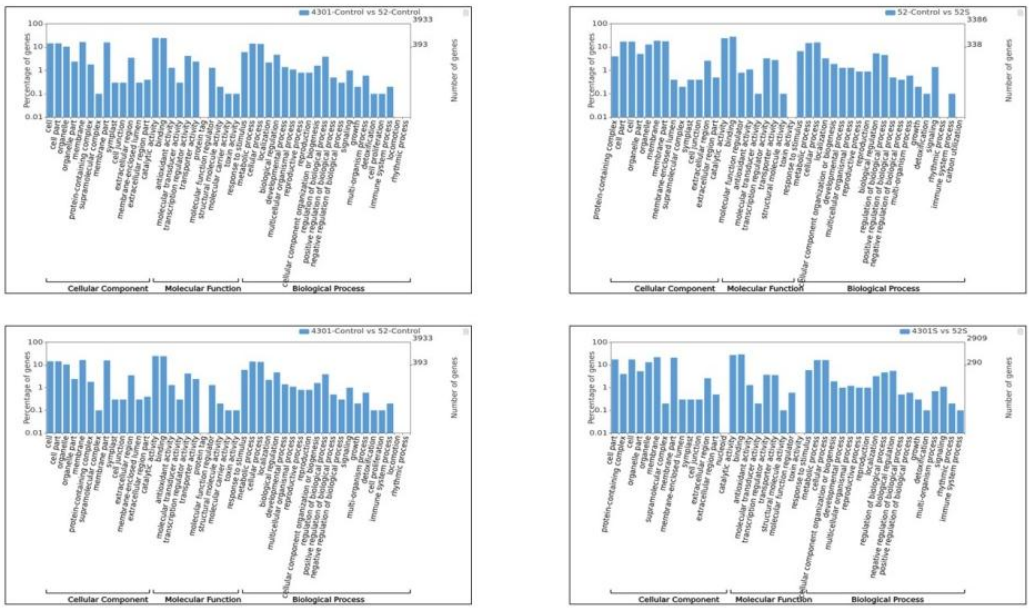


Fig 4.19: Gene annotation of Differentially Expressed Genes (DEGs) identified in various comparative analyses. The gene annotation provides insights into the biological functions, molecular functions and cellular functions associated with the DEGs.

4.7.4 KEGG analysis:

The KEGG pathway analysis also sheded light on the distinctive molecular responses to cold stress SKAU 52 and SKAU 4301, compared to their respective controls at 25°C. In SKAU 52, under cold stress, there is a noteworthy upregulation in pathways such as "Metabolic pathways" (169 genes), "Biosynthesis of secondary metabolites" (108 genes), "Plant-pathogen interaction" (55 genes) and "Plant hormone signal transduction" (35 genes). Additionally, specific metabolic pathways like "Phenylpropanoid biosynthesis" (28 genes) and "MAPK signaling pathway - plant" (26 genes) exhibited significant alterations. Conversely, downregulated pathways include "Photosynthesis - antenna proteins" (14 genes), "Necroptosis" (20 genes) and "RNA polymerase" (3 genes), indicating potential adjustments in cellular processes and interactions with pathogens.

In contrast, SKAU 4301, shows distinct responses. Under cold stress, upregulated pathways encompass "Metabolic pathways" (50 genes), "Biosynthesis of secondary metabolites" (33 genes), "Protein processing in endoplasmic reticulum" (1 gene), "Plant-pathogen interaction" (4 genes) and "Plant hormone signal transduction" (6 genes). Meanwhile, downregulated pathways involve a broad suppression, with "Metabolic pathways" (154 genes) and "Biosynthesis of secondary metabolites" (85 genes) showing substantial downregulation. Other affected pathways include "Protein processing in endoplasmic reticulum" (62 genes) and "Phenylpropanoid biosynthesis" (32 genes), indicating potential disruptions in cellular stress responses and secondary metabolite production.

4.7.5 Differentially expressed genes under cold stress:

In our investigation of SKAU 52 under cold stress compared to its control counterpart, we meticulously mined the differential gene expression data to unearth key cold-responsive genes shaping the genotype's adaptive strategies. Among the notable findings, TraesCS1D03G0703900 (Wcor18), TraesCS2A03G1018600 and TraesCS2B03G1138100, associated with cold-

responsive protein WCOR15-2B, demonstrated infinite fold changes. Furthermore, TraesCS3D03G0862400 (DHN33), TraesCS5A03G1181100 (Wcor615) and TraesCS5B03G1052800 (RAB15) showcased infinite fold changes, underscoring their significance as key players in the cold acclimation and dehydrin-mediated protective mechanisms of SKAU 52.

Contrastingly, our examination of the cold-susceptible wheat genotype revealed distinct expression patterns under cold stress compared to its control at 25°C. Among the noteworthy findings, TraesCS2D03G0363200 exhibited a 2.83-fold upregulation, suggesting its involvement in the genotype's response to cold stress. Notably, TraesCS3D03G0862400 (DHN33), TraesCS5A03G1006400 (RAB15) and TraesCS5D03G0953400 (RAB15) displayed downregulation. Additionally, TraesCS5A03G1181100 (Wcor615) exhibited a significant downregulation (-4.21-fold), indicating a potential impact on the cold acclimation process. Moreover, TraesCS5A03G1181200 (Wrab17.1) demonstrated a substantial downregulation (-8.76-fold), suggesting alterations in the expression of LEA/RAB-related COR proteins. TraesCS6A03G0900100 (Cold shock protein CS66) and TraesCS6B03G0794800 (Wcor410c) displayed notable downregulation (-4.50-fold and -3.34-fold), respectively.

Particularly, in the comparative analysis between the cold-tolerant wheat genotype SKAU 52 and the cold-susceptible genotype SKAU 4301 under cold stress, distinct expression profiles emerged, highlighting key differences in their responses. Notably, TraesCS2A03G0346000 (TRITD_2Av1G053010) displayed a -2.47-fold downregulation in SKAU 52, suggesting its role in the genotype's susceptibility to cold stress. TraesCS2B03G0465100 (TRITD_2Bv1G064680) exhibited a substantial -4.21-fold downregulation. The dehydrin-encoding gene TraesCS3D03G0862400 (DHN33) showed an infinite upregulation in SKAU 52, emphasizing its critical role in cold tolerance. Additionally, TraesCS5A03G1181100 (Wcor615) and TraesCS5A03G1181200 (Wrab17.1) demonstrated 5.28-fold and 6.72-fold upregulation, respectively, in SKAU 52.

Conversely, TraesCS6A03G0900100 (Cold shock protein CS66) and TraesCS6A03G0901500 (wzy2) exhibited infinite upregulation in SKAU 52. TraesCS7B03G0953600 (Cold acclimation induced protein 2-1) displayed a 5.12-fold upregulation in SKAU 52, suggesting its active participation in cold acclimation processes. Similarly, TraesCS7B03G1305200 (Wcor726) exhibited an infinite upregulation, emphasizing its role in the genotype's response to cold stress.

4.8 Proteomics to study differentially expressing proteins in contrasting wheat genotypes for cold tolerance

Comaparative proteomics analysis of SKAU 52 and SKAU 4301 led to the identification of 1939 proteins. Under control conditions of SKAU 52, 1060 proteinwere observed. Following exposure to cold stress, SKAU 52 exhibited an altered protein profile with 1168 identified proteins. In contrast, SKAU 4301 displayed 1073 proteins under control conditions and 1154 proteins after cold stress.

An insightful aspect of the analysis was the identification of unique proteins to each condition. In the control state of SKAU 52, 9 proteins were uniquely identified, under cold stress, 14 proteins were found exclusively in this genotype Similarly, SKAU 4301 exhibited 4 proteins exclusively in its control state and 4 proteins uniquely expressed after exposure to cold stress (Figure 6). The identified proteins were distributed in the 48 biological pathways (Figure 6). These pathways encompassed diverse cellular processes, redox reactions and polyamine metabolism to abiotic stress responses. Under stress, both genotypes exhibit enhanced amino acid synthesis and degradation pathways, with SKAU_52 showing more pronounced changes. Carbohydrate metabolism, particularly the Calvin cycle, decreases under cold stress. Notably, protein synthesis and folding pathways remain active while protein degradation increases, particularly in SKAU_4301. The distinct molecular signatures observed in each genotype, especially the increased activity of redox reactions, polyamine metabolism and

abiotic stress pathways in SKAU_52, underscore the complex and genotype-specific nature of stress responses.

Under control conditions, 236 proteins exhibited significant differences between SKAU 52 and SKAU 4301, highlighting inherent disparities of the proteome. Upon exposure to cold stress, SKAU_52 demonstrated alterations in 326 proteins compared to its control counterpart, emphasizing its dynamic response to environmental challenges. Among the significant proteins, various notable stress-related proteins, including cold shock protein-1 exhibited a substantial increase (2.5 fold change) in SKAU 52 after cold stress. Also, upregulation of key redox-regulating genes, such as Thioredoxin, Glutaredoxin, peroxidases and Superoxide Dismutase (21.1 to 21.6-fold change) was observed. A striking 11.6-fold increase in Lipid Transfer Protein 6 expression was also observed in SKAU_52 after cold stress. In addition, proteins associated with photosynthesis, amino acid metabolism and protein synthesis showed a significant increase in cold tolerant genotype following cold stress.

In parallel, SKAU 4301 exhibited differential expression in 172 proteins under cold stress compared to its control. Most proteins are involved in the gluconeogenesis/glyoxylate cycle and metal handling showed increased upregulation. Among the significant proteins related to cold tolerance, only few signaling protiens including RAB GTPase homolog A4D and proline, showed increased expression in cold susceptible genotype.

Cross-genotype comparison under cold stress revealed 211 proteins with significant variations. The cold-tolerant genotype (SKAU 52) exhibited increased expression of proteins related to cold tolerance, such as lipid transfer protein 6, Flavoprotein wrbA, peroxidases, glutathione S-transferase TAU 24, catalase 1, copper/zinc superoxide dismutase 1, hydroxyproline-rich glycoprotein family protein, signaling calcium protein like Calcium-binding EF-hand family protein and calcium-sensing receptor. Additionally, many proteins involved in carbohydrate metabolism, protein metabolism, tetrapyrrole synthesis and other

cellular processes responding to cold stress were highly expressed in SKAU 52. These findings underscore the complex, genotype-specific nature of stress responses in these wheat varieties.

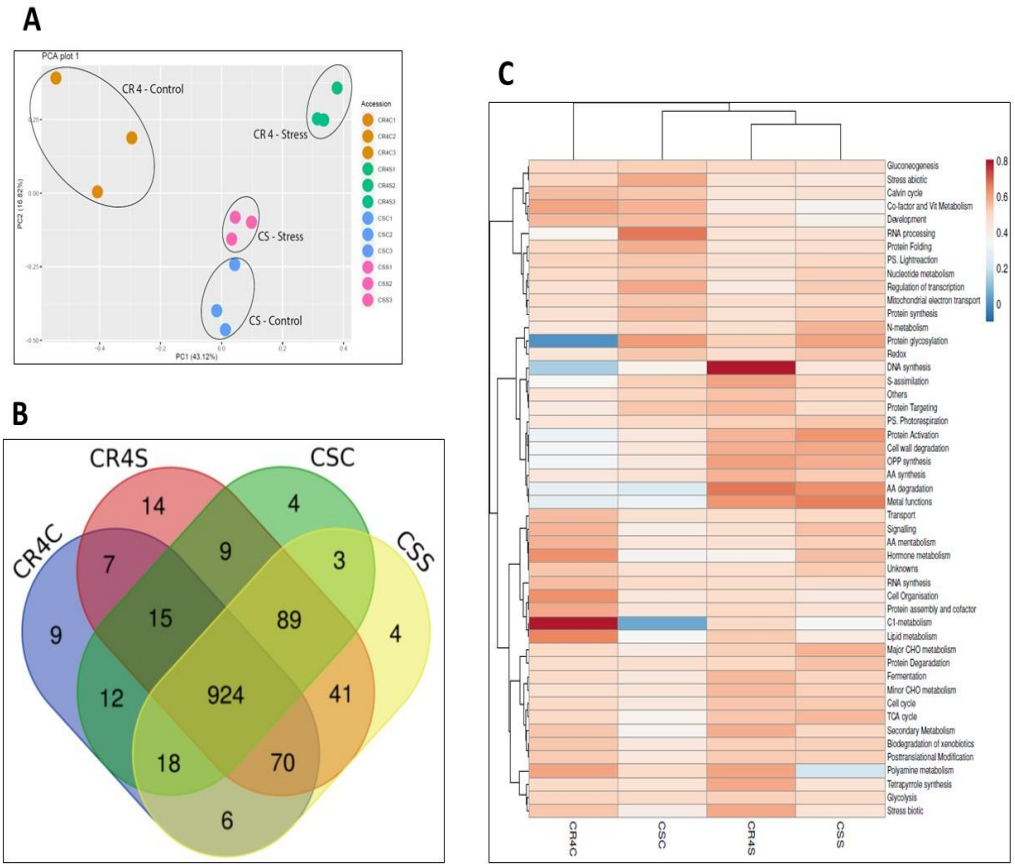


Fig. 4.20: **Figure showing (A) Principle Component Analysis comparing protein expression profiles of cold-tolerant (SKAU_52) and cold-susceptible (SKAU_4301) wheat genotypes under control and cold stress conditions (B) Venn diagram depicting the overlap and unique protein sets between SKAU_52 and SKAU_4301 under control and cold stress conditions,(C) Heat map displaying the biological functions associated with differentially expressed proteins in cold-tolerant and cold-susceptible wheat genotypes under control and cold stress conditions.**

Chapter-5

DISCUSSION

The present study was aimed to understand the mechanism underlying the response of wheat to cold stress by identifying changes in the morphology, biochemistry, physiology, metabolome, lipidome, in a diverse set of wheat germplasm. The findings of the different experiments are discussed in light of the available literature under appropriate headings below.

5.1 Morphological and physio-biochemical response of wheat germplasm to cold stress

Cold stress is a significant abiotic factor that severely affects crop productivity and poses a major challenge to agricultural systems, particularly in regions with low temperatures. Wheat, one of the world's most important cereal crops, is particularly susceptible to the detrimental effects of cold stress during various growth stages, including germination, seedling establishment and reproductive development (Hassan *et al.,* 2021). In response to the growing demand for cold-tolerant wheat varieties, extensive efforts have been made to understand the genetic basis of cold stress tolerance and to develop effective strategies for screening and selecting cold-tolerant genotypes (Rinalducci *et al.,* 2011; Díaz *et al.,* 2019; Zhao *et al.,* 2020; Wang *et al.,* 2023). Cold stress tolerance in wheat is a complex trait, governed by multiple genetic and physiological mechanisms, including the regulation of gene expression, enzymatic activity and osmoprotectant accumulation (Hassan *et al.,* 2021; Manasa *et al.,* 2022).

5.1.1 Cold tolerance in the field

Screening large populations of wheat genotypes for cold stress tolerance is of paramount importance, especially in regions prone to low temperatures, as it directly impacts crop productivity and food security (Li *et al.,* 2010; Mohammadi *et al.,* 2015). In this study, field screening of large diverse set of wheat genotypes,

in the challenging climatic conditions of Wadura, District Baramulla, Kashmir, India, provided valuable insights into the distribution of cold stress tolerance among the screened wheat genotypes over two consecutive years, "2020-21 and 2021-22". It is well-documented that wheat cultivars often exhibit a range of responses to cold stress (Li *et al.*, 2010; Zhao *et al.*, 2019). The prevalence of moderate cold tolerance among the majority of the genotypes, across the two years in our study underscores the stability of this trait in the evaluated genotypes, reinforcing the importance of moderate cold tolerance as a baseline trait in wheat breeding programs. The identification of genotypes with a cold score of 1, indicating cold tolerance, is a promising finding of this study. The observed increase in the number of cold-tolerant genotypes from 2020-21 to 2021-22, may be attributed to the relatively milder cold conditions during the year 2020-21, allowing for a broader spectrum of genotypic responses to cold stress. Remarkably, genotypes exhibiting exceptional cold tolerance, with a cold score of 0 over the two years hold substantial promise for breeding programs focused on developing highly cold-tolerant wheat varieties. Their ability to thrive under extreme cold stress conditions suggests that they possess valuable genetic traits for cold tolerance that can be leveraged to enhance the resilience of wheat crops to adverse weather conditions (Lopes *et al.*, 2015). On the flip side, a significant number of genotypes, exhibited susceptibility to cold temperatures, as indicated by cold scores of 3 and 4, underscore the importance of identifying and excluding these susceptible genotypes from breeding programs aimed at enhancing cold tolerance, as these genotypes are likely to suffer significant yield losses in areas with low temperatures. Overall, these cold screening findings of diverse wheat germplasm under the field conditions in this study contributes to the broader goal of improving wheat crop resilience in the face of increasingly variable and unpredictable climatic conditions.

5.1.2 Cell membrane stability under cold stress

While simple phenotyping or field screening methods are valuable for initial screening and identifying potential candidates, they may have limitations in terms of reliability and accuracy due to environmental variability, subjectivity in scoring and the inability to capture the full range of genetic and environmental interactions (Ghanem *et al.*, 2015). The results of our two-year field screening of wheat germplasm for cold stress tolerance, coupled with membrane stability assessment have provided more valuable information about the genetic basis of cold tolerance in wheat. Electrolyte Leakage Index (ELI) is a commonly used measure in plant physiology and stress research to assess membrane stability, particularly under abiotic stress conditions such as cold stress in plants (Svetlana *et al.,* 2023). Cold stress disrupts cell membranes, leading to increased electrolyte leakage (Sanghera *et al.,* 2011). The observed wide range of ELI values among the 350 selected wheat genotypes is a clear indicator of the substantial genetic diversity within the germplasm under study. This variation offers potential opportunities for selecting genotypes with enhanced membrane stability and cold stress tolerance. Furthermore, the highly significant variation among treatments (genotypes) in the ANOVA analysis underscores the strong influence of genotype on electrolyte leakage index (ELI) in field conditions. Genetic factors play a pivotal role in shaping a plant's response to environmental stressors and cold stress is no exception (Heidarvand *et al.,* 2010). The unique genetic makeup of each genotype dictates its ability to withstand cold stress and maintain membrane integrity. The strong association between cold tolerance and membrane stability is underpinned by the molecular and physiological mechanisms that underlie a plant's response to cold stress (Yanli *et al.,* 2023). In this study, the occurrence of highly resistant genotypes exhibiting the lowest mean ELI values, suggests better membrane stability and enhanced tolerance to cold stress. This finding aligns with the expectation that plants with superior cold tolerance have evolved mechanisms to mitigate membrane damage and, consequently, exhibit lower ELI values

(Amini *et al.,* 2021). Conversely, the genotypes in the HS group displayed the highest mean ELI values in the current study, indicates the lowest membrane stability under cold stress. These findings are consistent with the anticipated trend of increased membrane damage and reduced stability in more susceptible genotypes (Murata, 1990; Pukacki *et al.,* 1991). Furthermore, the higher variability observed within the more tolerant groups (HR, R and MR) is a reflection of adaptive plasticity, where genotypes within these groups exhibit diverse responses to varying cold stress conditions (Chevin *et al.,* 2017). Genotypes with higher cold tolerance possess the flexibility to respond to a wider range of cold stress intensities.

5.1.3 Biochemical analysis in response to cold stress

The comprehensive biochemical profiling undertaken in this study aligns with the overarching objective of unravelling the intricacies of cold stress tolerance mechanisms in wheat genotypes, by examining osmoprotectant accumulation (Proline) and antioxidant enzyme activities (such as SOD, CAT, APX, GPX) and oxidative damage by assessment of lipid peroxidation and hydrogen peroxidation responses of genotypes to low temperatures under different cold treatments. The substantial variation observed in biochemical parameters across different genotypes and treatments highlight the potential for identifying genotypes with superior cold stress resilience and the capacity to maintain membrane integrity, mitigate oxidative damage and regulate osmotic balance under varying cold stress scenarios. One-way ANOVA revealed significant variations in the mean values of each biochemical parameter across the treatments, indicating distinct effects on the plant's biochemistry. The protein levels which serve as indicators of cellular integrity and stress response (Flick *et al.,* 2012; Hamann, 2012), showed notable changes across the treatment levels. The control group (T0) exhibited relatively stable protein levels, indicating steady-state conditions under normal growth conditions. However, during the acclimation phase (T1), the protein levels significantly increased, suggesting the

activation of adaptive mechanisms to enhance stress tolerance. This increase in protein levels may be attributed to the synthesis of stress-related proteins and enzymes involved in cold acclimation processes (Mohsen *et al.,* 2015).

5.1.3.1 Reactive oxygen species (ROS) assays

The levels of malondialdehyde (MDA) and hydrogen peroxide (H_20_2), which are indicative of lipid peroxidation and oxidative stress, respectively (Sairam and Srivastava, 2000; Apostolova *et al.,* 2008), exhibited contrasting patterns across the treatments in the current study. The control group (T0) displayed relatively low levels of MDA and H_2O_2, signifying minimal oxidative damage. It is obvious, that under normal conditions, wheat genotypes maintain membrane integrity and redox homeostasis (Awasthi *et al.,* 2015). However, during cold stress without acclimation (T3), the MDA and H_2O_2 levels were found significantly increased, reflecting heightened oxidative stress. This finding aligns with previous research that has demonstrated the adverse effects of rapid cold stress on membrane integrity and redox balance (Dreyer *et al.,* 2018). In contrast, the acclimation phase (T1) and cold stress after acclimation (T2) showed lower MDA and H_2O_2 levels than T3, indicating that acclimation provides some level of protection against oxidative damage. It has been reported earlier researchers that during acclimation, plants undergo physiological adjustments, such as the accumulation of osmoprotectants and activation of antioxidant defenses (Thomashow, 2001). These adaptations likely contribute to the observed reduction in lipid peroxidation and ROS accumulation.

5.1.3.2 Antioxidants assays under cold stress

Under cold stress, antioxidant enzymes play a crucial role in mitigating oxidative stress by scavenging reactive oxygen species (ROS), thus conferring cold tolerance (Lascano *et al.,* 2001). The activities of APX, SOD, CAT and GPX were significantly influenced by the treatments in this study. The upregulation of APX, GPX and CAT leads to detoxification of hydrogen peroxide (H_2O_2), while

as SOD is responsible for dismutating superoxide radicals ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), a reactive oxygen species (ROS) generated during cold stress, thus reduces the potential for cellular damage under cold conditions (Gill *et al.,* 2010). The acclimation phase (T1) and cold stress after acclimation (T2) led to increased activities of these enzymes, indicating an enhanced antioxidant defense system. These findings suggest that acclimation primed the plant genotypes for improved antioxidant capacity, thereby enabling them to cope better with subsequent cold stress (Kim *et al.,* 2005; Kazemi *et al.,* 2013). Proline, an osmoprotectant and compatible solute, has been associated with enhanced cold stress tolerance, as it helps maintain cell turgor and stabilize cell structures (Hayat *et al.,* 2012). In our study, the control group (T0) and cold stress without acclimation (T3) showed relatively low proline levels, suggesting a limited response to osmotic stress. However, during the acclimation phase (T1) and cold stress after acclimation (T2), proline levels increased significantly. This indicates that acclimation promotes the accumulation of proline. Consistent with our biochemical profiling results, the correlation analysis of the biochemical parameters also revealed meaningful associations that support our understanding of cold stress tolerance in wheat. Consistent with previous studies (Xu *et al.,* 2013), we found positive correlations between osmoprotectants (such as proline), protein and antioxidant enzyme activities (such as superoxide dismutase, catalase and peroxidase), suggesting a coordinated response between enzyme activity and protein synthesis. Moreover, we observed that the negative association between antioxidants and damage indices (MDA and H_2O_2), which is consistent with the concept that increased antioxidant capacity can lead to a reduction in ROS levels and oxidative stress (Xu *et al.,* 2013; Nejadsadeghi *et al.,* 2014; Ashraf *et al.,* 2019; Hasanuzzaman *et al.,* 2019).

The physiological measurements between the cold tolerant and cold susceptible genotypes depicted the contrasting responses between the two groups. In this study, best five cold-tolerant genotypes among the studied genotypes for

each treatment were identified, using several key criteria based on the field cold screening data, ELI and biochemical data. First, genotypes exhibiting lower cold score values (0) and low ELI values were regarded as having improved cold tolerance. Additionally, the selection process favored genotypes demonstrating low MDA value, low H_2O_2 content and higher activities of important antioxidant enzymes, including APX, SOD, CAT and GPX, as these enzymes play a crucial role in mitigating oxidative damage. Lastly, genotypes characterized by increased accumulation of proline, a compatible solute with cryoprotective properties, were given preference. For cold-susceptible genotypes, certain characteristics were used to distinguish them from cold-tolerant lines. These included higher cold score values, indicating increased susceptibility to cold stress and elevated ELI values, indicating compromised membrane stability under cold conditions. Furthermore, cold-susceptible genotypes exhibited higher levels of MDA and H_2O_2, indicating higher oxidative damage. The activities of antioxidant enzymes, such as APX, SOD, CAT and GPX, were comparatively lower in cold-susceptible genotypes, suggesting a reduced capacity to counteract oxidative stress. Furthermore, the comparison between cold-tolerant and cold-susceptible genotypes based on the data obtained in this study revealed significant differences between cold-tolerant and cold-susceptible genotypes in response to cold stress conditions. Overall, cold-tolerant genotypes exhibited higher levels of antioxidants, such as SOD CAT, APX, GPX, proline, while as, in cold susceptible genotypes the opposite trend was observed. For Treatment T1 and T2, the tolerant genotype exhibited higher protein levels, proline and antioxidants enzymatic activity as compared to the susceptible genotypes. This indicates genotype-dependent variations in these biochemical expression in response to different treatments. Furthermore, the susceptible genotype showed increased levels of malondialdehyde (MDA), H_2O_2, as compared to tolerant genotypes, in all the treatments indicating higher oxidative stress, compared to the tolerant genotypes. These findings suggest that the susceptible genotype may be more susceptible to oxidative damage under the given experimental conditions.

5.1.3.3 Principal component analysis (PCA) for biochemical traits

Principal component analysis (PCA) is a widely used statistical technique that helps to uncover patterns and relationships within a dataset by reducing its dimensionality. In our study, we applied PCA to our biochemical data to gain insights into the underlying structure and identify key biochemical variables contributing to the observed variation. By performing PCA, we were able to condense the information from multiple biochemical variables into a smaller number of dominant principal components (PCs) while retaining the maximum amount of variation in the dataset. This dominant PC represents the major factors driving the variation in our dataset, offering profound understanding into the biochemical mechanisms underlying cold stress tolerance. Under normal conditions (T0), representing the control condition, APX and SOD exhibited exceptionally long vectors in the PCA biplot, signifying their pivotal roles in shaping the variance observed. This emphasizes the importance of antioxidant enzymes such as APX and SOD in maintaining cellular redox balance even under normal conditions. Transitioning to T1, which corresponds to the cold acclimation phase, Protein emerged as a significant contributor, as evidenced by the lengthiest vector in the PCA biplot at this time point. This shift highlights the dynamic adaptive responses occurring during the cold acclimation process, with an emphasis on protein-related mechanisms. Moving forward to T2, which represents the cold stress condition after acclimation and T3, involving cold stress without acclimation, Proline took center stage, exhibiting the longest vectors in these respective time points. The prominence of Proline suggests its crucial role as an osmoprotectant and stress-responsive metabolite, particularly during the active exposure to cold stress.

These findings collectively underscore the nuanced temporal dynamics of biochemical responses throughout the stress timeline. The observed shift in the contribution of key traits at different time points reflects the plant's adaptive

strategies, emphasizing the significance of distinct biochemical pathways during various phases of stress, from acclimation to active exposure.

5.2 Differential response of targeted and untargeted metabolites to cold stress

5.2.1 Defense harmone analysis for cold tolerance

In elucidating the metabolomic responses of wheat genotypes SKUA_52 (cold tolerant) and SKUA_4301 (cold susceptible) to cold stress, our study revealed intriguing insights into the activation of defense mechanisms. The observed significant variation in defense hormone levels, including Salicylic Acid (SA), Jasmonic Acid (JA), Abscisic Acid (ABA), JA-Isoleucine (JA-ILE) and cis-Octadecanoid-derivative (cis-OPDA), between the cold-tolerant wheat genotype SKUA_52 and the cold-susceptible genotype SKUA_4301 in response to cold stress can be attributed to the intricate signaling networks involved in plant stress responses, particularly in the context of cold stress. The intricate crosstalk between these hormones and their downstream signaling pathways contributes to the modulation of biochemical and physiological processes, ultimately influencing the cold stress tolerance mechanisms in these wheat genotypes (Zhao *et al.*, 2019; Cheong *et al.*, 2019).

SA is a well-known signalling molecule primarily associated with the activation of defense responses against biotic stresses, particularly in the context of systemic acquired resistance (SAR). While SAR is traditionally linked to biotic stress, recent research has highlighted the involvement of SA in abiotic stress responses, including cold stress (Taşgín *et al.*, 2003; Wang *et al.*, 2021). In the cold-tolerant genotype SKUA_52, the observed higher levels of SA suggest a multifaceted role for SA in orchestrating defense responses by regulating key physiological and biochemical processes that contribute to the enhanced cold tolerance. The pronounced increase in SA levels in the cold-tolerant genotype SKUA_52 is in agreement with studies demonstrating the upregulation of SA

during cold stress in various plant species (Ignatenko *et al.,* 2019). This substantiates the notion that SA plays a pivotal role in fortifying plants against adverse environmental conditions, including low temperatures. Likewise, jasmonic Acid, a well-known player in the jasmonate signalling pathway, has been implicated in the regulation of defense responses against both biotic and abiotic stresses (Repkina *et al.,* 2021). The substantial rise in JA levels, particularly in SKUA_52 (stressed), suggests an active involvement of JA in the defense mechanisms that confer cold tolerance in our cold tolerant genotype. Conversely, the observed lower JA levels in SKUA_4301 indicate a differential regulation of JA in the cold-susceptible genotype, possibly contributing to a less effective defense response in it to cold stress.

Similarly, abscisic acid has long been recognized as a central regulator in abiotic stress responses, including cold stress. The significant surge of ABA levels in SKUA_52, indicate its involvement in the cold tolerance response in this genotype. This finding aligns with earlier studies demonstrating the upregulation of ABA in response to cold stress (Kovács *et al.,* 2010). The elevated ABA levels in SKUA_52 may contribute to its adaptive strategies under cold stress, while the comparatively lower levels in SKUA_4301 suggest a potential limitation in its stress adaptation mechanisms.

The intricate relationship between JA and its derivative, JA-Isoleucine, is well-documented in plant defense mechanisms (Karki *et al.,* 2013). In SKUA_52, the higher levels of JA-ILE under cold stress indicate its potential involvement in mediating cold tolerance. These findings resonate with the earlier studies, elucidating the role of JA-related compounds in shaping plant responses to adverse environmental conditions, including cold stress (Karki *et al.,* 2013). Similarly, the significant increase in cis-Octadecanoid-derivative in SKUA_52 under cold stress aligns with the known role of this compound as a precursor in the biosynthesis of JA. The elevated levels of cis-OPDA in SKUA_52 under cold stress suggest its participation in the cold stress response. SKUA_4301, with

comparatively lower levels, may exhibit a distinct regulatory pattern, potentially contributing to its susceptibility to cold stress. This finding is in agreement with research highlighting the activation of JA biosynthetic pathways during cold stress in plants (Pigolev *et al.*, 2018). It reinforces the idea that the JA pathway, from precursor to downstream signalling molecules, is intricately involved in the cold stress responses observed in SKUA_52.

5.2.2 Flavanoid analysis for cold tolerance

The comprehensive LC-MS analysis of flavonoid profiles in wheat genotypes SKUA_52 and SKUA_4301 under cold stress has revealed distinct accumulation patterns of selected flavonoids, namely Naringenin, Rutin, Quercitin, Petunidin, Peonidin and Malvidin. These Flavanoids are known for its antioxidant properties and contribute to the scavenging of reactive oxygen species generated under cold stress, thereby mitigating oxidative damage and play crucial roles in enhancing the cold tolerance (Schulz *et al.,* 2016). Notably, the cold-tolerant genotype SKUA_52 consistently exhibited higher levels of these flavonoids under cold stress compared to the cold-susceptible genotype SKUA_4301. This heightened accumulation was evident across multiple flavonoids, with Naringenin, Rutin, Quercitin, Petunidin, Peonidin and Malvidin all showing significantly elevated levels in SKUA_52_stressed compared to both SKUA_4301_stressed and SKUA_4301_Control. These results suggest a potential association between increased flavonoid levels and enhanced cold tolerance in SKUA_52 after cold stress. The observed upregulation of flavonoids in the cold-tolerant genotype underscores their potential as molecular markers for cold stress resilience in wheat.

5.2.3 Untargeted metabolite analysis

The untargeted GC-MS metabolomic analysis has unveiled profound insights into the metabolic dynamics of the cold-tolerant genotype, SKUA-52, under cold stress conditions. Among the different metabolites identified,

carbohydrates emerged as the most abundunt metabolites in this study. Carbohydrates serve as crucial energy sources and signaling molecules in various cellular processes and under cold stress, plants often undergo metabolic adjustments to ensure energy balance and cope with the adverse conditions (Javadian *et al.*, 2010). The upregulation of sucrose in SKUA-52 under cold stress reflects a strategic metabolic adjustment aimed at enhancing osmotic protection, maintaining energy balance and activating signaling pathways to promote cold tolerance in this genotype. This finding is in consistent with the studies demonstrating, that sucrose accumulation contributes to osmotic adjustment, maintaining cell turgor and enhancing cold tolerance in Arabidopsis (Zhang *et al*., 2018).

The observed increase in amino acids, including oxyproline, in the cold-tolerant genotype SKUA-52, represents a pivotal aspect of the plant's metabolic reprogramming under cold stress, with implications for stress resilience. Amino acids play multifaceted roles in plant metabolism and their augmented abundance aligns with established mechanisms associated with stress-responsive protein synthesis (Hu *et al*., 2022). The observed upregulation of amino acids in SKUA-52 supports the notion that these metabolites play a crucial role in enhancing cold tolerance. The broader observation of a distinctive metabolic signature in the cold-tolerant genotype, as indicated by the upregulation of specific metabolites, corresponds with research emphasizing the importance of metabolic adjustments in plant cold tolerance. Chen *et al*. (2016) highlighted that plants with an ability to modulate their metabolite profiles exhibit enhanced cold tolerance. The findings in SKUA-52 resonate with this concept, underscoring the significance of the identified metabolites in contributing to its cold tolerance phenotype (Chen *et al.*, 2016).

5.3 Differential response of membrane lipids to cold stress

Lipids, essential components of cellular membranes, play a crucial role in maintaining membrane fluidity, integrity and functionality, especially under stress conditions such as cold stress (Golizadeh *et al.,* 2019). The degree of saturation in fatty acid chains of lipids significantly influences their physical properties and, consequently, the response of plants to environmental stresses including cold stress. Unsaturated lipids, characterized by the presence of double bonds in their fatty acid chains, are pivotal for cold tolerance (Golizadeh *et al.,* 2019). These unsaturated lipids contribute to membrane flexibility and fluidity, allowing the adjustment of membrane properties to cold stress. The introduction of desaturase genes responsible for synthesizing unsaturated fatty acids led to improved membrane fluidity and, consequently, enhanced cold tolerance Nejadsadeghi *et al.,* 2015). The up-regulation of unsaturated lipids in our cold tolerant genotype (SKUA-52) reflects a strategic adjustment in its lipidomic composition, enhancing membrane flexibility and stability under cold stress conditions. This lipidomic signature contributes to the plant's overall ability to thrive in cold environments.

The observed down-regulation of saturated lipids in SKUA-52, such as LPC(16:0), PC(34:0), LPG(16:0), LPC(15:0) and PE(34:0), indicates a dynamic adjustment in the composition of these lipids in response to cold stress. This down-regulation likely contributes to maintaining membrane fluidity, a critical aspect of cold adaptation. Findings from a study by Yu *et al.* (2014) on the wheat crop highlighted that the reduction in saturated lipids under cold stress positively correlates with increased membrane fluidity. The down-regulation of saturated lipids is an adaptive strategy to prevent membrane rigidity and maintain optimal cellular function under cold conditions. The up-regulation of unsaturated lipids, including DGDG(34:3), DGDG(36:6), PC(35:2), PC(32:1), PE(32:2), PC(36:3), PC(35:1), PC(33:4), LPC(18:3), PC(36:6), PC(34:4), PC(36:4), PC(38:2), PC(33:3), PC(32:2) and PC(33:2), indicates an active response to cold stress. These lipids likely contribute to maintaining membrane fluidity, stability and

functionality under low-temperature conditions. A study by Cheong *et al. (*2019) demonstrated that up-regulation of unsaturated lipids, particularly phosphatidylglycerol (PG) species, is crucial for maintaining photosynthetic efficiency and overall cold tolerance in the wheat crop. The increased abundance of unsaturated lipids supports membrane flexibility and resilience to cold-induced stresses.

The detailed lipidomic analysis provides strong evidence that the up-regulation of unsaturated lipids and the concurrent down-regulation of saturated lipids are key molecular mechanisms contributing to cold tolerance in the resistant genotype SKUA-52. These findings align with established literature on lipid-mediated cold tolerance mechanisms, further validating the significance of lipidomic adaptations in crop plants exposed to cold stress (Szalai *et al.,* 2001).

5.4 Gene expression of ICE-CBF-COR pathway by qRT-PCR

The ICE-CBF-COR pathway, also known as the C-repeat binding factor (CBF) pathway, is a crucial molecular signalling pathway in plants, particularly in response to cold stress. This pathway plays a central role in regulating the expression of genes that contribute to cold acclimation and enhanced tolerance to low temperatures. The key components of the ICE-CBF-COR pathway include Inducer of CBF Expression (ICE), CBF transcription factors and COR (Cold-Regulated) genes (Guo *et al.,* 2019). The qRT-PCR analysis of cold tolerance-related genes in our study belonging to the ICE-CBF-COR pathway provides insights into the molecular mechanisms underlying the differential cold stress response in the wheat genotypes SKUA_52 (cold-tolerant) and SKUA_4301 (cold-susceptible). TaICE1D, a key regulator in the ICE-CBF-COR pathway, showed significant differential expression between SKUA_52 (stressed) and SKUA_4301 (stressed) genotypes. TaCOR genes, specifically TaCOR3b, TaCOR5a and TaCOR3d, exhibited substantial upregulation in SKUA_52 (stressed), with fold changes of 11.16, 8.18 and 6.72, respectively. These findings underscore the significance of these COR (Cold-Regulated) genes in the cold

response of the tolerant genotype. Such upregulation indicates an active participation of these genes in the cold acclimation process, potentially contributing to enhanced cold tolerance in SKUA_52. Similar observations were reported in studies by Jin *et al*. (2018) and Hwarari *et al*. (2022), who emphasized the pivotal role of COR genes in the response to cold stress as their upregulation is associated with the accumulation of protective proteins and osmoprotectants, contributing to improved cold tolerance.

Among the CBF (C-repeat Binding Factor) genes, TaCBF11b showed minimal fold change (0.001), suggesting limited differences in expression between the two genotypes. On the contrary, TaCBF2d exhibited significant upregulation in SKUA_52 (stressed) with a fold change of 3.630. These findings suggest a nuanced regulatory role for specific CBF genes in the cold response of wheat, with TaCBF2d potentially playing a more prominent role in SKUA_52. The diverse roles of CBF genes in cold tolerance were highlighted by Kume *et al*. (2005), emphasizing those different CBF genes may have specific functions in the cold response and their differential expression contributes to the overall cold tolerance of the plant. TaICE2B, TaCBF11b, TaCBF2d and TaICE1D exhibited significant upregulation in SKUA_52 (stressed), further supporting the notion that these genes contribute to the enhanced cold tolerance observed in the cold-tolerant genotype. The relative expression levels (1.7, 4.1, 3.6 and 3.9, respectively) indicate a coordinated upregulation of multiple key genes in the ICE-CBF-COR pathway.

The qRT-PCR results reveal a complex interplay of gene expression in response to cold stress, highlighting the significance of specific COR and CBF genes in the cold-tolerant genotype, SKUA_52. These findings align with established literature on the ICE-CBF-COR pathway, supporting the idea that the coordinated expression of multiple genes within this pathway contributes to the overall cold tolerance observed in wheat.

5.5 Inheritance of cold tolerance in wheat

The examination of inheritance patterns through hybridization experiments provides valuable insights into the genetic basis of cold stress resistance in wheat genotypes. Two distinct crosses, SKAU_52 (cold tolerant) x SKAU_4301 (cold susceptible) and SKAU_44 (cold tolerant) x SKAU_1422 (cold susceptible), were analyzed to assess the segregation of cold tolerance and susceptibility in the F2 generation.

In the cross of SKAU_52 x SKAU_4301 and SKAU_44 x SKAU_1422, the observed distribution in the F2 generation deviates slightly from the expected 3:1 ratio, resulting in a chi-square (χ^2) value of 3.096 and 2.52, respectively. Despite this deviation, the calculated χ^2 value was less than the tabulated value of 3.84, indicating that the observed results are not significantly different from the expected Mendelian segregation ratios. The closeness of the calculated χ^2 value to the tabulated value suggests that the inheritance of cold stress resistance in the SKAU_52 x SKAU_4301 and SKAU_44 x SKAU_1422 crosses is governed by single dominant gene and these findings aligns reasonably well with Mendelian principles (Sofalian *et al.,* 2006). The minor discrepancy may be attributed to factors such as genetic modifiers or environmental influences, but overall, the results support the hypothesis of Mendelian inheritance. This finding resonates with earlier study of Limin and Fowler (1993), who revealed that cold hardiness in bread wheat is controlled by both additive and dominant genes, a concept reflected in the present study's observed distribution patterns. Likewise, Skinner *et al.* (2008) investigated freezing tolerance in F_2-derived F_4 populations, demonstrated that freezing sensitivity is influenced by strongly dominant genetic factors. The observed inheritance patterns in the current study may echo these findings, suggesting the presence of dominant genes in governing cold stress resistance. Overall, these findings contribute to our understanding of the heritability of cold stress resistance traits in wheat, providing a foundation for future research on crop improvement strategies.

5.6 Transcriptomic analysis for cold tolerance

Transcriptome analysis showcased a remarkable divergence in gene expression patterns under cold stress, with SKAU 52 exhibiting more upregulated genes than SKAU 4301 suggesting an active and potentially more effective adaptive mechanism, reinforcing its enhanced cold stress tolerance. The transcription factor analysis unraveled a complex regulatory landscape, where key families such as NAC, ERF, bHLH and C2H2 emerged as major players in both genotypes. Notably, SKAU 52 displayed a diverse repertoire of TFs, including the crucial NAC family, indicative of an elaborate regulatory network orchestrating its response to cold stress (Diao *et al*., 2022). Notably, the NAC (NAM, ATAF1/2 and CUC2) family of TFs emerged as a key regulator in both the cold-tolerant genotype SKAU 52 and the cold-susceptible genotype SKAU3 4301. NAC TFs are known to regulate various stress-responsive genes, playing a central role in plant responses to environmental challenges, including cold stress. Additionally, ERF TFs are well-documented for their involvement in ethylene-mediated responses and have been implicated in enhancing cold tolerance in plants(Ritonga *et al*.,2021). The abundance of ERF (Ethylene Response Factor) family proteins, particularly in SKAU 52, underscores their significance in activating stress signaling pathways. ERF TFs are well-documented for their involvement in ethylene-mediated responses and have been implicated in enhancing cold tolerance in plants. These TF families, along with others such as bHLH (basic Helix-Loop-Helix) and C_2H_2, MYB-related, WRKY and HSF, contribute to the fine-tuning of gene expression, enabling wheat plants to adapt and thrive under cold stress conditions (Sharma *et al*., 2020).

In the cold-tolerant wheat genotype SKAU 52, the upregulation of genes associated with protein-containing complexes, cell parts and membranes under cold stress aligns with the well-documented cellular response to cold acclimation. Cold-tolerant plants often enhance membrane stability and cellular structure to withstand low temperatures. The increased enzymatic and binding responses,

along with upregulated antioxidant and transcription regulator activities, suggest a robust defense mechanism (Sareen and Joshi, 2023). Enhanced enzymatic activities may facilitate metabolic adjustments, while upregulated antioxidant activities counteract reactive oxygen species produced during stress. Transcriptional regulation indicates a proactive approach to modulating gene expression for stress adaptation. Conversely, SKAU 4301's distinct changes may signify a different set of adaptive mechanisms or an altered response to stress, potentially involving rearrangements in cellular components and intensified enzymatic activities to cope with cold-induced challenges. Moreover, In SKAU 52 under cold stress, the upregulation of pathways related to "Metabolic pathways," "Biosynthesis of secondary metabolites," and "Plant-pathogen interaction" reflects an active metabolic reprogramming and a strategic preparation for potential pathogenic challenges associated with cold stress. The alterations in pathways like "Phenylpropanoid biosynthesis" and "MAPK signaling pathway - plant" indicate a sophisticated regulatory network involved in adapting to cold stress (Song *et al*., 2022; Pareek *et al*., 2017). Downregulation of pathways like "Photosynthesis - antenna proteins," "Necroptosis," and "RNA polymerase" demonstrates a prioritization of resources away from energy-intensive processes toward essential functions. In SKAU 4301, the upregulation of pathways suggests an effort to adapt to cold stress, though the broad suppression of essential pathways may indicate compromised metabolic activities and potential vulnerabilities to stress-induced disruptions.

Also, the in-depth analysis of differentially expressed genes (DEGs) highlighted key players in its adaptive response to cold stress. The infinite fold changes observed in Wcor18, WCOR15-2B, Wcor615, DHN33, Cbf18 and many genes involved in the lipid metabolism, phenylpropanoid pathway, trehalose synthesis underscore their pivotal roles in the genotype's cold stress response. These genes likely contribute to the activation of crucial pathways involved in cold acclimation and dehydrin-mediated protective mechanisms. The ICE-CBF-

COR pathway plays a crucial role in cold tolerance in wheat. The ICE genes are inducers of CBF expression, which in turn activate the transcription and expression of CBF genes (Liu *et al*., 2022; Hwarari *et al*., 2022). The CBF proteins bind to the C-repeat/Dehydration Responsive Element (CRT/DRE) in the promoter region of cold-regulated (COR) genes, leading to their transcriptional activation. Additionally, the DHN (dehydrin) and RAB (responsive to ABA) genes are also involved in cold tolerance in wheat. These genes are part of the molecular mechanisms that enable wheat plants to acclimate to cold stress and improve their cold tolerance (Welin *et al*., 1994; Shakirova *et al*., 2009; Hao *et al*., 2022).

5.7 Differential proteomic response due to cold stress

The proteomic investigation of cold-tolerant (SKAU_52) and cold-susceptible (SKAU_4301) wheat genotypes provided a comprehensive understanding of the molecular mechanisms underlying their differential responses to cold stress. In total, 1939 proteins were identified.

One key observation was the dynamic alteration in protein profiles in SKAU_52 after exposure to cold stress. The increased number of identified proteins (1168) compared to control conditions (1060) indicates a robust and specific response to cold stress. In contrast, SKAU_4301 exhibited a more modest change, with 1154 proteins after cold stress compared to 1073 in control conditions. This disparity suggests increased adaptability and responsiveness of SKAU_52 under cold stress suggesting cold-tolerant phenotype.

Pathway analysis revealed 48 pathways across both genotypes under control and stress conditions, providing a comprehensive overview of the cellular processes involved. The increased activity of redox reactions, polyamine metabolism and abiotic stress pathways in SKAU_52 is particularly noteworthy. These pathways have been previously implicated in conferring cold tolerance in plants, aligning with the findings of earlier studies (Singh *et al*., 2018; Gao *et al*.,

2020). The upregulation of amino acid synthesis and degradation pathways under stress, with SKAU_52 exhibiting more pronounced changes, is consistent with reports linking enhanced amino acid metabolism to cold tolerance.

Carbohydrate metabolism, especially the Calvin cycle, decreased under stress in both genotypes, indicating a shift in energy utilization. Similar response was observed by the study performed by Majumder and co-worker suggesting an energy-saving strategy during cold stress (Majumder *et al*., 2020). The consistent activation of protein synthesis and folding pathways in both genotypes and increased protein degradation in SKAU_4301 suggests a robust protein homeostasis mechanism in SKAU_52, contributing to its cold tolerance.

The comparison of protein expression patterns between genotypes highlighted inherent disparities, with 236 proteins showing significant differences under control conditions. SKAU_52's dynamic response to cold stress was evident, with alterations in 326 proteins compared to its control counterpart. Notable upregulation of stress-related proteins, including cold shock protein-1 and key redox-regulating genes in SKAU_52, further supports its enhanced cold tolerance. These findings align with earlier studies linking cold shock proteins and redox regulation to cold tolerance in plants (Dreyer *et al*., 2018; Hossain *et al*., 2018). The upregulation of proteins related to cold tolerance in SKAU_52, such as lipid transfer protein 6, Flavoprotein wrbA, peroxidases and calcium-related proteins, aligns with previous studies. Lipid transfer proteins have been implicated in membrane remodeling during cold stress in rice (Zhao *et al*., 2020), barley (Choi *et al*., 2015) and maize (Wei *et al*., 2014). Further the upregulation of peroxidases and calcium-related proteins in our cold tolerant genotype underscores their roles in mitigating oxidative stress, a common consequence of cold exposure (Tang *et al*., 2020). The limited upregulation of signaling proteins in the cold-susceptible genotype further highlights its compromised ability to mount an effective response. These findings provide valuable insights into the molecular basis of wheat cold tolerance and align with and contribute to the

existing body of knowledge in plant cold stress responses. Further studies can build upon these results to unravel the precise functions of identified proteins and pathways, paving the way for targeted strategies to enhance cold tolerance in crops.

Chapter-6

SUMMARY AND CONCLUSIONS

The summary and conclusions of the study entitled "**Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas,"** are presented below. This chapter encapsulates the key outcomes and insights gained from an extensive exploration of the genetic, physiological and breeding aspects related to cold tolerance in wheat within the unique environmental conditions of the Western Himalayas.

1. Field screening of large diverse set of wheat genotypes, in the challenging climatic conditions of Wadura, Kashmir, India, provided valuable insights into the distribution of cold stress tolerance among the screened wheat genotypes over two consecutive years, 2020 and 2021. Majority of wheat genotypes screened for cold tolerance exhibited a moderate resistance (cold score 2), with a limited number classified as cold-tolerant (score 1) or highly cold-tolerant (score 0), offering potential for breeding more resilient wheat varieties.

2. The results of our two-year field screening of wheat germplasm for cold stress tolerance, coupled with membrane stability assessment have provided more valuable information about the genetic basis of cold tolerance in wheat. The observed wide range of ELI values among the 350 selected wheat genotypes is a clear indicator of the substantial genetic diversity within the germplasm under study. This variation offers potential opportunities for selecting genotypes with enhanced membrane stability and cold stress tolerance.

3. The 350 wheat genotypes were grouped into five categories (HR, R, MR, S and HS) based on their cold scores in the field screening. A significant difference in mean ELI values among these groups was obseved. The

mean EC values of the genotypes in the highly resistant (HR) group were the lowest (14.2 µS/cm), indicating better membrane stability, while the mean EC values of the genotypes in the highly susceptible (HS) group were the highest (90.6 µS/cm), indicating the lowest membrane stability.

4. Analysis of 50 diverse wheat genotypes under controlled cold stress conditions revealed significant variations in stress-responsive biochemical parameters, malondialdehyde (MDA) levels, hydrogen peroxide (H_20_2) levels, protein content, antioxidant enzyme activities *viz,* ascorbate peroxidase (APX) activity, superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPX) activity and osmoprotectant *viz,* proline at different cold treatments viz, T0, T1, T2 and T3, where (T0=control, T1= acclimation phase at 4℃ for 14 days, T2=cold stress at -5℃ after acclimation and T3=cold stress at -5℃ without acclimation treatments, respectively)

5. One-way ANOVA revealed significant variations in the mean values of each biochemical parameter across the treatments, indicating distinct effects on the plant's biochemistry. Notably, protein levels, indicative of cellular integrity and stress response, exhibited dynamic changes across treatment levels. The acclimation phase (T1) triggered a significant increase in protein levels, suggesting the activation of adaptive mechanisms to enhance stress tolerance.

6. MDA and H_2O_2 levels, indicators of lipid peroxidation and oxidative stress, exhibited contrasting patterns. The control group (T0) displayed low levels, signifying minimal oxidative damage. Cold stress without acclimation (T3) led to a significant increase in MDA and H202, indicating heightened oxidative stress. However, acclimation (T1) and cold stress after acclimation (T2) showed lower levels than T3, suggesting that acclimation provides protection against oxidative damage.

7. Antioxidant enzymes (APX, SOD, CAT, GPX) plays a crucial role in mitigating oxidative stress during cold stress. The activities of these enzymes were significantly influenced by the treatments, with increased activities observed during acclimation (T1) and cold stress after acclimation (T2). This indicates an enhanced antioxidant defense system, suggesting that acclimation primes plant genotypes for improved antioxidant capacity.

8. The study employed various criteria, including field cold screening data, ELI and biochemical data, to identify cold-tolerant genotypes. Lower cold scores, low ELI values, low MDA, higher antioxidant enzyme activities and increased proline accumulation were key criteria. Cold-susceptible genotypes exhibited opposite trends, indicating their reduced capacity to counteract oxidative stress. Genotypes like SKUA_52, SKUA_44, SKUA_116, SKUA_127, SKUA_118, SKUA_6 with cold score 0, elevated antioxidants and reduced damage showed exceptional cold tolerance, marking them as promising candidates for breeding resilient wheat varieties.

9. The metabolomic investigation into the responses of wheat genotypes SKUA_52 (cold tolerant) and SKUA_4301 (cold susceptible) to cold stress provided valuable insights into the activation of defense mechanisms, particularly highlighting the modulation of key defense hormones including Salicylic Acid (SA), Jasmonic Acid (JA), Abscisic Acid (ABA), JA-Isoleucine (JA-ILE) and cis-Octadecanoid-derivative.

10. SKUA_52, identified as cold-tolerant, exhibited notable increases in defense hormone levels, including Salicylic Acid, Jasmonic Acid, Abscisic Acid, JA-Isoleucine and cis-Octadecanoid-derivative, underscoring its robust defense mechanisms against low temperatures. In contrast, the cold-susceptible genotype, SKUA_4301, displayed distinct metabolomic dynamics in response to cold stress. These findings emphasize the

genotype-specific nature of metabolomic responses, providing valuable information for targeted breeding efforts aimed at enhancing cold stress resilience in wheat varieties.

11. The comprehensive LC-MS analysis unveiled distinctive profiles of key flavonoids, including Naringenin, Rutin, Quercitin, Petunidin, Peonidin and Malvidin. Notably, SKUA_52 consistently demonstrated elevated levels of these flavonoids when subjected to cold stress, presenting a stark contrast to the cold-susceptible SKUA_4301. These findings underscore the potential of flavonoids as molecular markers associated with cold stress resilience in wheat.

12. The untargeted metabolite analysis revealed significant changes in metabolite profiles under cold stress conditions and identified genotype-specific variations in response. The cold-tolerant genotype, SKUA_52 exhibited dynamic metabolic adaptations, with specific metabolites like disaccharides, monosaccharides, amino acids, polyamines playing key roles in response to cold stress.

13. Lipidomics investigation by GC-MS revealed distinct lipid dynamics and profiles in our two contrasting wheat genotypes SKUA_52 and SKUA_4301. Cold stress induced substantial changes in the expression of specific lipids, especially saturated and unsaturated lipids, highlighting the profound influence of cold stress on lipid metabolism. The cold-tolerant genotype, SKUA_52, exhibited up-regulation of certain unsaturated lipids, including DGDG, PC, MGDG, PE and PG species, suggesting their role in enhancing membrane flexibility and fluidity, crucial for cold tolerance.

14. Our study delved into the molecular mechanisms underlying the differential cold stress responses in wheat genotypes, focusing on the ICE-CBF-COR pathway. The coordinated upregulation of specific genes within

the ICE-CBF-COR pathway in the cold-tolerant genotype highlights the importance of these genes in conferring cold tolerance.

15. Through qRT-PCR analysis, we investigated the expression patterns of cold tolerance-related genes in the cold-tolerant SKAU_52 and cold-susceptible SKAU_4301 genotypes. While the key regulator TaICE1D did not show significant differential expression, TaCOR genes (TaCOR3b, TaCOR5a and TaCOR3d) exhibited substantial upregulation in SKAU_52 under cold stress, emphasizing their role in cold acclimation and improved tolerance. Among the CBF genes, TaCBF11b displayed minimal fold change, while TaCBF2d showed significant upregulation in SKAU_52, suggesting nuanced regulatory roles for specific CBF genes in the cold response. The coordinated upregulation of TaICE2B, TaCBF11b, TaCBF2d and TaICE1D further underscores their collective contribution to enhanced cold tolerance in SKAU_52.

16. The analysis of hybridization experiments, specifically the cross between SKAU_× SKAU_4301 and SKAU_44 × SKAU_1422, suggests that cold tolerance in wheat may be governed by a single dominant gene. Despite a minor deviation in the F2 generation, the close alignment with Mendelian principles and the absence of significant differences in the calculated chi-square value support the hypothesis of a dominant genetic factor influencing cold stress resistance traits. This implies a potentially straightforward genetic basis for cold tolerance in this particular wheat cross.

17. Transcriptome analysis showcased a remarkable divergence in gene expression patterns under cold stress, with SKAU 52 exhibiting more upregulated genes than SKAU 4301 suggesting an active and potentially more effective adaptive mechanism, reinforcing its enhanced cold stress tolerance.

18. The infinite fold changes observed in Wcor18, WCOR15-2B, Wcor615, DHN33, Cbf18 and many genes involved in the lipid metabolism, phenylpropanoid pathway, trehalose synthesis underscore their pivotal roles in the SKUA_52 genotype's cold stress response.

19. The proteomic investigation of cold-tolerant (SKAU_52) and cold-susceptible (SKAU_4301) wheat genotypes provided a comprehensive understanding of the molecular mechanisms underlying their differential responses to cold stress. Notable upregulation of stress-related proteins, including cold shock protein-1 and key redox-regulating genes in SKAU_52, further supports its enhanced cold tolerance.

20. Overall, these findings provided the holistic understanding of the complex interactions shaping cold tolerance in wheat. Field screening indicated a range of cold stress tolerance among wheat genotypes, with potential for breeding resilient varieties. Biochemical analyses highlighted genetic diversity and identified criteria for selecting cold-tolerant genotypes. Controlled stress conditions demonstrated the importance of acclimation for enhanced stress tolerance. Metabolomic, flavonoid and lipidomic analyses provided molecular markers for resilience. Molecular investigations into the ICE-CBF-COR pathway suggested coordinated gene regulation and hybridization experiments hinted at a potential single dominant gene influencing cold tolerance. These findings offer a comprehensive understanding for targeted breeding of cold-tolerant wheat varieties in the Western Himalayan context.

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Annexure I

Cold screening of 4560 genotypes in the field during the year 2020-21 and year 2021-22.

Genotype	Cold Score 2020-21	Cold score 2021-22	Genotype	Cold Score 2020-21	Cold score 2021-22	Genotype	Cold Score 2020-21	Cold score 2021-22	Genotype	Cold Score 2020-21	Cold score 2021-22	Genotype	Cold Score 2020-21	Cold score 2021-22
SKUA_1	3	3	SKUA_913	4	1	SKUA_1825	4	3	SKUA_2737	2	2	SKUA_3649	1	2
SKUA_2	3	3	SKUA_914	3	2	SKUA_1826	2	3	SKUA_2738	2	2	SKUA_3650	2	1
SKUA_3	4	2	SKUA_915	3	1	SKUA_1827	4	2	SKUA_2739	2	1	SKUA_3651	2	1
SKUA_4	4	3	SKUA_916	4	1	SKUA_1828	3	2	SKUA_2740	2	1	SKUA_3652	2	1
SKUA_5	3	3	SKUA_917	2	1	SKUA_1829	3	2	SKUA_2741	3	2	SKUA_3653	2	2
SKUA_6	2	2	SKUA_918	4	1	SKUA_1830	4	2	SKUA_2742	2	2	SKUA_3654	1	2
SKUA_7	3	2	SKUA_919	4	2	SKUA_1831	3	2	SKUA_2743	2	2	SKUA_3655	2	3
SKUA_8	4	3	SKUA_920	3	2	SKUA_1832	3	2	SKUA_2744	3	2	SKUA_3656	1	1
SKUA_9	4	2	SKUA_921	3	1	SKUA_1833	3	2	SKUA_2745	2	2	SKUA_3657	3	1
SKUA_10	3	2	SKUA_922	4	1	SKUA_1834	4	2	SKUA_2746	1	3	SKUA_3658	3	1
SKUA_11	2	2	SKUA_923	4	2	SKUA_1835	2	3	SKUA_2747	3	2	SKUA_3659	2	3
SKUA_12	3	3	SKUA_924	3	1	SKUA_1836	3	3	SKUA_2748	2	1	SKUA_3660	2	3
SKUA_13	4	3	SKUA_925	4	2	SKUA_1837	3	2	SKUA_2749	4	1	SKUA_3661	2	1
SKUA_14	4	3	SKUA_926	3	2	SKUA_1838	3	2	SKUA_2750	2	2	SKUA_3662	3	2
SKUA_15	1	3	SKUA_927	3	1	SKUA_1839	3	2	SKUA_2751	2	3	SKUA_3663	4	1
SKUA_16	1	2	SKUA_928	2	3	SKUA_1840	2	2	SKUA_2752	2	3	SKUA_3664	4	1
SKUA_17	3	3	SKUA_929	3	3	SKUA_1841	4	2	SKUA_2753	3	3	SKUA_3665	2	1
SKUA_18	4	3	SKUA_930	4	2	SKUA_1842	2	2	SKUA_2754	2	1	SKUA_3666	3	2
SKUA_19	4	3	SKUA_931	2	1	SKUA_1843	3	3	SKUA_2755	4	2	SKUA_3667	3	2
SKUA_20	3	2	SKUA_932	3	2	SKUA_1844	3	2	SKUA_2756	3	1	SKUA_3668	2	3
SKUA_21	3	3	SKUA_933	4	2	SKUA_1845	2	2	SKUA_2757	3	2	SKUA_3669	4	3
SKUA_22	4	2	SKUA_934	3	1	SKUA_1846	2	2	SKUA_2758	3	2	SKUA_3670	3	3
SKUA_23	4	3	SKUA_935	3	1	SKUA_1847	3	1	SKUA_2759	2	2	SKUA_3671	1	2
SKUA_24	4	2	SKUA_936	4	1	SKUA_1848	4	2	SKUA_2760	2	2	SKUA_3672	3	2
SKUA_25	4	2	SKUA_937	4	2	SKUA_1849	4	2	SKUA_2761	2	2	SKUA_3673	2	2
SKUA_26	3	3	SKUA_938	3	1	SKUA_1850	4	3	SKUA_2762	2	2	SKUA_3674	3	1
SKUA_27	3	3	SKUA_939	1	1	SKUA_1851	3	3	SKUA_2763	3	2	SKUA_3675	3	1
SKUA_28	1	2	SKUA_940	1	2	SKUA_1852	3	2	SKUA_2764	2	2	SKUA_3676	3	1
SKUA_29	3	2	SKUA_941	1	1	SKUA_1853	1	2	SKUA_2765	3	3	SKUA_3677	4	1
SKUA_30	2	3	SKUA_942	2	1	SKUA_1854	2	3	SKUA_2766	1	2	SKUA_3678	4	0
SKUA_31	4	2	SKUA_943	3	1	SKUA_1855	2	2	SKUA_2767	3	2	SKUA_3679	4	0

SKUA_32	2	2	SKUA_944	3	2	SKUA_1856	2	2	SKUA_2768	3	3	SKUA_3680	3	0
SKUA_33	3	2	SKUA_945	3	1	SKUA_1857	3	3	SKUA_2769	4	3	SKUA_3681	4	0
SKUA_34	4	3	SKUA_946	3	1	SKUA_1858	2	2	SKUA_2770	1	3	SKUA_3682	3	3
SKUA_35	3	3	SKUA_947	2	2	SKUA_1859	4	2	SKUA_2771	4	2	SKUA_3683	3	2
SKUA_36	4	3	SKUA_948	3	3	SKUA_1860	3	2	SKUA_2772	4	3	SKUA_3684	4	2
SKUA_37	3	2	SKUA_949	3	3	SKUA_1861	3	3	SKUA_2773	4	2	SKUA_3685	4	1
SKUA_38	4	2	SKUA_950	4	3	SKUA_1862	4	2	SKUA_2774	3	3	SKUA_3686	3	1
SKUA_39	4	2	SKUA_951	3	1	SKUA_1863	2	1	SKUA_2775	4	4	SKUA_3687	2	2
SKUA_40	3	3	SKUA_952	4	1	SKUA_1864	2	2	SKUA_2776	1	3	SKUA_3688	3	2
SKUA_41	1	3	SKUA_953	3	2	SKUA_1865	3	2	SKUA_2777	2	4	SKUA_3689	4	1
SKUA_42	1	3	SKUA_954	3	1	SKUA_1866	2	2	SKUA_2778	2	4	SKUA_3690	4	3
SKUA_43	0	2	SKUA_955	2	2	SKUA_1867	2	2	SKUA_2779	2	3	SKUA_3691	3	3
SKUA_44	0	2	SKUA_956	4	2	SKUA_1868	3	1	SKUA_2780	1	3	SKUA_3692	4	2
SKUA_45	2	2	SKUA_957	3	1	SKUA_1869	2	1	SKUA_2781	3	3	SKUA_3693	3	2
SKUA_46	0	2	SKUA_958	4	1	SKUA_1870	3	1	SKUA_2782	4	4	SKUA_3694	3	1
SKUA_47	1	1	SKUA_959	2	1	SKUA_1871	3	1	SKUA_2783	4	3	SKUA_3695	2	2
SKUA_48	2	2	SKUA_960	3	2	SKUA_1872	4	2	SKUA_2784	2	3	SKUA_3696	3	3
SKUA_49	3	2	SKUA_961	3	3	SKUA_1873	4	1	SKUA_2785	4	2	SKUA_3697	2	2
SKUA_50	1	2	SKUA_962	4	2	SKUA_1874	3	1	SKUA_2786	4	3	SKUA_3698	3	2
SKUA_51	1	1	SKUA_963	2	3	SKUA_1875	4	2	SKUA_2787	3	3	SKUA_3699	2	1
SKUA_52	1	1	SKUA_964	3	2	SKUA_1876	2	2	SKUA_2788	2	3	SKUA_3700	3	2
SKUA_53	3	2	SKUA_965	4	2	SKUA_1877	2	1	SKUA_2789	2	2	SKUA_3701	4	2
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SKUA_859	2	2	SKUA_1771	4	2	SKUA_2683	3	3	SKUA_3595	1	2	SKUA_4507	4	0
SKUA_860	1	2	SKUA_1772	2	1	SKUA_2684	3	3	SKUA_3596	2	1	SKUA_4508	2	0
SKUA_861	3	1	SKUA_1773	3	1	SKUA_2685	3	3	SKUA_3597	4	1	SKUA_4509	2	1
SKUA_862	3	1	SKUA_1774	3	1	SKUA_2686	4	3	SKUA_3598	3	1	SKUA_4510	2	1
SKUA_863	2	1	SKUA_1775	3	2	SKUA_2687	4	2	SKUA_3599	2	1	SKUA_4511	1	1
SKUA_864	4	1	SKUA_1776	4	3	SKUA_2688	4	4	SKUA_3600	2	2	SKUA_4512	2	2
SKUA_865	3	3	SKUA_1777	4	1	SKUA_2689	3	2	SKUA_3601	3	2	SKUA_4513	4	1
SKUA_866	2	3	SKUA_1778	4	1	SKUA_2690	2	3	SKUA_3602	1	2	SKUA_4514	4	1
SKUA_867	3	3	SKUA_1779	4	2	SKUA_2691	3	3	SKUA_3603	2	2	SKUA_4515	3	1
SKUA_868	1	1	SKUA_1780	2	3	SKUA_2692	3	1	SKUA_3604	4	1	SKUA_4516	2	1
SKUA_869	4	3	SKUA_1781	3	1	SKUA_2693	3	1	SKUA_3605	4	2	SKUA_4517	2	1
SKUA_870	4	1	SKUA_1782	2	1	SKUA_2694	3	2	SKUA_3606	4	2	SKUA_4518	4	1
SKUA_871	3	1	SKUA_1783	4	1	SKUA_2695	3	2	SKUA_3607	4	1	SKUA_4519	4	2
SKUA_872	2	1	SKUA_1784	3	1	SKUA_2696	3	2	SKUA_3608	1	1	SKUA_4520	3	2
SKUA_873	2	3	SKUA_1785	3	2	SKUA_2697	3	2	SKUA_3609	3	1	SKUA_4521	4	1
SKUA_874	3	2	SKUA_1786	3	2	SKUA_2698	3	3	SKUA_3610	3	0	SKUA_4522	2	1
SKUA_875	2	2	SKUA_1787	4	3	SKUA_2699	3	2	SKUA_3611	2	1	SKUA_4523	4	1
SKUA_876	1	2	SKUA_1788	4	2	SKUA_2700	3	2	SKUA_3612	1	1	SKUA_4524	2	2
SKUA_877	3	2	SKUA_1789	3	4	SKUA_2701	3	3	SKUA_3613	4	1	SKUA_4525	2	1
SKUA_878	3	3	SKUA_1790	2	3	SKUA_2702	3	3	SKUA_3614	2	2	SKUA_4526	2	1
SKUA_879	2	3	SKUA_1791	4	2	SKUA_2703	3	4	SKUA_3615	2	3	SKUA_4527	3	1
SKUA_880	2	1	SKUA_1792	3	3	SKUA_2704	3	2	SKUA_3616	3	2	SKUA_4528	3	1
SKUA_881	1	1	SKUA_1793	3	3	SKUA_2705	4	2	SKUA_3617	3	1	SKUA_4529	3	2
SKUA_882	3	1	SKUA_1794	4	3	SKUA_2706	2	1	SKUA_3618	3	2	SKUA_4530	4	2
SKUA_883	3	1	SKUA_1795	3	3	SKUA_2707	3	1	SKUA_3619	2	2	SKUA_4531	4	2
SKUA_884	2	2	SKUA_1796	4	3	SKUA_2708	2	1	SKUA_3620	4	1	SKUA_4532	4	1
SKUA_885	3	3	SKUA_1797	4	2	SKUA_2709	4	3	SKUA_3621	4	3	SKUA_4533	4	1
SKUA_886	2	2	SKUA_1798	4	4	SKUA_2710	4	3	SKUA_3622	1	3	SKUA_4534	4	1
SKUA_887	4	2	SKUA_1799	4	2	SKUA_2711	3	2	SKUA_3623	2	2	SKUA_4535	4	2
SKUA_888	4	1	SKUA_1800	3	1	SKUA_2712	3	2	SKUA_3624	1	2	SKUA_4536	3	1
SKUA_889	3	1	SKUA_1801	3	2	SKUA_2713	2	2	SKUA_3625	3	1	SKUA_4537	4	1

SKUA_890	3	2	SKUA_1802	4	3	SKUA_2714	3	2	SKUA_3626	3	1	SKUA_4538	3	0
SKUA_891	3	1	SKUA_1803	3	3	SKUA_2715	1	2	SKUA_3627	4	2	SKUA_4539	3	2
SKUA_892	2	0	SKUA_1804	4	3	SKUA_2716	2	2	SKUA_3628	3	3	SKUA_4540	3	3
SKUA_893	2	0	SKUA_1805	3	2	SKUA_2717	2	2	SKUA_3629	2	1	SKUA_4541	3	2
SKUA_894	2	1	SKUA_1806	3	3	SKUA_2718	2	2	SKUA_3630	1	2	SKUA_4542	3	2
SKUA_895	3	3	SKUA_1807	3	4	SKUA_2719	1	2	SKUA_3631	1	1	SKUA_4543	3	1
SKUA_896	2	2	SKUA_1808	3	3	SKUA_2720	4	2	SKUA_3632	1	1	SKUA_4544	3	1
SKUA_897	3	1	SKUA_1809	2	3	SKUA_2721	4	2	SKUA_3633	4	1	SKUA_4545	3	0
SKUA_898	3	3	SKUA_1810	2	3	SKUA_2722	1	2	SKUA_3634	2	0	SKUA_4546	1	2
SKUA_899	2	2	SKUA_1811	3	2	SKUA_2723	2	2	SKUA_3635	3	0	SKUA_4547	4	2
SKUA_900	2	3	SKUA_1812	3	2	SKUA_2724	3	2	SKUA_3636	4	0	SKUA_4548	2	2
SKUA_901	2	1	SKUA_1813	4	3	SKUA_2725	3	2	SKUA_3637	3	0	SKUA_4549	2	2
SKUA_902	3	1	SKUA_1814	3	2	SKUA_2726	3	2	SKUA_3638	4	0	SKUA_4550	2	2
SKUA_903	2	2	SKUA_1815	4	3	SKUA_2727	3	2	SKUA_3639	3	1	SKUA_4551	4	2
SKUA_904	3	1	SKUA_1816	3	2	SKUA_2728	2	2	SKUA_3640	1	1	SKUA_4552	2	2
SKUA_905	4	0	SKUA_1817	3	2	SKUA_2729	2	3	SKUA_3641	1	2	SKUA_4553	4	2
SKUA_906	3	2	SKUA_1818	3	3	SKUA_2730	2	3	SKUA_3642	1	2	SKUA_4554	3	2
SKUA_907	2	3	SKUA_1819	4	3	SKUA_2731	1	3	SKUA_3643	2	2	SKUA_4555	2	1
SKUA_908	4	1	SKUA_1820	3	2	SKUA_2732	1	1	SKUA_3644	2	1	SKUA_4556	3	1
SKUA_909	4	1	SKUA_1821	2	1	SKUA_2733	2	2	SKUA_3645	2	1	SKUA_4557	3	1
SKUA_910	4	1	SKUA_1822	2	2	SKUA_2734	3	3	SKUA_3646	1	2	SKUA_4558	4	1
SKUA_911	3	2	SKUA_1823	3	2	SKUA_2735	2	3	SKUA_3647	1	2	SKUA_4559	4	1
SKUA_912	2	3	SKUA_1824	3	3	SKUA_2736	3	3	SKUA_3648	2	2	SKUA_4560	4	2

Annexure II

List of metabolites identified in SKUA_52 (Stressed) in comparison with SKUA_52 (control) by GC-MS during the current study. The values were derived from the peak area of the corresponding metabolites in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples.

Metabolite	Class	SKUA_52 (Stressed)	SKUA_52 (control)	Log2 Value	p-VALUE
N-Feruloylputrescine	Phenolamine	8451035	1772892.667	2.523706064	0.00282292
5-oxoproline	Amino Acid And Its Derivatives	1050392	254845	2.054343388	0.042730461
1-Methyladenine	Nucleotide And Its Derivates	30219100.33	11510114.83	1.71246104	0.000164191
Naringenin 7-O-glucoside	Flavonoids	1197840	370310	1.682703162	6.22E-10
Chrysoeriol	Flavonoids	3683681	1168193.667	1.651899872	0.019243138
2,3-Dihydroxy-2-methylpropanoic acid	Organic acids and derivatives	7298150.333	2650486.333	1.448154607	0.017102457
2,5-Furandione	Organic oxygen compounds	264649.6667	101195.3333	1.392217243	0.003706184
7-Methylxanthine	Nucleotide and its derivates	21255030.33	10081407.67	1.097556898	0.018440296
Sucrose	Carbohydrates	22457806.33	11718270.67	0.926540491	0.028547274
DL-Glyceraldehyde, tris(trimethylsilyl) ether	Carbohydrates	9828558.667	5362566.333	0.862651853	0.036746861
D-Fructose	Carbohydrates	17721842	9872746.5	0.842320542	0.012011569
N-p-Coumaroylagmatine	Phenolamine	191964	108323.5	0.81274223	0.016605556

Arabinofuranose	Carbohydrates	4659117	2661266.833	0.791101579	0.003509357
1-Hexacosanol	Alcohols	701019	415696.5	0.746546882	0.032970627
Galactitol	Carbohydrates	59184507.33	35006040.5	0.743546838	0.015170713
Acetin	Esters	205903.3333	122503.1667	0.739987406	0.010045381
3-Heptanol	Alcohols	531609.6667	320307.6667	0.721749317	0.001976141
D-Pantothenic acid	Vitamins	10056837.67	6116014.5	0.717106803	0.000508292
alpha-Arabinopyranose	Carbohydrates	637231	391758.5	0.69690173	4.12E-10
3-Hydroxybutyric acid	Organic acids and derivatives	384217.6667	244039.5	0.668529897	0.032599127
beta-D-Glucopyranose	Carbohydrates	10660234	6986775.667	0.592037812	0.00930042
Melibiose	Carbohydrates	430685469.7	285763931.3	0.59180575	0.004528042
Erythrono-1,4-lactone	Carbohydrates	17988752.67	12094549	0.582809646	0.032796762
Methyl galactoside	Carbohydrates	3293468	2241512.5	0.546505646	0.001049037
Methyl alpha-D-glucofuranoside	Carbohydrates	450358	307860.3333	0.543811545	0.012464417
Maltitol, nonakis(trimethylsilyl) ether	Carbohydrates	821637.6667	584892.8333	0.511324014	0.00064249
beta-D-Galactopyranoside	Carbohydrates	2310112.667	1746094.167	0.404068572	0.023053717
2-hydroxy-2-methyl-1-phenylpropane	Organic oxygen compounds	1416535.333	1102183	0.375145404	0.006160723
4-Guanidinobutyric Acid	Organic Acid And Its Derivatives	35891436.33	28365052.17	0.336840383	0.008457918
2-Pyrrolidinone	Organic oxygen compounds	2171901.333	1740681.167	0.315556924	0.027756687
5'-Deoxy-5'-(Methylthio)Adenosine	Nucleotide And Its Derivates	9247519.333	7517056	0.28296433	0.004528042
1-Monomyristin	Fatty acids and conjugates	12423715	10180352.83	0.264933854	0.029078134
(3-Hydroxy-4-	Others	3860786.667	3247082.333	0.251434699	0.047619416

methoxyphenyl)ethylene glycol					
1-Butanamine	Amines	1022393.333	872669	0.221114739	0.008624271
Adonitol	Carbohydrates	736740	626025.5	0.213817849	0.032599127
L-Tryptophan	Amino Acid And Its Derivatives	129800678.7	110430576.2	0.212302789	0.003295346
alpha-Linolenic acid	Carbohydrates	335122518.3	290009180.8	0.20187487	0.004714463
L-Tyrosine	Amino Acid And Its Derivatives	12609121	10936305.17	0.197782665	0.009059481
N-Feruloylspermidine derivative	Phenolamine	1109970.667	1000576.333	0.154657698	0.017349176
1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol	Carbohydrates	69309650.33	63142115.33	0.121006407	0.00211892
1-Cyclohexylethanol	Alcohols	822212.6667	789730.5	0.073887432	0.034682243
3-(p-Fluorobenzoyl)-propionic acid	Organic acids and derivatives	1130183	1092706.5	0.03564744	0.004528042
beta-D-Lactose	Carbohydrates	92184861.33	92147223.17	0	0.00383072
4-Hydroxybutanoic acid	Organic acids and derivatives	93818.66667	100003.3333	-0.129122619	0.000653689
2,3-Butanediol	Carbohydrates	751921.6667	860118.3333	-0.149587533	0.01117518
1,2,3-Butanetriol	Carbohydrates	876111.6667	1029096.667	-0.221468636	6.22E-07
Ethylene glycol	Organic oxygen compounds	210207.6667	282324.3333	-0.416332705	0.002205007
Galactinol, nonakis(trimethylsilyl) ether	Carbohydrates	497748.6667	678272	-0.424946704	0.005408895
Durohydroquinone	Organic oxygen compounds	119173	175484.3333	-0.561014173	0.027401533
4-Ketoglucose	Carbohydrates	68369	105139.1667	-0.616579685	0.00064249
meso-Erythritol	Carbohydrates	882021.3333	1429592.5	-0.752045847	0.020823857
1,3-Bis(trimethylsilyloxy)pentane	Organic oxygen compounds	1070946	1827261.167	-0.811546547	0.027756687

Nobiletin	Flavonoids	3740827	14295884.17	-1.94912496	0.006581652
2-alpha-Mannobiose	Carbohydrates	6150794	32646776.33	-2.531727718	0.006581652
Acrylic acid	Organic acids and derivatives	675112.3333	4225943.833	-2.674981297	0.042627325
3-alpha-Mannobiose	Carbohydrates	15587982.33	102142190.2	-2.730583913	0.011988177
8-Gingerol 1	Phenols	291104.3333	1127232.333	-2.980497259	0.000297418
Coniferin	Phenols	344036.6667	2784101	-3.717940543	0.047700221
Chrysoeriol 5-O-hexoside	Flavonoids	209742.3333	1909803.667	-3.7649069	0.020181394
3-Hydroxy-2-(trimethylsilyl)oxy)propyl palmitate	Organic oxygen compounds	1060466.333	18268007.83	-4.117267595	0.015720767
5,6-Dihydro-5-Methyluracil	Nucleotide And Its Derivates	108134.3333	2365021.5	-4.776628043	0.001913691
5-Methylcytosine	Nucleotide And Its Derivates	883930.6667	19532224.83	-4.790682582	0.020823857
Maltose	Carbohydrates	5132640	173857735.7	-5.133800853	0.001913691
L-(+)-Erythrulose, tris(trimethylsilyl) ether	Carbohydrates	350357.6667	8873140.5	-5.930134101	0.000432508
L-(+)-Threose	Carbohydrates	1506942.667	90390142.67	-6.600157937	0.029089841

Annexure III

List of metabolites identified in SKUA_52 (Stressed) in comparison with SKUA_4301 (stressed) by GC-MS during the current study. The values were derived from the peak area of the corresponding metabolites in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples.

Metabolite	Class	SKUA_52 (stressed)	SKUA_4301 (stressed)	Log 2FC	P-value
Sucrose	Carbohydrates	22457806.33	978735	4.5193	0.007453
beta-D-Allopyranose	Carbohydrates	3219240.333	247063.3333	3.7027	0.038401
Monolaurin	Fatty acids and conjugates	312803577.7	26646853	3.5247	0.044623
DL-Glyceraldehyde, tris(trimethylsilyl) ether	Carbohydrates	9828558.667	896574	3.4423	0.015815
Tricin 5-O-hexosyl-O-hexoside	Flavonoids	31601172.67	3336333.333	3.2177	0.045952
N', N'', N'''-DiFeruloyl,Sinapoylspermidine	Phenolamine	6286986	668586	3.2121	0.020675
Spermidine derivative	Phenolamine	11957662	1352182.333	3.1006	0.002472
D-Fructose	Carbohydrates	17721842	2023651	3.0888	0.003079
Dulcitol	Carbohydrates	11975266	1464864.667	2.9971	0.044202
Biopterin	Organoheterocyclic compounds	12287251.33	1539657.333	2.9638	0.023197
β-Nicotinamide Adenine Dinucleotide	Nucleotide And Its Derivates	6749562	857376	2.9466	0.001998
N', N''-DiFeruloylspermidine	Phenolamine	1207767.333	155236.6667	2.9317	0.043851
Silane, [(1-methoxy-1,3-propanediyl)bis(oxy)]bis[trimethyl-	Organic oxygen compounds	157526081.3	22206509	2.8057	0.002742

Arabinofuranose	Carbohydrates	4659117	663416.6667	2.7953	0.005671
D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 2)	Carbohydrates	2700827	400353.6667	2.7681	0.00651
D-(+)-Ribono-1,4-lactone (R,S,R)-	Carbohydrates	2279975.333	344516.6667	2.7507	0.005862
Dimethyl hexopyranosiduronate	Carbohydrates	602795.6667	97406.33333	2.6564	0.043611
Galactitol	Carbohydrates	59184507.33	10827573.67	2.4389	0.003066
1-Hexacosanol	Alcohols	701019	130374	2.4235	0.000771
Glycerol	Organic oxygen compounds	3967698	763175.6667	2.3979	0.001902
beta-D-Galactofuranose	Carbohydrates	6198637	1201000.667	2.3925	0.002619
D-Glucopyranose	Carbohydrates	114580805	22459201.33	2.3784	0.040037
3-Heptanol	Alcohols	531609.6667	109005.6667	2.3381	0.04081
L-Homoserine	Amino Acid And Its Derivatives	5130231.333	1092328.667	2.3004	0.049364
D-Pantothenic acid	Vitamins	10056837.67	2175191.333	2.2853	0.004603
D-Psicopyranose(isomer 2)	Carbohydrates	28500386	6227630.333	2.2762	0.000524
alpha-Arabinopyranose	Carbohydrates	637231	146286	2.2359	0.003099
D-(+)-Xylose, 4TMS derivative	Carbohydrates	811899.6667	188325.3333	2.2286	0.003401
L-Tryptophan	Amino Acid And Its Derivatives	129800678.7	91060473.67	0.5129	0.000771
Hypoxanthine	Nucleotide And Its Derivates	1368937.333	962196.6667	0.5116	0.001902
1-Butanamine	Amines	1022393.333	722944.6667	0.5074	0.002619
L-Pipecolic Acid	Amino Acid And Its Derivatives	1624110.333	1150430.667	0.5068	0.040037
Glucose	Carbohydrates	438354716	313644153	0.5011	0.04081
alpha-Linolenic acid	Fatty acids and conjugates	335122518.3	244895843.3	0.4909	0.049364
Glycerol monostearate	Organic oxygen	57384773	42131308	0.4892	0.004603

	compounds				
L-Tyrosine	Amino Acid And Its Derivatives	12609121	9263489.333	0.4889	0.000524
D-Fructose, 6-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-alpha-D-glucopyranosyl]-1,3,4,5-tetrakis-O-(trimethylsilyl)-	Carbohydrates	378769027	282715166	0.4817	0.003099
Talose	Carbohydrates	217407.3333	8610225.667	-4.6023	0.007589
5,6-Dihydro-5-Methyluracil	Nucleotide And Its Derivates	108134.3333	4621908.667	-4.7249	0.003966
5-Methylcytosine	Nucleotide And Its Derivates	883930.6667	38180519	-4.7313	0.000559
Diosmetin	Flavonoids	2050691.667	92953729.33	-4.7745	0.001743
L-(+)-Erythrulose, tris(trimethylsilyl) ether	Carbohydrates	350357.6667	17395923.33	-4.8463	0.002634
L-Rhamnose	Carbohydrates	239903	12274954	-4.8694	0.002726
4-Hydroxybutanoic acid	Organic acids and derivatives	93818.66667	5305988	-4.9516	0.004486
Hydroquinone	Organic oxygen compounds	256298.3333	14980192.67	-4.9812	0.042592
Maltose, octakis(trimethylsilyl) ether, methyloxime	Carbohydrates	5132640	342582831.3	-5.1277	0.043153
di-C,C-hexosyl-methylluteolin	Flavonoids	5103967.333	528772444.7	-5.8816	0.000943
3-Hydroxy-2-(trimethylsilyl)oxy)propyl palmitate	Organic oxygen compounds	1060466.333	115475549.3	-5.9324	0.00211
L-(+)-Threose, tris(trimethylsilyl) ether, methyloxime	Carbohydrates	1506942.667	179273342.7	-6.0298	0.002188

Annexure IV:

List of metabolites identified in SKUA_4301 (Stressed) in comparison with SKUA_4301 (control) by GC-MS during the current study. The values were derived from the peak area of the corresponding metabolites in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples

Metabolite	Class	SKUA_4301 (stressed)	SKUA_4301 (control)	Log2 FC	P-value
D-Fructose, TMS	Carbohydrates	282715166	56262000.67	2.609811186	0.000121
Vitamin B2	Vitamins	77641208	15836400	2.561453287	0.0408102
Melibiose	Carbohydrates	140842393	38196393.67	1.89268749	0.0104426
N-Acetylglucosamine 1-phosphate	Others	4963160.667	1396580.333	1.833104045	0.0126105
5-Methylcytosine	Nucleotide And Its Derivates	38180519	11656830.67	1.702060644	0.0027262
L-Rhamnose	Carbohydrates	12274954	3803750	1.693878618	0.0002416
N-Acetylaspartate	Amino Acid And Its Derivatives	8175325.667	3762374.667	1.124792564	0.0030988
Coniferin	Phenols	5224165.333	2508680.333	1.069108676	0.0159769
L-Pipecolic Acid	Amino Acid And Its Derivatives	1150430.667	575790.6667	0.999611686	0.0001916
Glucose	Carbohydrates	313644153	187983900	0.740703944	0.0038131
Diosmetin	Flavonoids	92953729.33	70695568.67	0.38980443	0.0026344
1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol	Carbohydrates	56974580.33	53130710.67	0.097292386	0.0058619
D-(+)-Cellobiose	Carbohydrates	370258022	360293500.3	0.032912486	0.043851
di-C,C-hexosyl-methylluteolin	Flavonoids	528772444.7	514868222.7	0.032060437	0.0034006
D-Trehalose	Carbohydrates	217448555.3	256183032.3	-0.200616492	0.0017435
Galactopyranose	Carbohydrates	1483964.667	1754481.667	-0.214791222	0.0115979
D-Glucopyranose	Carbohydrates	22459201.33	27551001.67	-0.356250055	0.0077006
3-Hydroxy-2-(trimethylsilyl)oxy)propyl palmitate	Organic oxygen compounds	115475549.3	159526994	-0.479440929	0.0370469

Chrysoeriol	Flavonoids	6652706.333	10318806.33	-0.611417703	0.0436114
alpha-D-Lactose	Carbohydrates	49514133.33	81319857.33	-0.795271703	0.0056706
1,2,3-Butanetriol	Carbohydrates	1182081.667	1958373.667	-0.934219007	0.020675
Chrysoeriol 5-O-hexoside	Flavonoids	3609865	6943818.333	-0.951868414	0.0202949
methylNaringenin C-pentoside	Flavonoids	14666920.67	25665700	-1.156057707	0.0026188
L-Sorbopyranose (1S,2R,3S)	Carbohydrates	2378610.667	4309731.333	-1.174854531	0.044202
(3-Hydroxy-4-methoxyphenyl)ethylene glycol	Others	2633378	5945525.667	-1.481231118	0.0377076
Erythrono-1,4-lactone	Carbohydrates	6200345.333	19091352.67	-1.63811619	0.002472
D-Ribopyranose	Carbohydrates	165762.3333	552559.3333	-1.739359706	0.049364
D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 2)	Carbohydrates	400353.6667	1430626.333	-1.819602715	0.004603
Ethylene glycol	Organic oxygen compounds	354441	1305704.667	-1.9004914	0.010823
Durohydroquinone	Organic oxygen compounds	231795.6667	881352	-1.957748186	0.0020626
N', N''-DiSinapoylspermidine	Phenolamine	2348980	8761394	-1.999270271	0.0006195
D-(+)-Xylose, 4TMS derivative	Carbohydrates	188325.3333	732685.6667	-2.001388056	0.0005588
5-oxoproline	Amino Acid And Its Derivatives	259298	1200350.667	-2.165558607	0.0143149
N-Acetyl-L-Tyrosine	Amino Acid And Its Derivatives	2897975	12997830	-2.468152046	0.02713
Arabinose	Organic acids and derivatives	274080.6667	1849015	-2.755125226	0.0446229
Dimethyl hexopyranosiduronate	Carbohydrates	97406.33333	1057949	-3.515512937	0.0061817
1-Butanamine	Amines	722944.6667	10035028	-3.821295844	0.0083675
D-Fructose	Carbohydrates	2023651	42481162.67	-4.480905344	0.0007705
3-Heptanol	Alcohols	109005.6667	2524531	-4.481002791	0.0030792
N,N-Dimethylglycine	Amino Acid And Its Derivatives	237057.3333	6071386	-5.397395886	0.0217672
1-Hexacosanol	Alcohols	130374	23423765.67	-7.437734282	0.0039656
beta-Sitosterol	Organic oxygen compounds	477514	97255040	-8.579409428	0.0005242
N6,N6-dimethyladenine	Nucleotide and its derivates	902734	448386512.3	-10.65181392	0.0323116

Annexure V:

List of metabolites identified in SKUA_52 (control) in comparison with SKUA_4301 (control) by GC-MS during the current study. The values were derived from the peak area of the corresponding metabolites in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples.

Metabolite	Class	SKUA_52 (control)	SKUA_4301 (control)	Log2 FC	P-value
D-Fructose, TMS	Carbohydrates	3307420.5	562620.6667	9.19326729	0.000139
5-Methylcytosine	Nucleotide And Its Derivates	19532224.83	1165683.667	4.070694104	0.024392
N-Acetylglucosamine 1-phosphate	Others	11374781.17	1396580.333	3.013914828	0.041234
Melibiose	Carbohydrates	285763931.3	38196393.67	2.887776859	0.020853
D-Lactose	Carbohydrates	78175832	14047958.33	2.457637254	0.044802
Ascorbic acid	Carbohydrates	5945962.5	1133511	2.394062269	0.036629
Vitamin B2	Vitamins	68906276.5	15836400	2.109732525	0.027636
1-Methyladenine	Nucleotide And Its Derivates	11510114.83	2803310	2.038206828	0.044802
8-Gingerol 1	Phenols	1127232.333	379539	1.567457171	0.037732
D-Glucopyranose	Carbohydrates	68520003.17	27551001.67	1.298683735	0.020387
L-Pipecolic Acid	Amino Acid And Its Derivatives	1387270.5	575790.6667	1.261414429	0.031019
2,3-Dihydroxy-2-methylpropanoic acid	Organic acids and derivatives	2650486.333	1320551.333	1.017391334	0.031396
Glucose	Carbohydrates	375999434.5	187983900	1.008798042	0.002188
beta-D-Lactose	Carbohydrates	92147223.17	51244110.67	0.80795127	0.004002
Biochanin A 7-O-glucoside	Flavonoids	29428762.67	16718930.67	0.802817736	0.005051
L-Rhamnose	Carbohydrates	6257428.5	3803750	0.710010281	0.022625
2-Pyrrolidinone	Organic oxygen compounds	1740681.167	1125314	0.619307313	0.027372
D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer	Carbohydrates	1410222.5	1032005.667	0.461391612	0.011329

2)					
N-Acetylaspartate	Amino Acid And Its Derivatives	5104471	3762374.667	0.432251033	0.00211
Coniferin	Phenols	2784101	2508680.333	0.154992694	0.00275
D-(-)-Ribofuranose, tetrakis(trimethylsilyl) ether (isomer 2)	Carbohydrates	1550590.333	1430626.333	0.104067206	0.033976
D-Arabinose, tetrakis(trimethylsilyl) ether, ethyloxime (isomer 2)	Carbohydrates	997097.5	984157.3333	0.027384317	0.000808
Galactopyranose	Carbohydrates	1657128	1754481.667	-0.106021513	0.022885
D-Ribopyranose	Carbohydrates	437561.6667	552559.3333	-0.321379679	0.00018
beta-D-Allopyranose	Carbohydrates	1733151.833	2526327.667	-0.52949664	0.026077
D-(+)-Xylose, 4TMS derivative	Carbohydrates	500112.5	732685.6667	-0.543705081	0.02546
D-(+)-Cellobiose	Carbohydrates	221700133	360293500.3	-0.570557311	0.014701
Erythrono-1,4-lactone	Carbohydrates	12094549	19091352.67	-0.624802109	0.00964
Diosmetin	Flavonoids	47502210.5	70695568.67	-0.631194692	0.024196
(3-Hydroxy-4-methoxyphenyl)ethylene glycol	Others	3247082.333	5945525.667	-0.792699269	0.019237
di-C,C-hexosyl-methylluteolin	Flavonoids	266938206	514868222.7	-0.946502179	0.003077
2,5-Furandione	Organic oxygen compounds	101195.3333	196309	-0.954288598	0.006839
D-Trehalose	Carbohydrates	109132138.7	256183032.3	-1.21064472	0.008673
N', N''-DiSinapoylspermidine	Phenolamine	3437224.167	8761394	-1.352275183	0.000943
methylNaringenin C-pentoside	Flavonoids	9704690.333	25665700	-1.415860446	0.034418
D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether	Carbohydrates	644948.3333	1941801.667	-1.594504252	0.041211
Dimethyl hexopyranosiduronate	Carbohydrates	350101	1057949	-1.628543949	0.006402
alpha-D-Lactose	Carbohydrates	26066802.33	81319857.33	-1.670062898	0.009338
L-Sorbopyranose (1S,2R,3S)	Carbohydrates	1305001.833	4309731.333	-1.739907111	0.043153
Chrysoeriol 5-O-hexoside	Flavonoids	1909803.667	6943818.333	-1.857004703	0.000313

D-Fructose	Carbohydrates	9872746.5	42481162.67	-2.165602276	0.047084
D-Lactose, octakis(trimethylsilyl) ether, methyloxime	Carbohydrates	2805765.5	13459579.33	-2.612312671	0.011363
Arabinose	Organic acids and derivatives	286563	1849015	-2.617030563	0.019785
Ethylene glycol	Organic oxygen compounds	282324.3333	1305704.667	-2.617581246	0.027938
Durohydroquinone	Organic oxygen compounds	175484.3333	881352	-2.652090827	0.000901
2-alpha-Mannobiose, octakis(trimethylsilyl) ether, methyloxime	Carbohydrates	32646776.33	159051456	-2.665068882	0.00964
5-oxoproline	Amino Acid And Its Derivatives	254845	1200350.667	-2.749572647	0.004403
1-Butanamine	Amines	872669	10035028	-3.509350065	0.003265
N,N-Dimethylglycine	Amino Acid And Its Derivatives	471584	6071386	-3.614810201	0.015069
N-Acetyl-L-Tyrosine	Amino Acid And Its Derivatives	1795435.333	12997830	-3.824650692	0.021928
3-Heptanol	Alcohols	320307.6667	2524531	-3.97916563	0.019035
Chrysoeriol	Flavonoids	1168193.667	10318806.33	-4.140369139	0.009282
1-Hexacosanol	Alcohols	415696.5	23423765.67	-4.142608401	0.016702
3-Hydroxybutyric acid	Organic acids and derivatives	244039.5	2117323.667	-4.172316792	0.020336
3-Hydroxy-2-(trimethylsilyl)oxy)propyl palmitate	Organic oxygen compounds	18268007.83	159526994	-4.177356569	0.008673
D-(-)-Rhamnose, tetrakis(trimethylsilyl) ether, methyloxime (syn)	Carbohydrates	965867.6667	16217407	-4.805503521	0.012369
beta-Sitosterol	Organic oxygen compounds	1250415.333	97255040	-6.678786127	0.001812
N6,N6-dimethyladenine	Nucleotide and its derivates	1352205	448386512.3	-7.823768429	0.042592
D-2-Hydroxypentanedioic acid	Amino Acid And Its Derivatives	328141.5	85601893.67	-8.448822145	0.003232
DL-Glyceraldehyde, bis(trimethylsilyl) ether, ethyloxime	Carbohydrates	482031.8333	561791990	-9.756960808	0.015497

Annexure-VI

List of lipids identified in SKUA_52 (control) in comparison with SKUA_4301 (control) by GC-MS during the current study. The values were derived from the peak area of the corresponding lipids in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples.

Lipid	SKUA_52 (control)	SKUA_4301 (control)	Log2 FC	P-value
LPC(24:4)	49720	36687	1.355248453	0.007452826
PC(32:1)	112951	42277	2.671689098	0.038401469
PC(33:4)	52651	469397	0.112167313	0.044622886
PC(35:3)	119148	238592	0.499379694	0.015815374
PC(36:1)	212308	98319	2.159379164	0.045952008
PC(36:6)	210643	543826	0.387335287	0.020674956
PC(37:5)	246849	259017	0.953022388	0.002471956
PC(38:3)	44546	37807	1.178247414	0.003079167
PE(32:2)	1305663	1303505	1.001655536	0.044201998
PE(33:3)	158804	145360	1.092487617	0.023197102
PE(35:4)	138801	4320292	0.03212769	0.001998379
PG(34:4)	30592	31971	0.956867161	0.043850991
PI(36:2)	117452	431088	0.272454812	0.002742327

Annexure VII

List of lipids identified in SKUA_52 (stressed) in comparison with SKUA_52 (control) by GC-MS during the current study. The values were derived from the peak area of the corresponding lipids in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples.

LIPID CLASS	SKUA_52(stressed)	SKUA_52(Control)	Log 2 FC	P-value
PC(34:0)	10002	51470	0.194326792	0.038401469
PC(38:5)	18495	84930	0.217767573	0.044622886
LPG(16:0)	1893553	6269160	0.302042538	0.015815374
PC(38:6)	35817	62585	0.572293681	0.045952008
PC(39:4)	26181	48876	0.535661674	0.020674956
LPC(15:0)	184955	259935	0.71154327	0.002471956
PE(34:0)	17211	20734	0.830085849	0.003079167
PC(35:3)	1533848	1758467	0.872264307	0.044201998
PC(34:1)	113494	119148	0.952546413	0.023197102
DGDG(34:3)	180861	186571	0.969395029	0.001998379
DGDG(36:6)	639247	629836	1.014941985	0.043850991
PC(35:2)	1706309	1569959	1.086849402	0.002742327
PC(32:1)	47278	37114	1.273858921	0.005670562
PE(32:2)	153848	112951	1.362077361	0.006510207
PC(36:3)	1978169	1305663	1.51506859	0.005861879
PC(35:1)	395572	233746	1.69231559	0.043611353
PC(33:4)	219896	99667	2.206307002	0.003065694
LPC(18:3)	124416	52651	2.363032041	0.000770549
PC(36:6)	133993	52751	2.540103505	0.001901748
PC(34:4)	554137	210643	2.630692689	0.002618796
PC(36:4)	287970	107926	2.668217112	0.040037498
PC(38:2)	121659	43749	2.780840705	0.040810204
PC(33:3)	92402	29393	3.143673664	0.049363978
PC(32:2)	165432	47118	3.511014899	0.004602966
PC(33:2)	491071	136057	3.609303454	0.000524193
PC(35:4)	160824	44100	3.646802721	0.003098839

Annexure VIII

List of lipids identified in SKUA_4301 (stressed) in comparison with SKUA_4301 (control) by GC-MS during the current study. The values were derived from the peak area of the corresponding lipids in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples

LIPID CLASS	SKUA_4301(stressed)	SKUA_4301(Control)	Log 2FC	P-value
PC(35:4)	155388	153388	1.01303883	0.000121
PC(32:0)	83189	221189	0.376099173	0.04081
PC(36:0)	1131470	1111470	1.017994188	0.010443
PC(34:2)	91934	59434	1.54682505	0.012611
PC(34:3)	156655	224155	0.698869086	0.002726
PC(35:5)	699620	689620	1.01450074	0.000242
PE(40:4)	44700	42600	1.049295775	0.003099
PG(34:4)	33471	31971	1.046917519	0.015977
LPC(15:0)	33522	22522	1.488411331	0.000192
LPC(16:0)	100766	54266	1.856890134	0.003813
PC(34:0)	46353	94853	0.488682488	0.002634
PE(34:0)	1876820	1874320	1.001333817	0.005862
LPC(18:3)	28990	27490	1.054565296	0.043851
PC(32:1)	44777	42277	1.059133808	0.003401

PC(34:1)	122930	112930	1.088550429	0.001743
PC(35:1)	266148	263648	1.00948234	0.011598
PC(35:2)	140435	138435	1.014447213	0.007701
PE(33:1)	415800	414300	1.003620565	0.037047
PE(33:3)	146360	145360	1.006879472	0.043611
LPC(18:1)	471032	468532	1.005335815	0.005671
LPC(24:4)	39187	36687	1.068144029	0.020675
PC(36:1)	100419	98319	1.021359046	0.020295
PE(32:1)	21827065	21824565	1.00011455	0.002619
PE(33:2)	10535651	10510651	1.00237854	0.044202
PE(34:1)	2935235	2932735	1.000852447	0.037708
PE(38:1)	283033	280533	1.008911608	0.002472
PE(38:2)	279601	277012	1.009346166	0.049364
PE(38:6)	103110	200110	0.515266603	0.004603
PI(34:2)	371112	3696120	0.100405831	0.010823
PI(36:2)	432588	431088	1.003479568	0.002063
PI(36:5)	272292	270292	1.007399405	0.000619

Annexure IX

List of lipids identified in SKUA_52(stressed) in comparison with SKUA_4301 (stressed) by GC-MS during the current study. The values were derived from the peak area of the corresponding lipids in data files generated by GC-MS and represented the relative content of corresponding metabolites. The values can be only compared within the same metabolites between different samples

LIPID CLASS	SKUA_52(stressed)	SKUA_4301(stressed)	Log 2FC	P-value
DGDG(34:3)	639247	232514	2.749283914	0.020853171
DGDG(36:3)	87453	85816	1.019075697	0.044801554
DGDG(36:6)	1706309	961230	1.775130822	0.036628962
DGDG(38:6)	484301	1279789	0.378422537	0.027636324
LPC(17:0)	21816	48867	0.446436245	0.044801554
MGDG(36:4)	368375	110331	3.338816833	0.037731662
MGDG(36:6)	353609	342523	1.03236571	0.02038657
PC(32:0)	103363	83189	1.242508024	0.031018835
PC(33:3)	165432	245432	0.674044134	0.031395654
PC(34:2)	234869	91934	2.554756673	0.002188346
PC(34:3)	411037	156655	2.623835818	0.004002254
PC(34:4)	287970	85017	3.387204912	0.00505058
PC(35:3)	113494	241092	0.470749755	0.022624867
PC(35:4)	432612	155388	2.78407599	0.027371701
PC(35:5)	955483	699620	1.365717104	0.011328616
PC(36:0)	691837	1131470	0.611449707	0.002109879
PC(36:3)	395572	240196	1.646871721	0.002750418
PC(36:6)	554137	546326	1.014297324	0.033976466
PC(37:5)	256059	262517	0.975399688	0.000807641
PC(38:3)	75443	38807	1.944056485	0.022885325
PC(38:4)	372769	3635255	0.102542738	0.000180105

PC(38:5)	1893553	654152	2.894668212	0.026076777
PC(38:6)	26181	874802	0.029927915	0.025459666
PC(39:4)	184955	561072	0.32964575	0.014700607
PC(39:6)	1005240	992477	1.012859744	0.009640164
PE(34:2)	202791	80294	2.525605898	0.024196247
PE(35:4)	334844	4327792	0.077370631	0.0192365
PE(35:6)	634589	139873	4.536894183	0.003077478
PE(36:6)	930923	580056	1.604884701	0.006839466
PE(38:3)	1723373	277393	6.212748699	0.008672822
PE(38:4)	101097	85197	1.18662629	0.000942705
PE(40:4)	83534	44700	1.868769575	0.034417978
PG(34:4)	103388	33471	3.088882914	0.041210723
PG(36:5)	1087803	289187	3.761590251	0.006402471
PG(36:6)	465246	150593	3.089426467	0.009338245
PG(36:7)	86805	127877	0.678816363	0.043153083
PI(36:4)	237910	275410	0.863839367	0.000313269
SQDG(38:4)	321458	1709301	0.18806401	0.047084076

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. Upendra Kumar Balyan, Professor, Plant Sciences, Mahatma Jyotiba Phule Rohikhhand University, Bareilly, Uttar Pradesh, India during Viva-Voce examination held on 07-03-2024 have been incorporated in the manuscript entitled **"Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas"** submitted by **Ms. Sofora Jan (Regd. No. 2020-975-D)**

(Dr. Reyazul Rouf Mir)
Chairman
Advisory Committee

Personal Information

AREAS OF INTEREST:

- Molecular Breeding, Crop Biotechnology, Applied Genomics, Transcriptomics

TECHNICAL SKILLS:

- **Laboratory Techniques:** DNA and RNA extraction, genotyping, qRT-PCR, GC-MS, LC-MS, spectrophotometry.
- **Metabolomics and Transcriptomics:** Expertise in metabolomics and transcriptomics methodologies.
- **Field Phenotyping:** Skilled in trait phenotyping in the field.
- **Statistical Analysis:** Proficient in R-software for statistical analysis.
- **Data Management:** MS Excel for data analysis and visualization.
- Competent in using primer designing tools, TB-Tools, Metaboanalyst for metabolomics data analysis and graphics softwares like origin-pro, Biorender.

EDUCATION

2020- 2024: Ph. D., Genetics & Plant Breeding, with 8.93 OGPA.

Thesis title: "Integrated Metabolomics and Genomics studies in Response to Cold Stress in a Core Set of Wheat (*Triticum aestivum* L.) in Western Himalayas".

2017-2019: M. Sc. Genetics & Plant breeding, with **9.19** OGPA.

Thesis title: "**Transcriptomics for Differential Expression of Genes for Seed micronuurtrients (Fe and Zn) in Common Beans (*Phaseolus vulgaris*)"**

2012-2017: B. Sc. (Agriculture) with **8.24**

MEDALS/AWARDS

1. Received **Highest Marks Award Certificate** in B. Sc for getting highest marks in B. Sc., Agriculture.
2. Received **Highest Marks Award Certificate** in M. Sc for getting highest marks in M. Sc., Genetics & Plant Breeding.
3. **Best poster presentation award** during 19th J&K Agriculture Science Congress organized by University of Kashmir, March, 2018.
4. **Received Certificate of Appreciation and Medal** for scoring highest merit in Ph.D (UET) conducted by SKUAST-K, in 2020.

5. **Received Merit certificate** for securing Third Position in written test assessment conducted during Training Program on "Rejigging the concept of Genetics' with germaneness to translational research" (03-10-August, 2020)
6. Winner of 2021 **Wiley's Early Career Research Competition** for best publication.
7. Qualified **ICAR-NET** 2021.
8. **Best Oral presentation award** on "Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas" in Ist international Symposium on Cereals for food security and Climate Resilience".
9. **Best Oral presentation award** on "Integrated Genomics, Physiology and Breeding for Understanding Mechanism of Cold Tolerance in Bread Wheat (*Triticum aestivum* L.) in Western Himalayas" in National Seminar ON Recent Advances in science and technology for Agriculture Sustainibility".
10. **Best oral presentation award on "**Genomics Assisted Approaches for Breeding Climate Resilient wheat for western Himalayas of Kashmir Valley" in International conference on Existing climate change scenario and its Arising Risks".
11. **Achiever Award_2023** for High Impact Publications by SKUAST-Kashmir.

PUBLICATIONS:

S:no	Publication
1	**Jan, S**., Kumar, S., Yousuf, M., Shafi, S., Majid, R., Khan, M. A.,..& Mir, R. R. (2023). Do diverse wheat genotypes unleash their biochemical arsenal differentially to conquer cold stress? A comprehensive study in the Western Himalayas. ***Physiologia Plantarum***, *175*(6), e14069.
2	**Jan, S**., Rustgi, S., Barmukh, R., Shikari, A. B., Leske, B., Bekuma, A., & Mir, R. R. (2023). Advances and opportunities in unraveling cold-tolerance mechanisms in the world's primary staple food crops. ***The plant genome,*** e20402.
3	Kumar, V., Sharma, H., Saini, L., Tyagi, A., Jain, P., Singh, Y., Balyan, P., Kumar, S., **Jan, S**., Mir, R.R. and Djalovic, I., 2022. Phylogenomic analysis of 20S proteasome gene family reveals stress-responsive patterns in rapeseed (Brassica napus L.). ***Frontiers in plant science,*** p.4269.

4	Sharma, D., Kumari, A., Sharma, P., Singh, A., Sharma, A., Mir, Z. A., **Jan S., …** & Kumar, S. (2023). Meta-QTL analysis in wheat: progress, challenges and opportunities. ***Theoretical and Applied Genetics,*** *136*(12), 1-25.
5	Kumar, S., Saini, D. K., Jan, F., **Jan, S**., Tahir, M., Djalovic, I.,... & Mir, R. R. (2023). Comprehensive meta-QTL analysis for dissecting the genetic architecture of stripe rust resistance in bread wheat. ***BMC genomics***, *24*(1), 1-19.
6	Choudhary N, Bawa V, **Jan S**, Singh B, Bhat MA, Paliwal R, Gupta A, Chitikineni A, Thudi M, Varshney RK, Mir RR (2021) Allelic Diversity, Structural Analysis and Genome-wide Association Study (GWAS) for Yield and Related Traits using Unexplored Common bean (Phaseolus vulgaris L.) Germplasm from Western Himalayas. **Frontiers in Genetics** (Plant Genomics)
7	Jan S, Khan MN, **Jan S**, Zaffar A, Rashid R, Khan MA, Sheikh FA, Bhat MA, Mir RR (2021) Trait Phenotyping and Molecular Marker Characterization of Barley (*Hordeum vulgare* L.) Germplasm from Western Himalayas. **Genetic Resources and Crop Evolution** https://doi.org/10.1007/s10722-021-01251-z
8	**Jan S**, Rather IA, Sofi PA, Wani MA, Sheikh FA, Bhat MA, Mir RR (2021) Characterization of common bean (*Phaseolus vulgaris* L.) germplasm for morphological and seed nutrient traits from Western Himalayas. **Legume Science (Wiley Online Library).**
9	Choudhary N, Anjali, Gupta M, Shafi S, **Jan S**, Mir A, Singh B, Mir RR (2021) Molecular Diversity and Nutriment studies of Common bean (Phaseolus vulgaris L.) from the Two Hot-Spots of Western Himalayas of Jammu and Kashmir. **Crop and Pasture Science.**
10	Nakeeb-Un-Nisa, Sakina, A., Sofi, N.R. Shikari, A.B., Mir R.R., **S, Jan**, Bhat M.A., *et al*. (2022) DNA marker-based diversity across rice genotypes and advanced breeding lines bred for temperate regions of North-West India. ***Molecular Biology Reports*** https://doi.org/10.1007/s11033-022-07609-5