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Deciphering Cold Stress Resilience: Multiomics Insights in Contrasting Wheat Genotypes From the Western Himalayas

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ABSTRACT

Cold stress threatens wheat productivity, particularly in regions with extreme climatic conditions. To elucidate the molecular mechanisms underlying wheat's response to cold stress, we performed a multiomics analysis integrating lipidomics, transcriptomics, proteomics and metabolomics. Our study focused on two wheat genotypes with contrasting cold tolerance levels, SKAU_52 (tolerant) and SKAU_4301 (susceptible) to capture genotype-specific responses under cold stress. Lipidomic analysis revealed significant changes in lipid composition, with unsaturated lipids such as digalactosyldiacyl glycerols (DGDGs) and monogalactosyldiacylglycerols (MGDGs) upregulated in response to cold stress. These lipids are associated with maintaining membrane fluidity, whereas saturated lipids were downregulated in the cold-tolerant genotype. Transcriptomics analysis provides a strong evidence that cold tolerance in wheat is governed by coordinated activation of the ICE-CBF-COR regulatory cascade, with the cold-tolerant genotype 'SKAU_52' showing stronger and more sustained induction across pathway tiers than the cold susceptible wheat genotype 'SKAU_4301'. Similarly, proteomic data highlighted differential abundance of proteins involved in antioxidative defence, osmotic adjustment and signal transduction, including late embryogenesis abundant (LEA) proteins. Metabolome assessment revealed substantial alterations in carbohydrate and amino acid metabolism, with sucrose and amino acids such as hydroxyproline identified as key contributors to cold tolerance. Additionally, defence hormones such as salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) exhibited genotype-specific regulation with higher accumulation in cold-tolerant genotype. Overall, this integrated multi-omics approach provides novel insights into the complex molecular mechanisms underlying cold stress adaptation in wheat, supporting the development of resilient wheat varieties capable of thriving in challenging cold environments.

Sofora Jan and Farkhandah Jan contributed equally to this work.

1 | Introduction

Wheat (*Triticum aestivum* L.) is a vital cereal crop produced globally between latitudes 30°–60° N and 27°–40° S and is cultivated at elevations of up to 3000 m above sea level (Deng et al. 2005). It is grown on more than 222 million hectares worldwide, with production reaching approximately 800 million metric tonnes in the 2024/25 cropping season, far exceeding global food needs (Ashraf et al. 2024). However, various abiotic stresses impact agricultural production (Rosenzweig et al. 2014). In particular, cold stress poses a significant threat to wheat productivity in temperate environments by adversely affecting plant growth, development and yield through various physiological and biochemical changes, including alterations in membrane fluidity, cellular structure and metabolic processes (Hassan et al. 2021).

India accounts for approximately 14% of the global wheat production (Jan et al. 2026). The country reported a record wheat production of 113.29 Metric Tons, with an average yield of 35.87 qt/ha (Jan et al. 2025). Wheat cultivation is primarily carried out in temperate climates, posing challenges in regions like the Western Himalayas of Jammu and Kashmir (J&K), India. In 2024-2025, Jammu and Kashmir contributed significantly to national wheat production, yielding 2.062 metric tonnes per hectare (https://data.desagri.gov.in). However, the unique agroclimatic conditions of J&K impose challenges, particularly due to extreme cold stress. Harsh winters and unpredictable temperature fluctuations create an environment where wheat plants must employ intricate survival strategies, beginning with acclimation processes necessary to adapt to these challenging conditions (Jan, Rustgi, et al. 2023).

Plant acclimation to cold stress involves a highly coordinated, multifaceted response at multiple molecular levels. At the genomic level, this includes not only changes in gene expression but also epigenomic modifications such as DNA methylation and chromatin remodelling, which modulate the accessibility and activity of cold-responsive genes. These genomic and epigenomic alterations are integrated with transcriptomic, lipidomic, proteomic and metabolomic adjustments, collectively enabling the plant to perceive, respond to and survive under low-temperature stress. In plants, the plasma membrane plays a central role in detecting and responding to cold stress by maintaining membrane fluidity and structural integrity at low temperatures (Solanke and Sharma 2008; Yadav 2010). Acting as the primary sensor, it perceives temperature fluctuations and coordinates adaptive responses, enhancing the plant's resilience and endurance in harsh cold environments. This sophisticated mechanism entails a complex interplay of molecular interactions and signalling pathways meticulously calibrated to preserve the plant's vitality and performance. Cold stress impacts membrane fluidity by altering the composition and organisation of lipids, thereby disrupting membrane-associated cellular functions and positioning the plasma membrane (PM)- the primary sensor of low-temperature stress (Knight and Knight 2012). In recent years, lipidomics has emerged as a valuable omics tool for revealing lipid composition and dynamics in response to various environmental stresses, including cold stress, in wheat. This lipid-centric approach provides a comprehensive understanding of the role of lipids in cellular membranes, signalling and metabolic reprogramming during cold acclimation (Zhang et al. 2020; Liu, Xin, et al. 2022; Gao et al. 2024). Homeoviscous adaptation, characterised by changes in lipid saturation levels to maintain membrane fluidity, has been explored through lipidomic analyses. For instance, Zhang et al. (2020) conducted lipidomic analysis in peanuts, revealing alterations in the abundance of various lipid classes, including glycerolipids, phospholipids and sphingolipids, in response to cold stress. This study highlighted the importance of lipid remodelling in maintaining membrane fluidity and integrity under low-temperature conditions. Summarising findings from different plant studies, Yu et al. (2021) reported alterations in the unsaturation levels of membrane lipids, such as monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs), in response to cold stress as a common theme.

Transcriptomic analysis, which captures early gene-expression changes triggered by environmental signals, has revealed a set of differentially expressed genes (DEGs) and key transcription factors that play central roles in cold tolerance (Li et al. 2018; Zhao et al. 2019; Díaz et al. 2019). These DEGs are associated with various metabolic pathways, including glutamate metabolism, starch and sucrose metabolism and plant hormone signal transduction (Tian et al. 2022). The DEGs are also involved in lipid metabolism and biosynthesis pathways. Additionally, this study explores the importance of metabolites and key enzymes involved in flavonol biosynthesis and sucrose and amino acid biosynthesis pathways for cold tolerance in wheat. This study also evaluated the role of major transcription factors, such as AP2/ERF, bZIP, NAC, WRKY, bHLH and MYB, in response to cold stress. These findings provide valuable insights into the molecular mechanisms underlying cold tolerance in wheat and can contribute to the development of cold-resistant genotypes. Proteomics explores the second layer of regulation in plant environmental responses, providing insights into differential protein expression, post-translational modifications and functional adaptations (Ghatak et al. 2017; Weckwerth et al. 2020). Proteins involved in sensing cold stress, such as cold-regulated (COR) genes and cold shock proteins (CSPs), play crucial roles in initiating the cold stress response (Yu et al. 2017). Kosová et al. (2021) utilised proteomics to identify cold-responsive proteins in wheat and found them to be involved in stress signalling, osmotic regulation and antioxidant defence mechanisms. Xu et al. (2022) also performed quantitative proteome profiling of wheat under cold stress and identified a key dehydrin protein (WDhn13) responsible for cold tolerance. Metabolomics, which surveys the third layer of regulation, provides a snapshot of the small molecules (secondary metabolites) involved in cellular processes, shedding light on metabolic adjustments during cold stress (Jan, Rustgi, et al. 2023). Previous studies have identified various metabolites that are important for cold stress in wheat, including P-coumaroyl putrescine, D-proline betaine and chlorogenic acid and osmolytes such as fructose, glucose, putrescine and shikimate. These metabolites were found to differentially accumulate in wheat under cold stress conditions (Zhao et al. 2019; Yang et al. 2020; Lv et al. 2022). Additionally, the levels of auxin, cytokinin and salicylic acid were found to increase under extreme low-temperature conditions (Cheong et al. 2019). Changes in the levels of these hormones were associated with changes in the expression of genes involved in synthesis, catabolism, transport and signal transduction pathways. Integrated analysis of gene expression and metabolite profiles revealed that abscisic acid (ABA) and jasmonic acid (JA) signalling and proline biosynthesis pathways were significantly modulated under cold acclimation and freezing treatments (Zhao et al. 2019).

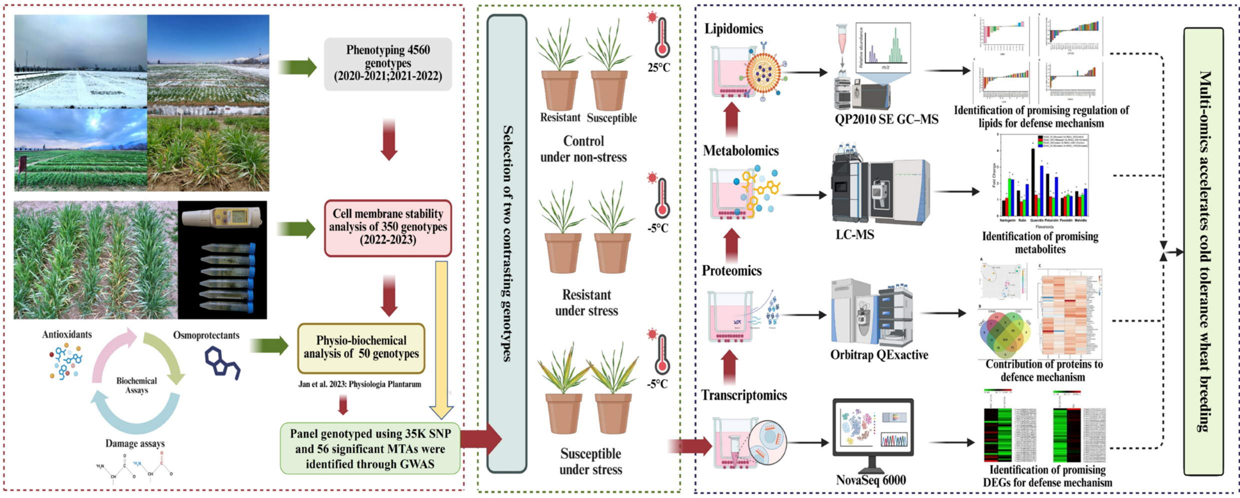

FIGURE 1 | Integrated phenotyping and multi-omics workflow for dissecting cold tolerance mechanisms in wheat. A large wheat panel (4560 genotypes) was phenotyped across multiple seasons (2020–2022), followed by stepwise screening through cell membrane stability (350 genotypes) and physio-biochemical analyses (50 genotypes). Two contrasting genotypes (cold-tolerant and cold-susceptible) were selected and evaluated under control (25°C) and cold stress (−5°C) conditions. Comprehensive lipidomics (GC–MS), metabolomics (LC–MS), proteomics (Orbitrap Q Exactive) and transcriptomics (NovaSeq 6000) analyses were conducted to identify key lipids, metabolites, proteins and differentially expressed genes associated with cold stress defence. The integrated multi-omics framework highlights molecular and biochemical regulators of cold tolerance, accelerating cold-tolerant wheat breeding.

In the context of the unique environmental challenges faced by crops in the Kashmir valley of J&K, India, understanding the molecular mechanisms governing cold tolerance in wheat has become paramount. This region experiences extreme cold stress, posing significant threats to wheat productivity and agricultural sustainability. Despite its agricultural significance, limited research has been conducted to unravel the molecular intricacies of cold stress response in wheat within this region. Given the lack of information, this study represents the first comprehensive investigation in India employing a multiomics approach to elucidate the mechanisms of cold tolerance in wheat. By analysing lipidomics, transcriptomics, proteomics and metabolomics, we aimed to uncover the complex molecular interactions associated with cold stress response in wheat. Through this holistic approach, we sought to decipher the dynamic interplay of genes, proteins and primary (lipids) and secondary metabolites involved in adaptation of wheat to cold stress, providing novel insights into the underlying mechanisms. This research contributes to the broader scientific understanding of cold stress responses in wheat by integrating multi-omics approaches, including lipidomics, transcriptomics, proteomics and metabolomics. Ultimately, our findings may have profound implications for global food security by informing the development of resilient wheat crop varieties capable of withstanding cold stress conditions.

2 | Materials and Methods

2.1 | Plant Material and Sample Collection

In our pursuit to unravel the molecular intricacies of cold tolerance in wheat, particularly in the challenging climatic conditions of the Kashmir Valley of J&K, India, we curated a subset of 350 genotypes for comprehensive investigation in our previous study (Jan, Kumar, et al. 2023). This subset stemmed from a diverse wheat germplasm panel of 4560 genotypes subjected to a multi-tiered cold screening process (Figure 1). Initially, a 2-year field evaluation was undertaken to assess the performance of these genotypes under cold/freezing stress. Following this, core diverse panel of 350 genotypes exhibiting superior field resilience were identified and evaluated for membrane stability through the electrolyte leakage index (ELI) under cold stress conditions (Jan, Kumar, et al. 2023). Subsequently, 50 selected genotypes (a mini-core set) were evaluated for various biochemicals related to lipid peroxidation, antioxidants (e.g., APX, GPX, CAT and SOD) and osmoprotectants such as proline (Jan, Kumar, et al. 2023).

Two genotypes, SKAU_52 (cold-tolerant; IC138387) and SKAU_4301 (cold-susceptible; GID6417112), emerged from this exhaustive cold/freezing stress screening process (Figure 1). Notably, the genotypes selected for the multiomics investigation were chosen based on their performance in the multi-tier screening for cold tolerance to elucidate the molecular mechanisms underlying cold tolerance. Since these selected genotypes are part of both a bigger panel of 4560 genotypes and a core set of 350 genotypes, the trait data of cold tolerance and related traits and 35 K SNP genotypic data were available with us. The trait data of cold/freezing stress, including data on cold tolerance recorded in the field and electrolyte leakage index for two consecutive years (2021–2022), were used together with 35 K SNP data for working out marker–trait associations (MTAs) for cold/freezing tolerance (Mir 2025; under preparation). The two genotypes selected during the present study differ for alleles at several important MTAs identified for cold tolerance (Mir 2025; under preparation). Plants of selected genotypes were grown in individual pots in a controlled growth chamber at SKUAST-K, FoA, Wadura. The selected genotypes were replicated in two sets, each consisting of two biological replicates. One set was designated the control group, while the other set was subjected to cold shock treatment at −5°C. At the 3-leaf stage, the plants in the treatment group were exposed to low temperature for 24 h to induce cold stress. While, the control group was maintained under normal growth conditions (25°C) without any cold shock. Following the cold shock, leaves from both the control and stress groups were carefully harvested and used for lipidome analysis, transcriptome analysis, proteome analysis and metabolome analysis.

2.2 | Lipidomics Using the GC–MS Approach

The lipid content was estimated according to the methods of Matyash et al. (2008), with minor modifications. Precisely, 300 mg of lyophilised tissue was ground to a fine powder. Subsequently, 3 mL of chilled methyl tert-butyl ether, methanol and water mixture prepared in a 6:3:1 proportion was added to the vial and shaken for an hour at 4°C. 750 μL of LC–MS-grade water was added to the suspension, vortexed and centrifuged for 15 min at 4°C. The upper organic layer containing the nonpolar lipids was subjected to GC–MS following standard procedures. Three biological replicates were used to obtain statistically relevant data.

Wheat sample extracts (1 μL) were analysed using a QP2010 SE GC–MS (Shimadzu, Kyoto, Japan) instrument equipped with an Rxi-5 ms capillary column (30 m × 0.25 mm with a 0.25 μm 5% diphenyl 95% dimethyl polysiloxane phase; Restek Corporation, Bellefonte, PA). Helium was used as the carrier gas. The GC injector temperature was maintained at 250°C, and the injector was operated in split ratio (1:5) mode with a helium flow rate of 1.00 mL/min. The initial GC column temperature was set to the boiling point of each solvent, which was held for 1 min, and then ramped to 300°C at 5°C/min with a 5 min hold at 300°C. The MS instrument was operated in electron impact ionisation mode at 70 eV and a 0.1 kV detector voltage. The ion source and MS interface temperatures were maintained at 200°C and 250°C, respectively. Mass spectra were obtained in full scan mode, with the mass range 40–500 amu scanned at a rate of 3333 scans/s. The peaks were identified and verified by comparison with the library of mass spectra (NIST). Lipids with significant differences in content were determined according to the following criteria: variable importance in the projection value ≥ 1 and fold-change ≥ 1.

2.3 | Transcriptome Sequencing

2.3.1 | RNA Extraction, Quality Check and Library Preparation

Transcriptome sequencing of the SKAU_52 and SKAU_4301 samples under control and cold stress conditions was performed using NovaSeq 6000 2X (150 bp) chemistry at NGB DIAGNOSTICS, Noida, India. RNA was extracted from the samples in triplicate and checked for quality using a NanoDrop 2500 (Thermo Fisher Scientific) and for purity and integrity with an Agilent 2100 system. First-strand cDNA was generated using random hexamer-directed reverse transcription, followed by the synthesis of second-strand cDNA. cDNA concentration was measured using a Qubit dsDNA Assay Kit, and the integrity of the cDNA was evaluated by agarose gel electrophoresis. Next, 500 ng of genomic DNA (gDNA) and 200 ng of cDNA were pooled for each sample to construct sequencing libraries with an insert size of 300–400 bp, using the NEBNext Ultra II DNA Library Prep Kit for Illumina (Cat. No. E7770; Biolabs New England), following the manufacturer's recommended protocol. The pooled libraries were subsequently sequenced on the S4 flowcell of the NovaSeq 6000 platform using 2 × 150 bp paired-end chemistry, enabling simultaneous high-resolution analysis of both genomic and transcriptomic features. The pooled libraries were indexed with unique adapter sequences to allow multiplexing, enabling multiple samples to be sequenced simultaneously in a single run. After sequencing, the samples were demultiplexed based on these unique indices, and the indexed adapter sequences were trimmed using CASAVA v1.8.2 software (Illumina Inc.). This process ensures that reads are correctly assigned to their respective samples, minimising cross-sample contamination and facilitating accurate downstream analysis.

2.3.2 | Read Statistics Read Quality Check

FastQC (version 0.11.9, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used for quality checking, which includes base quality score, sequence quality score, average base content per read and GC distribution in the reads. Universal Illumina adapters (AGATCGGAAGAGC) were removed using Trim Galore (version 0.6.7, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Sequences shorter than 20 bp were discarded. In addition to adapter removal, low-quality ends were trimmed from reads with a phred score of 20. The clean reads were mapped to the reference genome of *Triticum aestivum* (version 2.1, https://wheat-urgi.versailles.inrae.fr/Seq-Repository/Assemblies) through HISAT (version 2.2.1) using 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 were aligned to the genome using default parameters and quantify their expression. Differential expression analysis of the new transcripts that were assembled was performed using Cuffmerge with the default parameters. Transcripts with a length greater than 100 bp were used for further downstream analysis. Reads and the merged assembly were fed to Cuffdiff, which calculated the expression levels and tested the statistical significance of the observed changes with the default parameters. DEGs with an absolute (log2FC) ≥ 2 and a p value ≤ 0.05 were considered highly significant differentially expressed genes and isoforms. Gene sequences were mapped against the NR database using BLASTX (version 2.2.29+), and annotation was performed using the UniProt database (https://www.uniprot.org/). Sequence-based KEGG annotation was performed using KAAS (https://www.genome.jp/kegg/kaas/). Web Gene Ontology Annotation Plot (WEGO) (version 2.0, http://wego.genomics.org.cn/) was used for visualising and plotting the Gene Ontology results of highly significant DEGs and DEIs. GO enrichment analysis was performed using topGO (version 2.34.0), an R package for gene set enrichment analysis. The sequences were also mapped against the Plant Transcription Factor Database (version 5.0, http://planttfdb.gao-lab.org/). Cold-related genes were filtered using in-house shell scripts.

2.4 | Validation of Transcripts/Genes

Total RNA was isolated from 50 to 100 mg of flag leaf tissue of both genotypes using a Maxwell RSC Plant RNA Kit (Promega, United States) according to the recommendations of manufacturer. A RevertAid cDNA Synthesis Kit (Thermo Scientific, United States) was used for the synthesis of first-strand cDNA from the total extracted RNA. A set of 12 transcripts/genes was chosen for their expression validation. A primer list of the five differentially expressing transcripts/genes identified in our study is provided in Table S1 while the primer sequences of the remaining seven genes were taken from literature (Guo et al. 2019). The primers for transcripts identified during the present study, along with *Actin* as an endogenous control, were designed with the help of Primer Express Software v3.0.1 (Applied Biosystems) (Table S1). Quantitative real-time polymerase chain reaction was performed using a QuantStudio 6 Flex Real-Time PCR Detection System (Applied Biosystems) with PowerUp SYBR Green PCR Master Mix (Applied Biosystems) for 2 min at 50°C for UDG activation and 10 min at 95°C for initial denaturation, followed by 40 cycles of 15 s of denaturation at 95°C, 15 s of annealing at 53°C and extension at 72°C for 1 min. Fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method. The statistical significance of the differences among the transcripts was checked using a Student's *t*-test.

2.5 | Proteomics Analysis

For proteomics analysis, leaf tissue was freeze-dried in liquid nitrogen and ground to a fine powder using a mortar and pestle. The proteins were extracted, prefractionated (by loading 40 μg of total protein onto a 1D SDS-PAGE gel), trypsin digested and desalted (using 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 microgram of each sample (three replicates for each cell type) was loaded on a C18 reversed-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 (Milli-Q)) 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 an 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 by the rejection of unassigned and +1 charge states; and minimum signal threshold, 500.

2.5.1 | Peptide and Protein Identification

Raw data were searched with the SEQUEST algorithm in Proteome Discoverer version 1.3 (Thermo, Germany), as described previously (Ghatak et al. 2021). The raw data were also searched against the IWGSC (International Wheat Genome Consortium Sequences) database, which contains 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 included 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. The identified proteins were normalised using the normalised spectral abundance factor (NSAF) strategy (Paoletti et al. 2006). The statistical significance of the proteins in various comparisons was checked using Student's *t*-test.

2.5.2 | Metabolomics to Quantify Defence Hormones Using the LC–MS Approach

Defence phytohormones were quantified following the methods of Vadassery et al. (2012), with minor modifications. Precisely, 20 mg of lyophilised leaf samples were extracted using 1 mL methanol containing 40 ng mL^{-1} D6-jasmonic acid, 40 ng mL^{-1} D4-salicylic acid, 40 ng mL^{-1} D6-abscisic acid and 8 ng mL^{-1} jasmonic acid-[13C6] isoleucine conjugate as internal standards. The homogenised sample underwent 30 min of shaking and centrifugation at 14 000 rpm for 20 min at 4°C. After the supernatant was collected, the pellet was re-extracted with 500 μL of methanol by centrifugation. The supernatant was combined with the previous extract. All procedures were conducted at 4°C. The combined extracts were evaporated in a speed-vac at 25°C and redissolved in 500 μL of methanol. Analysis was carried out on an Exion LC (Sciex) UPLC system using a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent) coupled with a triple quadrupole-trap MS/MS system (Sciex 6500+) in negative ionisation mode. The elution profile was as follows: 0–0.5 min, 5% B; 0.5–9.5 min, 5%–42% B; 9.5–9.51 min, 42%–100% B; 9.51–12 min, 100% B; and 12.1–15 min, 5% B. Phytohormones were quantified relative to the signal of their corresponding internal standard concentrations using scheduled multiple reaction monitoring (MRM) with a detection window of 60 s, following the method of Vadassery et al. (2012).

2.5.3 | Metabolic Profiling and Quantification of Flavonoids by LC–MS

The flavonoids were extracted with 80% methanol overnight at room temperature under agitation. The resulting extracts were subjected to centrifugation, and the supernatants were transferred to a fresh reaction tube and then vacuum-dried at 65°C using a SpeedVac. Finally, the dried extracts were resuspended in 80% methanol. The analysis of target flavonoids in the plants was performed as described by Naik et al. (2022). Methanolic extracts were used for the quantification of individual flavonoids.

LC–MS analysis of the samples was carried out in a UPLC (Exion LC, Sciex) coupled to a triple quadrupole MS/MS system (QTRAP6500+; ABSciex) using electrospray ionisation. For positive ionisation, the voltage was set at 5500 V. The values of gas 1 and gas 2 (70 psi), curtain gas (40 psi), collision-assisted dissociation (medium) and temperature of the source (650°C) were used. A mass spectrometer was used in multiple reaction monitoring (MRM) mode for qualitative and quantitative analysis using analytical standards of flavonols (Merck, USA). Analyst software (version 1.5.2) was used for identification and quantitative analysis.

2.5.4 | Metabolic Profiling and Quantification of Untargeted Metabolites Using GC–MS

The method was developed following Schauer et al. (2005) with minor modifications. A 20–25 mg lyophilised plant sample was extracted with 480 μL of pure methanol, and 20 μL of 0.2 mg mL^{-1} ribitol (adonitol) was added as an internal standard. The mixture was vigorously shaken for 2 min and then heated at 70°C for 15 min. Thereafter, an equal volume of water was added, and the mixture was vigorously shaken, followed by the addition of 250 μL of chloroform and thorough mixing. This mixture was centrifuged at 2200× g for 10 min at room temperature (~25°C). The upper aqueous phase was removed and dried in a speed vacuum rotator at 35°C. The dried fraction was then redissolved in 40 μL of 20 mg mL^{-1} methoxamine hydrochloride in pyridine and incubated for 90 min at 37°C. After that, 60 μL of MSTFA (N-methyl-N-[trimethylsilyl] trifluoroacetamide) was added, and the mixture was incubated for 30 min at 37°C. After this derivatisation process, the sample was transferred to a GC–MS vial containing an insert. The injection volume was set at 0.2 μL, and the injection mode was set at split mode with a split ratio of 5. Gas chromatography–mass spectrometry (GC–MS) analysis for untargeted analysis consisted of a Shimadzu gas chromatograph (GC-2010 plus) coupled with a mass spectrometer (TQ 8050) and an autosampler (AOC-20s)-auto injector (AOC-20i). Analysis was conducted using an SH-Rxi-5Sil MS capillary column (30 m × 0.25 μm, 0.25 mm) (Restek Corporation, USA) and helium with a flow rate of 1 mL min^{-1} as the carrier gas. The method consisted of 80°C isothermal heating for 2 min, followed by a ramp rate of 5°C min^{-1} to 250°C, a hold of 2 min, a final ramp of 10°C min^{-1} and a hold time of 24 min. The total run time for GC–MS was 67 min with a solvent delay of 4.5 min. Chromatogram integration and mass spectra analysis were performed using GC–MS solution version 4.45 SP 1, and NIST14s and the WILEY8 spectral library were used for derivatised metabolite identification. Metabolites with significant differences in content were determined according to the following criteria: variable importance in the projection (VIP) value ≥ 1 and fold-change ≥ 1.

3 | Results

3.1 | Lipidomics in Response to Cold Stress

Lipidomic analysis of SKAU_52 and SKAU_4301 using GC–MS identified a total of 90 lipids, categorised into phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylglycerols (PGs), lysophosphatidylcholines (LPCs), phosphatidylinositols (PIs), digalactosyldiacylglycerols (DGDGs), monogalactosyldiacylglycerols (MGDGs), sulfoquinovosyldiacylglycerols (SQDGs) and lysophosphatidylglycerol (LPG) (Figure S1a). Comparative analysis of SKAU_52 (control) and SKAU_4301 (control) revealed that several lipids showed up and down regulation. For instance PE (35:4) showed several fold change down regulation while as PC (32:1) showed up-regulation (Figure 2a). While comparing the same two genotypes under various condition, we also noticed substantial changes in different lipids. For instance, comparative analysis between SKAU_52 (stressed) and SKAU_52 (control) revealed differential expression of lipids. The levels of saturated lipids, including LPC(16:0), PC(34:0), LPG(16:0), LPC(15:0) and PE(34:0) decreased significantly in response to cold stress, and a similar reduction was observed in unsaturated lipids like PC(35:3) and PC(34:1). Conversely, several unsaturated lipids were upregulated, with fold change values ranging from approximately 1.68–3.00 (Figure 2b). The comparative analysis of SKAU_4301 (stressed) and SKAU_4301 (control) conditions revealed differential accumulation of various lipids. The saturated lipid PC (32:0) exhibited downregulation, while PC (36:0) displayed slight upregulation. Among the unsaturated lipids, several showed diverse fold changes, indicating varied accumulation levels under stress conditions, with LPC (15:0) and LPC (16:0) displaying notable upregulation (Figure 2c). Similarly, comparative analysis between SKAU_52 (stressed) and SKAU_4301 (stressed) identified 33 differentially accumulated lipids. The levels of the saturated lipids PC (32:0) and LPC (17:0) and several unsaturated lipids decreased, while the levels of polyunsaturated lipids, including DGDG (36:3), PC (36:6), MGDG (36:6), PE (38:4), PC (38:6) and others, increased, with fold changes ranging from approximately 1.014–5.419 (Figure 2d).

3.2 | Transcriptome Analysis

3.2.1 | 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, a cold susceptible genotype 'SKAU_4301 (control)' and a cold tolerant genotype 'SKAU_52 (control)' displayed 17 598 958 and 16 224 928 reads, respectively, both within a sequence length range of 20–150 bp 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, respectively, 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%.

The analysis identified 7302 differentially expressed genes (DEGs). 3916 DEGs were identified in SKAU_4301 under cold stress, where 859 genes showed upregulation and 3057 genes showed downregulation. On the other hand, SKAU_52 exhibited 3386 DEGs under cold stress, with 2652 upregulated and 734 downregulated genes. A comparison between SKAU_ 4301 and SKAU_ 52 demonstrated a higher number of DEGs in SKAU_4301. Notably, cold tolerant SKAU_52 exhibited more upregulated genes under cold stress than the cold-susceptible SKAU_4301. Among these DEGs, 846 were common between both genotypes, and 2540 genes in SKAU_52 and 3070 genes in SKAU_4301 showed differential expression uniquely in either of the genotypes. Furthermore, inherent differences were observed between the two genotypes under control conditions,

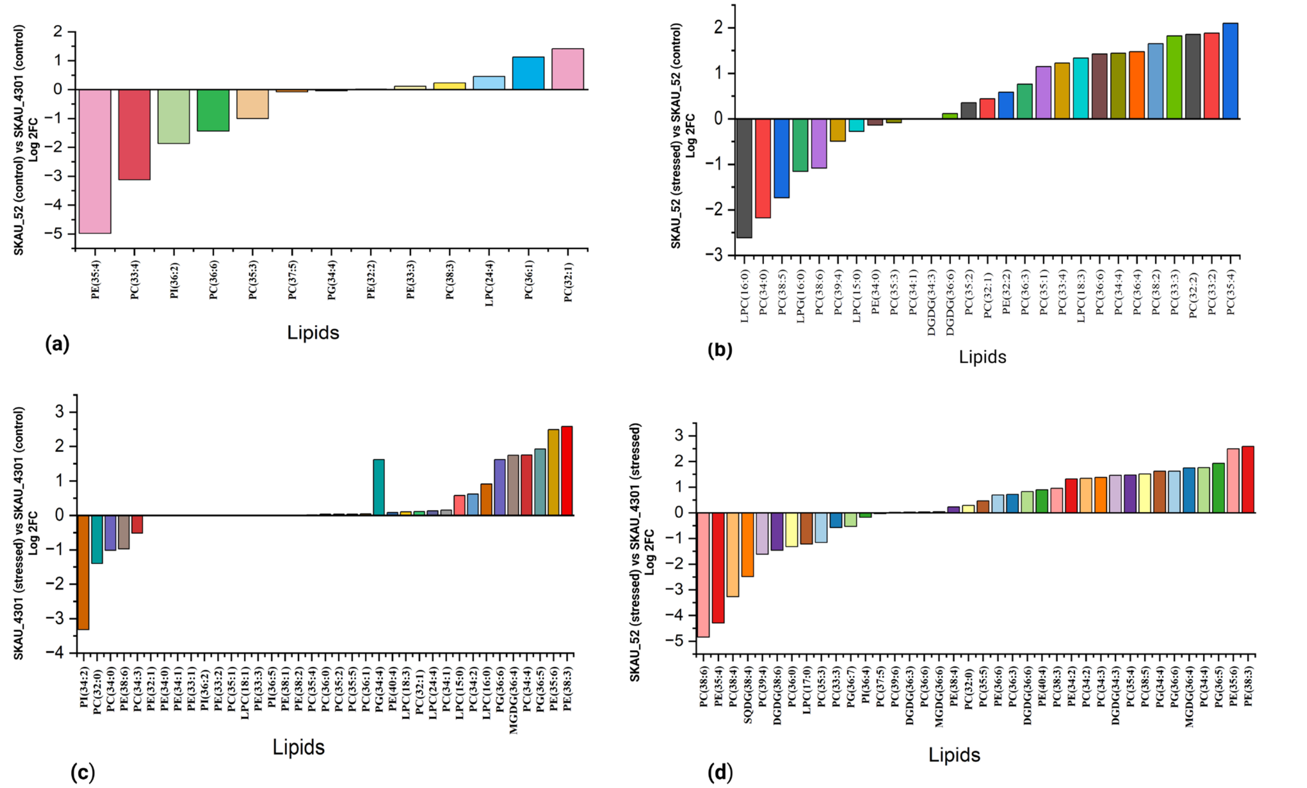

FIGURE 2 | Log2-fold changes (Log 2FC) in significant lipids between two genotypes SKAU_52 and SKAU_4301 used during the present study. The figure shows changes in lipids between: (a) SKAU_52 (control) vs SKAU_4301 (control), (b) SKAU_52 (stressed) vs SKAU_52 (control), (c) SKAU_4301 (stressed) vs SKAU_4301 (control) and (d) SKAU_52 (stressed) vs SKAU_4301 (stressed).

with 3938 common DEGs, including 314 upregulated DEGs and 3623 downregulated DEGs in SKAU_52. The top 30 DEGs between SKAU_4301(control) vs SKAU_52 (control), SKAU_52 (control) vs SKAU_52 (stresses), SKAU_4301(stressed) vs SKAU_52 (stressed) and SKAU_4301(control) vs SKAU_4301(stressed) are presented in Figure 3a–d.

3.2.2 | Transcription Factors

In our analysis of the cold-tolerant genotype SKAU_52 (stressed) vs SKAU_52 (control), we identified a total of 1655 differentially expressed transcription factors (TFs) under cold stress conditions. The NAC family TFs were the most prevalent, with 158 members. Other prominent TF families included ERF (141), bHLH (140), C_2H_2 (106) and MYB (88), while families such as WRKY, HSF and bZIP also played important roles in the genotype-specific response to cold stress (Table S2). In contrast, SKAU_4301 (stressed) vs SKAU_4301 (control) exhibited 1870 differentially expressed TFs, with a higher prevalence of bHLH (208), ERF (187) and NAC family TFs (173) (Table S2). Additionally, the C_2H_2, MYB-related, WRKY and B3 family TFs contributed significantly to the transcriptional response to cold stress. The common DEGs between the two genotypes highlighted the central roles of the ERF and NAC family TFs, along with the C_2H_2, bHLH and MYB family TFs (Table S3). Similarly, differentially expressing TFs were identified between SKAU_52 (control) vs SKAU_4301 (control) (Table S4) and SKAU_52 (stressed) vs SKAU_4301 (stressed) (Table S5).

3.2.3 | Gene Annotation

Gene Ontology analysis revealed distinctive molecular responses of SKAU_52 and SKAU_4301 under cold stress compared to respective controls kept at 25°C. Under control conditions, both genotypes shared common features, with membrane-related functions dominating the cellular component category. However, under cold stress, SKAU_52 exhibited significant upregulation of genes associated with protein-containing complexes, cell parts and membranes, indicating structural adaptations. The molecular function categories in SKAU_52 included enhanced enzymatic and binding responses, along with upregulated antioxidant and transcription regulator activities. Conversely, SKAU_4301 displayed distinct changes under cold stress, featuring alterations in cellular structure and intensified enzymatic and binding responses (Figure S2).

3.2.4 | KEGG Analysis

KEGG pathway analysis also revealed distinctive molecular responses to cold stress in SKAU_52 and SKAU_4301 compared to their respective controls at 25°C (Tables S6 and S7). In SKAU_52 (stressed) vs SKAU_52 (control), there was 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, such as 'phenylpropanoid biosynthesis' (28 genes) and 'MAPK signalling pathway – plant' (26 genes), exhibited

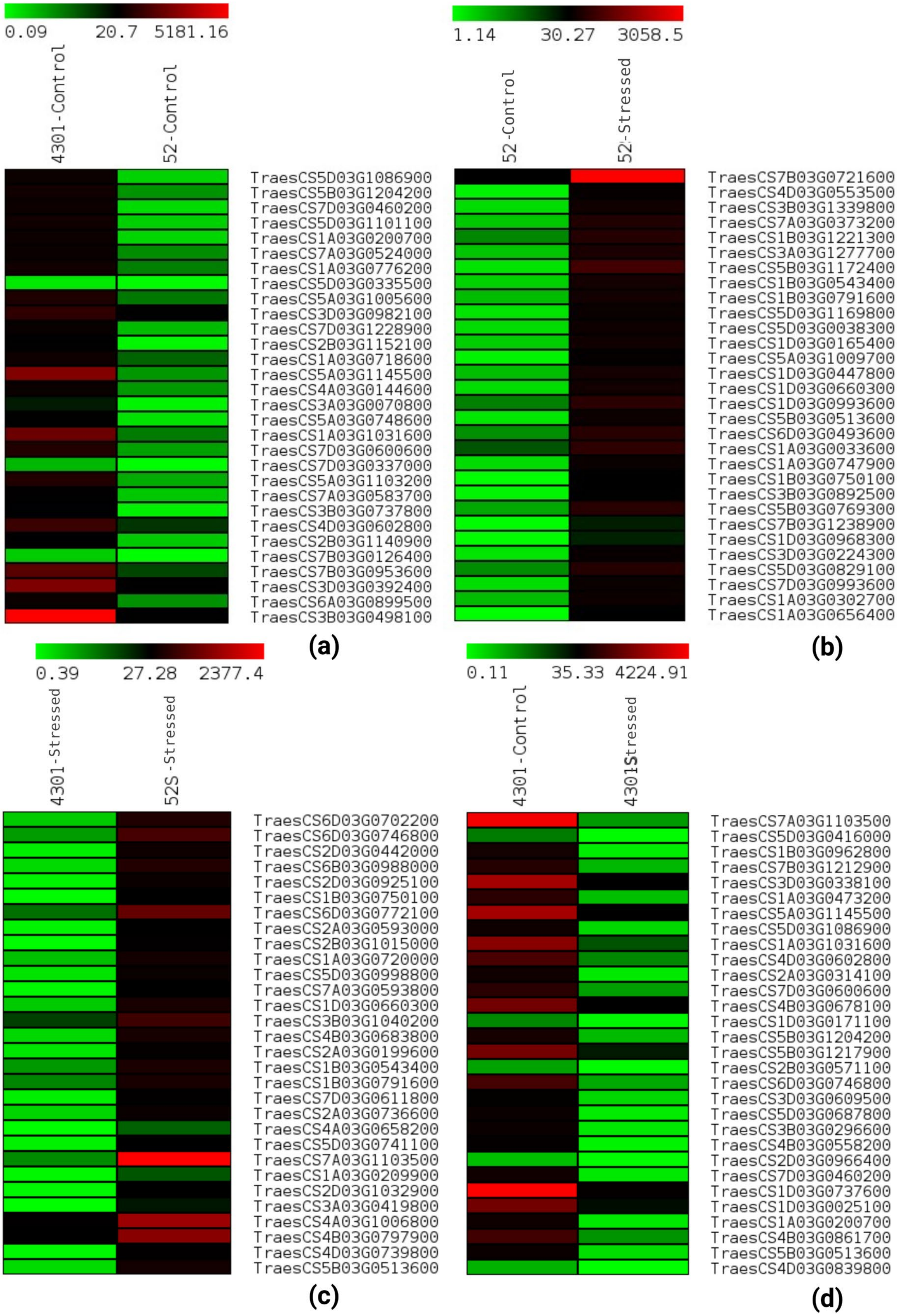

FIGURE 3 | Heatmaps depicting the expression profiles of the top 30 DEGs identified between the cold susceptible genotype 'SKAU_4301' and cold tolerant genotype 'SKAU_52'. The figure shows heat maps of top 30 DEGs between: (a) SKAU_4301(control) vs SKAU_52 (control), (b) SKAU_52 (control) vs SKAU_52 (stressed), (c) SKAU_4301(stressed) vs SKAU_52 (stressed) and (d) SKAU_4301(control) vs SKAU_4301(stressed). The colour gradient represents the level of gene expression, with red indicating upregulation and green indicating downregulation.

significant alterations. On the other hand, the downregulated pathways included 'photosynthesis – antenna proteins' (14 genes), 'necroptosis' (20 genes) and 'RNA polymerase' (3 genes), indicating potential adjustments in cellular processes and interactions with pathogens (Table S8).

In contrast, SKAU_4301 (stressed) vs SKAU_4301 (control) showed distinct responses. Under cold stress, the upregulated pathways included '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). Moreover, downregulated pathways with broad suppression included 'metabolic pathways' (154 genes) and 'biosynthesis of secondary metabolites' (85 genes). Other affected pathways included 'protein processing in endoplasmic reticulum' (62 genes) and 'phenylpropanoid biosynthesis' (32 genes), indicating potential disruptions in cellular stress responses and secondary metabolite production (Table S9).

3.2.5 | Differentially Expressed Genes Under Cold Stress

Comparative analysis of the gene expression patterns from SKAU_52 under cold stress relative to the corresponding control led to the identification of key cold-responsive genes contributing to the adaptive strategies of this genotype. Among the notable findings, *TraesCS1D03G0703900* (*WCOR18*), *TraesCS2A03G1018600* (*WCOR15-2A*), *TraesCS2B03G1138100* (*WCOR15-2B*), *TraesCS3D03G0862400* (*DHN33*), *TraesCS5A03G1181100* (*WCOR615*), *TraesCS1D01G369500* (*COR3D*), *TraesCS5B03G0791900* (*CBF3D*) and *TraesCS5B03G1052800* (*RAB15*) showed significant infinite fold changes, underscoring their significance as contributions to the cold acclimation and dehydrin-mediated protective mechanisms of SKAU_52 (Table S10).

In contrast, our examination of the cold-susceptible wheat genotype SKAU_4301 revealed distinct expression patterns under cold stress compared to the control (25°C). Among the noteworthy findings, *TraesCS2D03G0363200* (TRITD_2Av1G053010) exhibited a 2.83-fold upregulation, suggesting its involvement in the genotype's response to cold stress. Notably, *TraesCS3D03G0862400* (DHN33) and *TraesCS5A03G1006400* (RAB15) were significantly downregulated. 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. Additionally, *TraesCS6A03G0900100* (cold shock protein CS66) and *TraesCS6B03G0794800* (*WCOR*410c) also showed notable downregulations (−4.50-fold and −3.34-fold, respectively) (Table S11).

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 (Table S12). Notably, *TraesCS2A03G0346000* (TRITD_2Av1G053010) displayed a −2.47-fold downregulation in SKAU_52, suggesting its role in the reduced susceptibility/enhanced tolerance of this genotype to cold stress. Additionally, *TraesCS2B03G0465100* (TRITD_2Bv1G064680) exhibited a substantial decrease of 4.21-fold. The dehydrin-encoding gene *TraesCS3D03G0862400* (*DHN33*) showed infinite upregulation in SKAU_52, emphasising its critical role in cold tolerance. Additionally, *TraesCS5A03G1181100* (WCOR615), TraesCS5A03G1181200 (Wrab17.1) and *TraesCS6A03G0900100* (cold shock protein CS66) were also upregulated 5.28-fold, 6.72-fold and 5.48-fold, respectively, in SKAU_52. In addition, *TraesCS6A03G0901500* (wzy2) showed infinite upregulation in SKAU_52. The expression of *TraesCS7B03G0953600* (cold acclimation-induced protein 2–1) increased 5.12-fold in SKAU_52, suggesting its active participation in cold acclimation processes. Similarly, *TraesCS7B03G1305200* (*WCOR*726) and *TraesCS5A03G0758800* (*CBFIIIc*) exhibited infinite upregulation, emphasising their role in the response of the genotype to cold stress.

3.2.6 | Validation of Genes Associated with Cold Tolerance Using qRT-PCR

To validate the transcriptomic data, we selected five DEGs: WCOR615, WCOR726, WCOR18, RAB17 and LOC109758099 (an uncharacterised protein). Among the five DEGs, four DEGs (WCOR615, WCOR726, RAB17 and LOC109758099) showed the highest fold changes in the comparison between SKAU_52 (stressed) and SKAU_4301 (stressed), while WCOR18 showed the highest fold change in the comparison between SKAU_52 (stressed) vs SKAU_52 (control). Overall, all genes displayed variable expression patterns between the two genotypes, with most exhibiting substantially higher upregulation in SKAU_52 (Table S12).

Consistent with the RNA-seq results, both genotypes showed a decrease in WCOR615 expression under cold stress relative to the control, although the reduction was not statistically significant. In contrast, WCOR726 was significantly upregulated in both genotypes under cold stress i.e., 7.50-fold in the cold-tolerant SKAU_52 ($p < 0.01$) and 3.38-fold in the cold-susceptible SKAU_4301 ($p < 0.05$). The difference in expression between the two genotypes under stress was highly significant ($p < 0.01$). A similar trend was observed for WCOR18, with SKAU_52 exhibiting a highly significant 27.24-fold upregulation ($p < 0.01$) and SKAU_4301 showing a significant 12.16-fold increase ($p < 0.05$). The expression difference between genotypes under stress was also highly significant ($p < 0.01$). RAB17 expression increased in both genotypes (4.38-fold in SKAU_52 and 2.37-fold in SKAU_4301), but the upregulation was statistically significant only in SKAU_52 ($p < 0.01$). In contrast, the uncharacterised protein LOC109758099 was significantly upregulated in SKAU_4301 (7.6-fold, $p < 0.05$), while SKAU_52 showed a non-significant downregulation (0.65-fold). The difference in expression of this gene between the genotypes was also significant ($p < 0.05$) (Figure 4).

We also examined additional genes associated with cold acclimation and tolerance, including *TaCBF4B*, *TaCBF2D*, *TaICE2B*, *TaCOR5A*, *TaCOR3D*, *TaCOR3B* and *TaCBF11B* (Guo et al. 2019). These genes showed variation in expression between

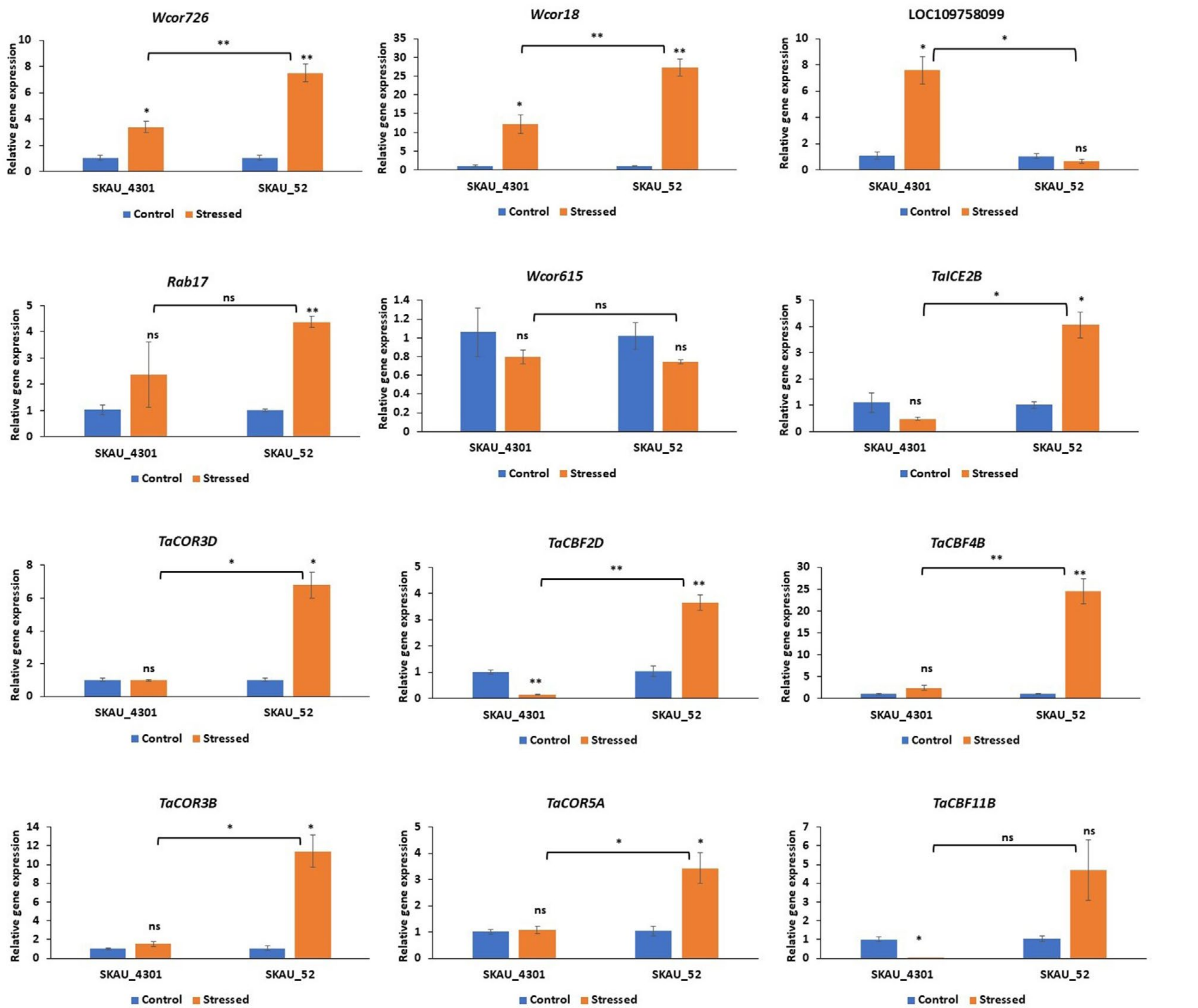

FIGURE 4 | Validation of highly upregulated cold-responsive transcripts using quantitative real-time polymerase chain reaction (qRT–PCR) in cold tolerant genotype 'SKAU_52 and cold susceptible genotype 'SKAU_4301'. Relative expression levels of *Wcor726, Wcor18, LOC109758099, Rab17, Wcor615, TaICE2B, TaCOR3D, TaCBF2D, TaCBF4B, TaCOR3B, TaCOR5A,* and *TaCBF11B* were analyzed in two contrasting wheat genotypes (SKAU_52 and SKAU_4301) under control and cold-stressed conditions. Bars represent mean ± standard error (SE) of biological replicates. Blue bars indicate control conditions, and orange bars indicate cold stress treatment. Asterisks denote statistically significant differences between treatments within each genotype ($p < 0.05$; $p < 0.01$), while "ns" indicates non-significant differences.

the two genotypes and were highly upregulated in SKAU_52 compared to SKAU_4301 under cold stress (Figure 4).

Under cold stress, *TaICE2B* expression increased significantly ($p < 0.05$) in SKAU_52, showing a 4.05-fold increase relative to its control. In contrast, SKAU_4301 displayed a non-significant decrease in *TaICE2B* expression (0.488-fold). The difference in the expression of this gene between the two genotypes was highly significant ($p < 0.05$) under stress. Similarly, *TaCBF4B* expression was strongly induced in SKAU_52, with a highly significant 24.5-fold upregulation ($p < 0.01$), whereas SKAU_4301 showed only a modest 2.5-fold increase. The difference in expression between the genotypes under cold stress was also highly significant ($p < 0.01$). The gene *TaCBF2D* followed a comparable pattern with significant upregulation in SKAU_52 (3.65-fold, $p < 0.01$), while SKAU_4301 showed a significant downregulation ($p < 0.01$) under stress in comparison to the control. The variation in expression between the two genotypes under cold conditions was likewise highly significant ($p < 0.01$).

For *TaCOR3D*, the cold-tolerant SKAU_52 exhibited a significant 6.80-fold increase in expression ($p < 0.05$), whereas SKAU_4301 showed almost no change (0.991-fold). This difference in expression between the two genotypes under cold stress was statistically significant ($p < 0.05$). SKAU_52 also demonstrated significant upregulation of *TaCOR3B* (11.405-fold) and *TaCOR5A* (3.426-fold), while SKAU_4301 exhibited only slight increases of 1.532-fold and 1.073-fold, respectively. The expression difference between genotypes for both genes under stress was also highly significant ($p < 0.05$). Finally, *TaCBF11B* expression increased markedly in SKAU_52 under cold stress (4.698-fold), whereas SKAU_4301 showed a significant reduction in its expression (0.001-fold) (Figure 4).

3.2.7 | Proteomics Analysis

Comparative proteome analysis of SKAU_52 and SKAU_4301 led to the identification of 1939 proteins. 1060 proteins were identified in SKAU_52 under the control conditions, while 1168 proteins were identified under cold stress. In contrast, 1073 proteins were identified under control conditions and 1154 proteins under cold stress in SKAU_4301.

An insightful aspect of the analysis was the identification of proteins unique to each treatment (Figure 5a,b,c). Nine proteins were uniquely identified in SKAU_52 (control). Whereas, 14 unique proteins were identified in SKAU_52 (stressed). Similarly, four proteins were exclusively identified in SKAU_4301 (control) and in SKAU_4301 (stressed) (Figure 5b). The identified proteins were distributed in 48 biological pathways (Figure 5c). These pathways encompass diverse cellular processes, redox reactions and polyamine metabolism in response to abiotic stress. Under stress, both genotypes exhibited enhanced amino acid synthesis and degradation, with SKAU_52 showing more pronounced changes. Carbohydrate metabolism, particularly the Calvin cycle, was downregulated under cold stress. 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 in the proteomes of two genotypes (Table S13). Upon exposure to cold stress, SKAU_52 demonstrated alterations in 326 proteins compared to its control counterpart, emphasising 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. Upregulation of key redox-regulating genes, such as thioredoxin, glutaredoxin, peroxidases and superoxide dismutase (21.1- to 21.6-fold change), was observed. A notable 11.6-fold

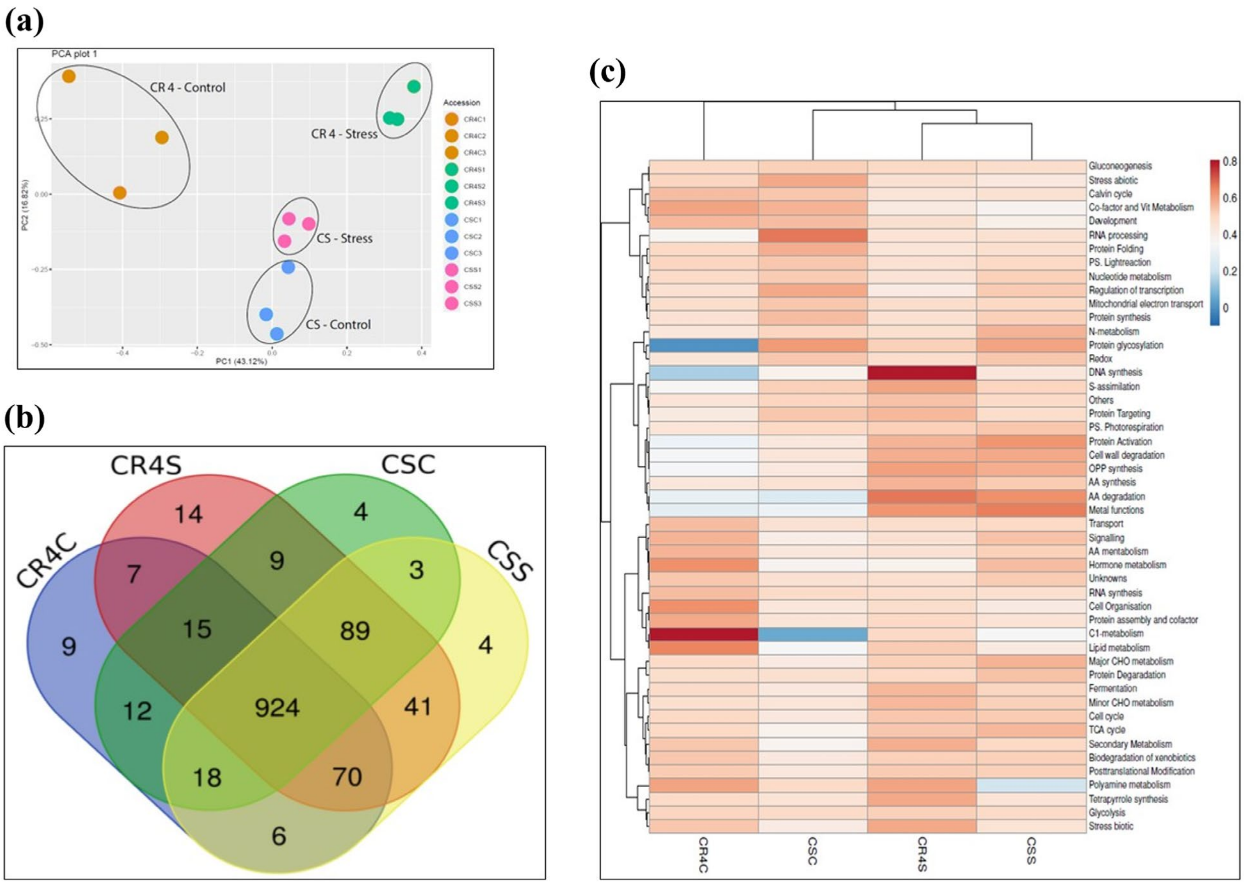

FIGURE 5 | Comparative proteomic profiling of cold-tolerant (SKAU_52) and cold-susceptible (SKAU_4301) wheat genotypes under control and cold stress conditions. (a) Principal component analysis (PCA) based on normalized protein abundance values showing clear separation of samples according to genotype and treatment. PC1 and PC2 represent the major sources of variance in the dataset. Biological replicates cluster together, indicating high reproducibility. (b) Venn diagram illustrating the distribution of identified proteins under control and cold stress conditions in both genotypes. Numbers indicate unique and shared protein sets among SKAU_52 control (CR4C), SKAU_52 stressed (CR4S), SKAU_4301 control (CSC), and SKAU_4301 stressed (CSS). (c) Hierarchical clustering heatmap showing functional categorization of differentially expressed proteins (DEPs) across genotypes and treatments. Functional groups were assigned based on biological processes. Color intensity represents relative enrichment or abundance levels (red indicates higher representation; blue indicates lower representation). Clustering was performed using hierarchical clustering based on similarity in expression patterns.

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 significantly increased in the cold-tolerant genotype following cold stress (Table S14).

In parallel, SKAU_4301 exhibited differential expression of 171 proteins under cold stress compared to the control. Most proteins involved in the gluconeogenesis/glyoxylate cycle and metal handling were increased. Among the signalling proteins related to cold tolerance, only a few, including RAB GTPase homologue A4D, exhibited significantly increased expression in the cold-susceptible genotype (Table S15).

Cross-genotype comparison under cold stress revealed that 211 proteins exhibited significant variation (Table S16). 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, signalling calcium protein such as 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 genotypes.

3.2.8 | Defence Hormone Analysis

Significant variations in defence hormone levels were detected among the cold-tolerant and cold-susceptible genotypes under the control (25°C) and cold stress (−5°C) conditions after 14 days of acclimation at 4°C (Figure S3a). Notably, the levels of salicylic acid (SA) and jasmonic acid (JA) differed among the genotypes under cold stress. SKAU_52 (stressed) vs SKAU_52 (control) exhibited higher SA (748.90 ng/g) and JA (355.11 ng/g) concentrations than SKAU_4301 (stressed) vs SKAU_4301 (control) (SA: 208.13 ng/g, JA: 207.97 ng/g). Abscisic acid (ABA) levels also differed, with SKAU_52 (stressed) vs SKAU_52 (control) (145.01 ng/g) surpassing SKAU_4301 (stressed) vs SKAU_4301 (control) (33.80 ng/g). Further analysis revealed higher concentrations of JA-isoleucine (JA-Ile) and cis-12-oxo-phytodienoic acid (cis-OPDA) in SKAU_52 (stressed) vs SKAU_52 (control) (JA-Ile: 330.59 ng/g, cis-OPDA: 96.13 ng/g) than in SKAU_4301 (stressed) vs SKAU_4301 (control) (JA-Ile: 115.27 ng/g, cis-OPDA: 29.90 ng/g). A comparative analysis of fold changes (FCs) between genotypes and treatments (Figure 6) highlighted significant differences. Fold changes (FC) difference in the levels of SA, JA, ABA, JA-Ile and cis-OPDA was 3.16, 2.10, 4.62, 2.99 and 4.45, respectively, for SKAU_52 (stressed) vs. SKAU_52 (control). Similar comparisons for SKAU_4301 (stressed) vs SKAU_4301 (control) indicated FCs of 1.37, 1.23, 1.52, 1.37 and 1.85. Comparing SKAU_52 (stressed) to SKAU_4301 (stressed) revealed FCs for SA, JA, ABA, JA-Ile and cis-OPDA as 3.59, 1.70, 4.49, 2.86 and 3.24 respectively, while SKAU_52 (control) and

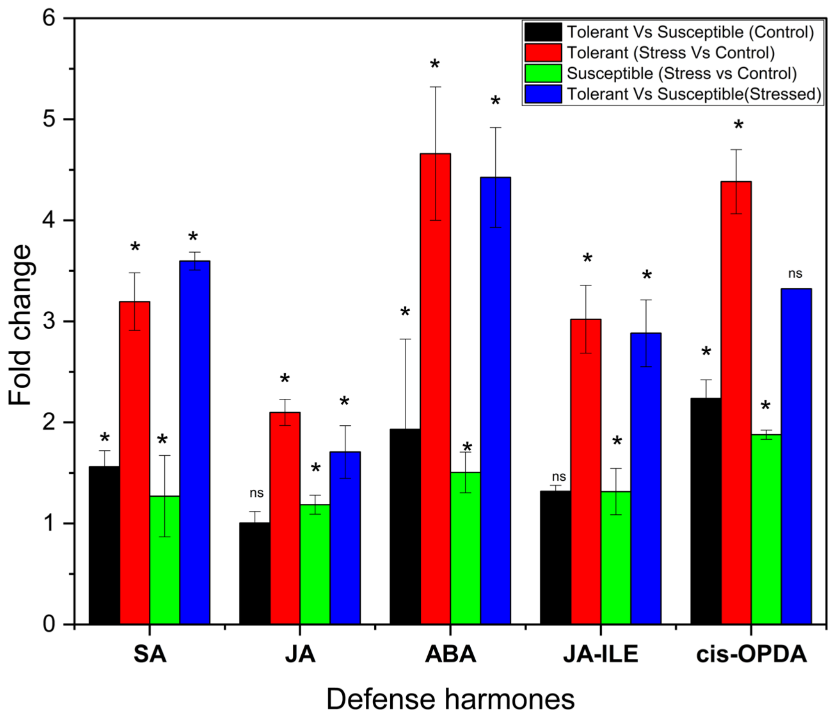

FIGURE 6 | Relative fold changes in defence hormone levels in cold-tolerant (SKAU_52) and cold-susceptible (SKAU_4301) wheat genotypes under cold stress (−5°C) and control conditions (25°C). Hormones analyzed include salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), jasmonoyl-isoleucine (JA-Ile), and cis-12-oxo-phytodienoic acid (cis-OPDA). Black bars represent tolerant vs. susceptible comparisons under control conditions; red bars represent stress vs. control comparisons within the tolerant genotype; green bars represent stress vs. control comparisons within the susceptible genotype; and blue bars represent tolerant vs. susceptible comparisons under cold stress conditions. Values are expressed as fold change relative to the respective control. Error bars indicate ± standard error of the mean. Asterisks denote statistically significant differences (*$p < 0.05$), and "ns" indicates non-significant differences.

SKAU_4301 (control) had FCs for SA, JA, ABA, JA-Ile and cis-OPDA as 1.56, 1.00, 1.83, 1.31 and 2.24, respectively (Figure 6).

3.2.9 | Flavonoid Analysis

Flavonoid analysis revealed differential expression in genotypes SKAU_52 and SKAU_4301 under control and cold stress conditions. SKAU_52 consistently displayed higher flavonoid levels than SKAU_4301, especially for naringenin, rutin, quercetin, petunidin, peonidin and malvidin under cold stress (Figure S3b). Comparative analysis using fold changes indicated significant upregulation or downregulation of all flavonoids (Figure 7).

Naringenin presented a significant fold change of 0.92 in SKAU_52 (stressed) vs. SKAU_52 (control) and 2.25 in SKAU_52 (stressed) vs. SKAU_4301 (stressed). Rutin exhibited a fold change of 1.58 in SKAU_52 (stressed) vs. SKAU_52 (control), 1.89 in SKAU_52 (stressed) vs. SKAU_4301 (stressed) and 0.8 in SKAU_4301 (stressed) vs. SKAU_4301 (control). Quercetin displayed a fold change of 4.15 in SKAU_52 (stressed) vs. SKAU_52 (control), 3.12 in SKAU_52 (stressed) vs. SKAU_4301 (stressed) and 1.2 in SKAU_4301 (stressed) vs. SKAU_4301 (control). Petunidin presented a moderate fold change of 2.6 in SKAU_52 (stressed) vs. SKAU_52 (control) and 2.4 in SKAU_52 (stressed) vs. SKAU_4301 (stressed). Peonidin showed a low fold change of 1.1 in SKAU_52 (stressed) vs. SKAU_52 (control) and 1.23 in SKAU_52 (stressed) vs. SKAU_4301 (stressed). Malvidin exhibited a fold change of 1.4 in SKAU_52 (stressed) vs. SKAU_52 (control) and 1.7 in SKAU_52 (stressed) vs. SKAU_4301 (stressed) (Figure 7).

3.2.10 | GC–MS Analysis of Untargeted Metabolites

Metabolomic analysis employing GC–MS was used to investigate alterations in the primary metabolome of SKAU_52 and SKAU_4301 under both control and cold stress conditions. Among 246 detected metabolites, carbohydrates (43.9%) and amino acids (7.3%) were predominant (Figure S1b). Comparative analyses revealed significant differences in metabolite expression profiles. For instance, in SKAU_52 (control) vs SKAU_52 (stressed), the abundance of 66 metabolites increased, including sucrose, glyceraldehyde, fructose, hydroxyproline and specific flavonoids, such as naringenin 7-O-glucoside and chrysoeriol (Figure S4 and Table S17). A comparison between SKAU_52 (stressed) vs SKAU_4301 (stressed) highlighted 43 upregulated metabolites, including sucrose, beta-D-allopyranose and monolaurin, along with the downregulation of metabolites such as talose, diosmetin and L-rhamnose (Table S18). Similarly, examination of SKAU_4301 (control) vs SKAU_4301 (stressed) revealed 12 upregulated metabolites, including D-fructose and vitamin B2 and 29 downregulated metabolites, including D-trehalose and chrysoeriol (Table S19). Finally, comparison of SKAU_52 (control) vs SKAU_4301 (control) revealed 60 differentially accumulated metabolites, showing upregulation of D-fructose and 5-methylcytosine and downregulation of metabolites such as chrysoeriol and beta-sitosterol (Table S20).

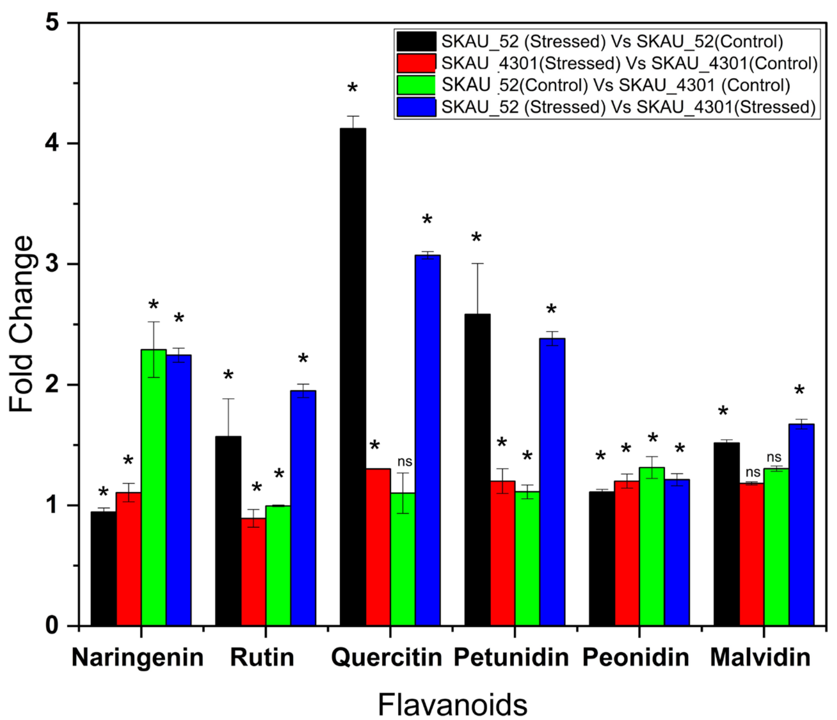

FIGURE 7 | Relative fold changes in flavonoid concentrations in cold-tolerant (SKAU_52) and cold-susceptible (SKAU_4301) wheat genotypes under cold stress (−5°C) and control conditions (25°C). Metabolites analyzed include naringenin, rutin, quercetin, petunidin, peonidin, and malvidin. Black bars represent stress vs. control comparisons within SKAU_52; red bars represent stress vs. control comparisons within SKAU_4301; green bars represent SKAU_52 vs. SKAU_4301 under control conditions; and blue bars represent SKAU_52 vs. SKAU_4301 under cold stress conditions. Values are expressed as fold change relative to the respective control. Error bars indicate ± standard error of the mean. Asterisks denote statistically significant differences (*$p < 0.05$), and "ns" indicates non-significant differences.

3.2.11 | Enrichment and Pathway Analysis

Metabolomic profiling of SKAU_52 (stressed) vs SKAU_52 (control) and SKAU_4310 (stressed) vs SKAU_4301 (control) revealed significant shifts across multiple metabolite classes, with several metabolites enriched in the cold tolerant genotype (SKAU_52). These included carbohydrates and carbohydrate conjugates, O-methylated flavonoids, pyrimidines and pyrimidine derivatives, alcohols and polyols, amines, indolyl carboxylic acids and derivatives, fatty acids and conjugates, pterins and derivatives, amino acids, peptides and analogues, benzenediols, purines and purine derivatives, hydroxycinnamic acids and derivatives and linoleic acids and derivatives.

Compared to those of the control, the pathway analysis of the top metabolites exhibiting high fold changes in the cold-tolerant genotype (SKAU_52) revealed a significant metabolic shift. Several metabolites demonstrated noteworthy fold changes, influencing diverse pathways (Table 1). N-Feruloylputrescine exhibited a 5.89-fold change in the arginine and proline metabolism pathway. There was a substantial 6.08-fold change in the level of 5-oxoproline, which is associated with glutathione metabolism. 1-Methyladenine showed a 2.63-fold change, while naringenin 7-O-glucoside and chrysoeriol exhibited 3.23-fold and 3.15-fold changes, respectively, impacting flavone and flavanol biosynthesis. 2,3-Dihydroxy-2-methylpropanoic acid exhibited a 2.75-fold change and 2,5-furandione demonstrated a significant

TABLE 1 | Metabolites significantly upregulated in the cold-tolerant wheat genotype SKAU_52 under cold stress compared to control, with the corresponding database identifiers, associated metabolic pathways and fold change values.

Metabolite	HMDB	PubChem	KEGG	SKAU_52 (stressed) vs SKAU_52 (control)	Fold change
N-Feruloylputrescine	HMDB0033463	92339985	C10497	Arginine and proline metabolism	5.894906
5-oxoproline	HMDB0000267	7405	C01879	Glutathione metabolism	6.083667
1-Methyladenine	HMDB0011599	78821	C02216	NA	2.625439
Naringenin 7-O-glucoside	HMDB0041761	5113660	NA	NA	3.234695
Chrysoeriol	HMDB0030667	5280666	C04293	Flavone and flavanol biosynthesis	3.153314
2,3-Dihydroxy-2-methylpropanoic acid	HMDB0002601	560781	NA	NA	2.753514
2,5-Furandione	HMDB0032523	7922	C19524	NA	3.603424
7-Methylxanthine	HMDB0001991	68374	C16353	Caffeine metabolism, biosynthesis of secondary metabolites, metabolic pathways	2.10834
Sucrose	HMDB0000258	5988	C00089	Starch and sucrose metabolism, galactose metabolism	4.476583
DL-Glyceraldehyde	HMDB0001051	751	C02154	Pentose phosphate pathway	1.832809
D-Fructose	HMDB0000660	439709	C02336	Starch and sucrose metabolism, galactose metabolism	1.795027
N-p-Coumaroylagmatine	HMDB0033460	528091	C04498	Biosynthesis of various plant secondary metabolites	1.772136
Arabinofuranose	HMDB0012325	440921	C06115	Pentose and glucuronate interconversions, ascorbate and aldarate metabolism, amino sugar and nucleotide sugar metabolism, metabolic pathways, biosynthesis of nucleotide sugars, ABC transporters	1.750714
L-Tryptophan	HMDB0000929	6305	C00078	Indole alkaloid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, tryptophan metabolism, glycine, serine and threonine metabolism, glucosinolate biosynthesis	1.686372
Galactitol	HMDB0000107	11850	C01697	Galactose metabolism, metabolic pathways, phosphotransferase system (PTS)	1.690694

3.60-fold change. 7-Methylxanthine displayed a 2.11-fold change, influencing pathways such as caffeine metabolism and the biosynthesis of secondary metabolites. The sucrose content exhibited a notable 4.48-fold change, impacting starch and sucrose metabolism and galactose metabolism (Table 1). Conversely, in the cold-susceptible genotype (SKAU_4301), pathway analysis of the top metabolites with high fold changes compared to those in the control (25°C) revealed substantial alterations in different metabolic pathways (Table 2).

4 | Discussion

4.1 | Differential Response of Membrane Lipids to Cold Stress

Lipids, which are essential components of cellular membranes, play a crucial role in maintaining membrane fluidity, integrity and functionality, especially under cold stress conditions (Golizadeh and Kumleh 2019). The degree of saturation of the fatty acid chains of lipids significantly influence their physical properties and, consequently, the response of plants to environmental stresses, including cold stress. Unsaturated lipids, characterised by double bonds in their fatty acid chains, are pivotal for cold tolerance (Golizadeh and Kumleh 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 synthesising unsaturated fatty acids led to improved membrane fluidity and, consequently, enhanced cold tolerance (Nejadsadeghi et al. 2019). The upregulation of unsaturated lipids in the cold-tolerant genotype, SKAU_52, reflects a strategic adjustment in its lipidomic composition, enhancing membrane flexibility and stability under cold stress conditions. This lipidomic signature contributes to the overall ability of plants to thrive in cold environments.

The observed downregulation of saturated lipids, such as LPC (16:0), PC (34:0), LPG (16:0), LPC (15:0) and PE (34:0), in SKAU_52 indicates a dynamic adjustment in the composition of these lipids in response to cold stress. This downregulation likely

TABLE 2 | Metabolites significantly upregulated in the cold-susceptible wheat genotype SKAU_4301 under cold stress compared to control, with corresponding database identifiers, associated metabolic pathways and fold change values.

Metabolite	HMDB	PubChem	KEGG	FC	Pathway SKAU_4301 (stressed) vs SKAU_4301 (control)
D-Fructose	HMDB0000660	439709	C02336	5.024975	Starch and sucrose metabolism
Vitamin B2	HMDB0000244	493570	C00255	4.902706	Riboflavin metabolism, metabolic pathways, Biosynthesis of cofactors, Vitamin digestion and absorption
Melibiose	HMDB0000048	6602503	C05402	3.687322	Galactose metabolism, metabolic pathways
N-Acetylglucosamine 1-phosphate	HMDB0001367	440272	C04256	3.553795	NA
5-Methylcytosine	HMDB0002894	65040	C02376	3.275377	Pyrimidine metabolism, metabolic pathways
L-Rhamnose	HMDB0000849	25310	C00507	3.227066	Fructose and mannose metabolism, Microbial metabolism in diverse environments
N-Acetylaspartate	HMDB0000812	65065	C01042	2.172916	Alanine, aspartate and glutamate metabolism
Coniferin	HMDB0013682	5280372	C00761	2.082436	Phenylpropanoid biosynthesis
L-Pipecolic acid	HMDB0000716	439227	C00408	1.998002	Lysine degradation, tropane, piperidine and pyridine alkaloid biosynthesis
Glucose	HMDB0000122	64689	C00221	1.668463	Glycolysis/Gluconeogenesis, Carbon metabolism
Diosmetin	HMDB0029676	5281612	C10038	1.314845	NA
1,2,3,4,5,6-Hexa-O-trimethelsilyl-myo-inositol	NA	NA	NA	1.072347	NA
D-(+)-Cellobiose	HMDB0000055	10712	C06422	1.027657	NA
di-C,C-hexosyl-methylluteolin	NA	NA	NA	1.027005	NA
D-Trehalose	HMDB0000975	7427	C01083	0.848802	Starch and sucrose metabolism

contributes to maintaining membrane fluidity, a critical aspect of cold adaptation. Findings from a study by Yu et al. (2014) on wheat highlighted that a reduction in saturated lipids under cold stress is positively correlated with increased membrane fluidity. The downregulation of saturated lipids is an adaptive strategy to prevent membrane rigidity and maintain optimal cellular function under cold conditions. The upregulation 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 the upregulation of unsaturated lipids, particularly those of phosphatidylglycerol (PG) species, is crucial for maintaining photosynthetic efficiency and overall cold tolerance in wheat crops. The increased abundance of unsaturated lipids supports membrane flexibility and resilience to cold-induced stress.

The detailed lipidomic analysis provides strong evidence that the upregulation of unsaturated lipids and the concurrent downregulation of saturated lipids represents key molecular mechanisms contributing to cold tolerance in the resistant genotype SKAU_52. These findings align with the established literature on lipid-mediated cold tolerance mechanisms, further validating the significance of lipidomic adaptations in crop plants exposed to cold stress.

4.2 | Transcriptomic Insights Into Cold Stress Response in Wheat

Transcriptome analysis revealed 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. Transcription factor (TFs) analysis revealed a complex regulatory landscape in which key families such as NAC, ERF, bHLH and C_2H_2 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. 2020). Notably, the NAC (NAM, ATAF1/2 and CUC2) family of TFs has emerged as a key regulator in both the cold-tolerant genotype SKAU_52 and the cold-susceptible genotype SKAU_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 higher abundance of ERF (ethylene response factor) family proteins, particularly in SKAU_52, underscores their significance in activating cold-induced signalling pathways. These TF families, along with others such as bHLH (basic helix–loop–helix), C_2H_2 (Cys2His2 zinc finger), 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. The stability of membranes and cellular structure of cold-tolerant plants often increase in response to low temperatures. The increased enzymatic and binding responses of proteins, along with upregulated activities of antioxidative enzymes such as CAT, SOD, APX and GPX, as well as transcriptional regulator activities, suggest a robust defence 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 is a proactive approach to modulate gene expression for stress adaptation. Conversely, the distinct changes in SKAU_4301 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 active metabolic reprogramming and a strategic preparation for potential pathogenic challenges associated with cold stress. The alterations in pathways such as '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 such as 'photosynthesis-antenna proteins,' 'necroptosis' and 'RNA polymerase' demonstrates a prioritisation of resources away from energy-intensive processes towards essential functions. In SKAU_4301, the upregulation of pathways suggested an effort to adapt to cold stress, although the broad suppression of essential pathways may indicate compromised metabolic activities and potential vulnerabilities to stress-induced disruptions.

Additionally, in-depth analysis of differentially expressed genes (DEGs) highlighted key players in the adaptive response of plants to cold stress. The infinite fold changes observed in *WCOR18*, *WCOR15-2B*, *WCOR615*, *DHN33*, *Cbf18* and many genes involved in lipid metabolism, the phenylpropanoid pathway and trehalose synthesis underscore their pivotal roles in the cold stress response of these wheat genotypes. These genes likely contribute to the activation of crucial pathways involved in cold acclimation and dehydrin-mediated protective mechanisms. The ICE-CBF-COR signalling cascade, a well characterised regulatory module conserved across cereals, plays a crucial role in cold tolerance in wheat. *ICE* genes are inducers of *CBF* expression, which in turn activates the transcription and expression of *COR* genes (Liu, Zhang, et al. 2022; Hwarari et al. 2022). 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. Recent functional genomics studies in wheat and barley revealed that the high expression of CBFs is strongly associated with chilling tolerance, delayed leaf senescence and improved winter survival, which further underscores the significance of this pathway (Zhang et al. 2025). Additionally, the *DHN* (dehydrin) and *RAB* (responsive to ABA) genes are also involved in cold tolerance in wheat. Dehydrins function as protective chaperones that help maintain protein and membrane stability during freezing-induced dehydration and their strong induction in tolerant genotypes is consistent with previous reports in cereals (Welin et al. 1994; Shakirova et al. 2009; Hao et al. 2022). RAB genes, many of which are regulated through ABA signalling, contribute to cold tolerance by supporting osmotic balance, limiting oxidative damage and sustaining cellular homeostasis under low temperatures (Shakirova et al. 2009). Recent transcriptomic and proteomic studies further highlight that strengthened ABA signalling enhances cold acclimation by promoting the accumulation of LEA proteins, including DHNs and RABs (Khan 2025). Overall, the collective upregulation of CBFs, CORs, dehydrins, ABA-responsive genes and pathways governing membrane stability and antioxidant metabolism provides a comprehensive molecular explanation for the cold tolerance observed in SKAU_52.

4.3 | Validation of Transcripts and Genes for Cold Tolerance Through qRT-PCR

A set of 12 transcripts/genes was validated for cold tolerance through qRT-PCR analysis. The analysis of results largely confirmed RNA-seq results. For instance, genes like WCOR726 and WCOR18 identified through RNAseq analysis showed strong concordance between RNA-seq and qRT-PCR validation data and therefore confirmed and reinforced their role in cold stress response in wheat. The analysis revealed that the cold tolerant genotype 'SKAU_52' exhibited significantly higher expression of key cold-responsive genes compared to the cold susceptible genotype 'SKAU_4301' suggesting a more robust activation of cold acclimation pathways. Further, the magnitude of upregulation in the cold tolerant genotype 'SKAU_52' for *TaCBF4B* (24.5-fold) and *WCOR18* (27.24-fold) highlights their potential as biomarkers for cold tolerance.

Further analysis highlighted the activation of ICE-CBR-COR pathway. For instance, strong induction of *TaICE2B* and *TaCBF* genes in SKAU_52 indicates efficient upstream signalling and transcriptional regulation under cold stress. This is also evident by the significant upregulation of downstream COR genes (*TaCOR3B*, *TaCOR3D*, *TaCOR5A*) in the cold tolerant genotype 'SKAU_52'. Further upregulation of 'RAB17', a well-known LEA protein gene in SKAU-52, suggests that RAB17 is involved in osmoprotection and membrane stabilisation during cold stress. One of the interesting genes 'LOC109758099' that displayed contrasting expression patterns between genotypes (upregulated in cold susceptible 'SKAU_4301' and downregulated in cold tolerant 'SKAU_52') indicate a possible genotype-specific role or stress signalling divergence. This gene could prove a novel candidate gene and warrants further functional characterisation in the near future.

In summary, our transcriptomics analysis provide a strong evidence that cold tolerance in wheat is governed by coordinated activation of the ICE-CBF-COR regulatory cascade, with the cold-tolerant genotype 'SKAU_52' showing stronger and more sustained induction across pathway tiers than the cold susceptible wheat genotype 'SKAU_4301'. Upstream signalling was evident from the significant upregulation of *TaICE2B* in SKAU_52, while SKAU_4301 showed a modest decline, consistent with models in which ICE transcription factors act as master regulators that trigger CBF transcription under low temperature stress (ICE → CBF → COR) (Guo et al. 2019). The identification of genes like *TaCBF4B*, *WCOR18* and *TaICE2B* in the present study can serve as molecular markers for cold tolerance in wheat breeding programs. The strong genotype specific expression differences provide a strong basis for molecular breeding programs aimed at enhancing cold tolerance through marker-assisted selection.

4.4 | Differential Proteome Response Under Cold Stress

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.

One of the 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 that under the 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 under control conditions. This disparity suggests greater adaptability and responsiveness of SKAU_52 under cold stress, indicating a cold-tolerant phenotype.

Pathway analysis revealed 48 functional 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, consistent 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 (Hildebrandt 2018).

Carbohydrate metabolism, especially the Calvin cycle, decreased under stress in both genotypes in this study, indicating a shift in energy utilisation. A similar response was observed in a study by Majumder and coworkers, 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 suggest 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. The dynamic response of SKAU_52 to cold stress was evident, with alterations in 326 proteins compared to those in its control counterpart. The 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 and Dietz 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 remodelling during cold stress in rice (Zhao et al. 2020), barley (Choi and Hwang 2015) and maize (Wei and Zhong 2014). Furthermore, the upregulation of peroxidases and calcium-related proteins in the cold-tolerant genotype underscores their roles in mitigating oxidative stress, a common consequence of cold exposure (Tang and Thompson 2020). The limited upregulation of signalling 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 existing knowledge on plant cold stress responses. Further studies can build upon these results to unravel the precise functions of the identified proteins and pathways, paving the way for targeted strategies to enhance cold tolerance in wheat.

4.5 | Differential Response of Targeted and Untargeted Metabolites to Cold Stress

By elucidating the metabolomic responses of the wheat genotypes SKAU_52 and SKAU_4301 to cold stress, our study revealed insights into the activation of defence mechanisms. Significant variations in defence hormones, including salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), jasmonic acid-isoleucine (JA-Ile) and cis-12-oxo-phytodienoic acid (cis-OPDA), were detected between SKAU_52 and SKAU_4301 under cold stress. This finding points to intricate signalling networks influencing plant stress responses, particularly in the context of cold stress (Zhao et al. 2019; Cheong et al. 2019). SA, which is traditionally associated with biotic stress, is present at relatively high levels in SKAU_52, suggesting its multifaceted role in enhancing cold tolerance by regulating key physiological and biochemical processes (Ignatenko et al. 2023). Similarly, the substantial increase in JA levels, particularly in SKAU_52 (stressed), implies the active involvement of JA in defence mechanisms contributing to cold tolerance. Conversely, lower JA levels in SKAU_4301 indicate potential differential regulation and a less effective defence response to cold stress. ABA, a central regulator of abiotic stress responses, was significantly increased in SKAU_52, contributing to its adaptive strategies under cold stress (Kovács et al. 2011), whereas its lower levels in SKAU_4301 suggest potential limitations in stress adaptation. The intricate relationship between JA and JA-isoleucine, along with the increase in cis-OPDA derivatives in SKAU_52, supports the involvement of the JA pathway in shaping cold stress responses, emphasising its intricate role in the cold tolerance of SKAU_52 (Karki et al. 2013; Pigolev et al. 2018).

Furthermore, the distinct accumulation patterns of selected flavonoids, namely, naringenin, rutin, quercetin, petunidin, peonidin and malvidin, in cold-tolerant and cold-susceptible genotypes illustrate the underlying mechanism of cold tolerance. Environmental stress can induce the biosynthesis of secondary metabolites, such as phenylpropanoids, flavonoids and flavone and flavonol (Masocha et al. 2020). These flavonoids are known for their antioxidant properties and contribute to the scavenging of reactive oxygen species generated under cold stress, thereby mitigating oxidative damage and playing crucial roles in enhancing cold tolerance (Schulz et al. 2016). Notably, SKAU_52 consistently exhibited higher levels of these flavonoids under cold stress than SKAU_4301, suggesting a potential association between increased flavonoid levels and enhanced cold tolerance in SKAU_52.

4.6 | Untargeted Metabolite Analysis

Untargeted GC–MS metabolomic analysis provided profound insights into the metabolic dynamics of the cold-tolerant genotype SKAU_52 under cold stress conditions. Carbohydrates, identified as the most abundant metabolites, serve as crucial energy sources and signalling molecules in cellular processes. In response to cold stress, SKAU_52 strategically upregulates sucrose, contributing to osmotic protection, maintaining energy balance and activating signalling pathways for enhanced cold tolerance, which is consistent with studies in *Arabidopsis* (Zheng et al. 2018). The observed increase in amino acids, including oxyproline, reflects a pivotal aspect of the plant's metabolic reprogramming under cold stress, supporting stress-resilient protein synthesis mechanisms (Stefl et al. 1978). While metabolic alterations can also occur in cold-susceptible genotypes, these changes are often less coordinated and fail to confer effective cold tolerance. In contrast, the distinctive metabolic signature of SKAU_52, with upregulated specific metabolites, highlights its enhanced stress-adaptive response and underscores the importance of these metabolites in contributing to the cold tolerance phenotype (Zhao et al. 2019). These findings support the concept that plants whose metabolite profiles are altered exhibit improved cold tolerance, highlighting the significance of the identified metabolites in contributing to the cold tolerance phenotype of SKAU_52.

4.7 | Integration of Multi-Omics with Genetic Background of Two Genotypes

The genetic architecture of the two contrasting wheat genotypes, SKAU_52 (cold-tolerant) and SKAU_4301 (cold-susceptible), was examined in the context of key marker–trait associations (MTAs)/genes/QTLs identified through genome-wide association study (GWAS) in an independent study (Mir 2025; under preparation). Integration of these genomic insights with multi-omics investigations (lipidomics, transcriptomics, proteomics and metabolomics) reveals a strong convergence, demonstrating that the two genotypes differ fundamentally at both genetic and molecular levels.

Specifically, we analysed allele states for 56 cold-tolerance MTAs in SKAU_52 and SKAU_4301. Among these, 31 SNPs exhibited identical alleles, while 18 SNPs showed compatible overlap due to IUPAC ambiguity. Importantly, seven SNPs (Affx-88654511 (1A), Affx-92190159 (7D), Affx-92426982 (1B), Affx-92674740 (1D), Affx-92862169 (7B), Affx-92943208 (4D) and Affx-92327484 (7B)) displayed completely divergent alleles, providing a concise set of genotype-discriminating markers (Figure S5). These seven MTAs represent robust candidates for marker-assisted selection (MAS) and breeding panel development to differentiate cold-tolerant from cold-susceptible backgrounds.

These seven MTAs have been identified using different GWAS models like BLINK and FarmCPU for both cold tolerance (year 2021–2022) and electrolyte leakage index (ELI%).

The concordance between allelic variation and multi-omics outputs suggests that these MTAs may be linked to genes regulating cold stress-responsive pathways, thereby influencing lipid remodelling, transcriptional reprogramming, protein accumulation and metabolite adjustments under stress. Collectively, these multilayered omics and genomics insights offer a coherent mechanistic explanation for the contrasting cold-tolerance phenotypes of SKAU_52 and SKAU_4301, reinforcing the value of integrative approaches for trait dissection and crop improvement.

5 | Conclusion

In conclusion, our integrative omics analysis elucidates the intricate molecular mechanisms underlying wheat response to cold stress, particularly in the contrasting genotypes SKAU_52 and SKAU_4301. The findings underscore the pivotal roles of lipids, transcripts, proteins, metabolites, flavonoids and defence hormones in orchestrating adaptive responses to cold stress. Sucrose, amino acids and specific defence hormones emerged as key contributors to cold tolerance, while alterations in lipid composition, differential expression of stress-responsive genes and protein accumulation highlight the complex nature of cold stress adaptation in wheat. Importantly, transcriptomic analysis coupled with qRT-PCR validation revealed strong activation of the ICE-CBF-COR pathway in SKAU_52, with significant upregulation of *TaICE2B*, *TaCBF4B*, *TaCBF2D*, *TaCBF11B* and downstream COR genes (*WCOR18*, *WCOR726*, *TaCOR3B*, *TaCOR3D* and *TaCOR5A*). These genes, along with RAB17, exhibited genotype-specific expression patterns, confirming their central role in cold acclimation. These insights provide valuable knowledge for developing resilient crop varieties capable of withstanding cold stress, ultimately contributing to sustainable agriculture in J&K region of India and other similar environments where extreme climatic conditions challenge wheat production.

Author Contributions

Sofora Jan (SJ) designed the experiment, conducted the experiment and wrote the manuscript. Farkhandah Jan (FJ) conducted the experiment and wrote the manuscript. Mukesh Rathore (MR), Yogita Singh (YS), and Prexha Kapoor (PK) data curation, analysis, review and editing of the manuscript. Palak Chaturvedi (PC), Arindam Ghatak (AG) and Palakurthi Ramesh (PR) data analysis, provided suggestions, review and editing of the manuscript. Upendra Kumar (UK), Manoj Prasad (MP), Wolfram Weckwerth (WW), Sundeep Kumar (SK), Sachin Rustgi (SR), Sanjay Kalia (SK) and Rajeev K. Varshney (RKV) provided genetic resources, funding, guidance, suggestions and helped in revising the manuscript. Reyazul Rouf Mir (RRM) conceptualised the experiment, provided supervision, guidance, suggestions and revised the manuscript. All authors contributed to manuscript revision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supporting material of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** pbi70594-sup-0001-DataS1.zip. **Figure S1:** Distribution of different classes of lipids (a) and metabolites (b) identified in cold-tolerant and cold-susceptible genotypes through GC–MS. **Figure S2:** Gene annotation of differentially expressed genes (DEGs) identified in various comparative analyses. Gene annotation provides insights into the biological functions, molecular functions and cellular functions associated with the DEGs. **Figure S3:** Heatmap representing defence hormone concentrations (a) and flavonoid concentrations (b) in cold-tolerant (SKAU_52) and cold-susceptible (SKAU_4301) wheat genotypes under cold stress (−5°C) and control conditions (25°C). The colour intensity reflects hormone levels, with warmer colours indicating higher concentrations. **Figure S4:** Log2 fold changes in untargeted metabolite profiles of SKAU_52 (control) vs SKAU_4301 (control) (a), SKAU_52 (stressed) vs SKAU_52 (control) (b), SKAU_52 (stressed) vs SKAU_4301 (stressed) (c) and SKAU_4301 (stressed) vs SKAU_4301 (control) (d) plants. **Figure S5:** Allelic state variation across cold-tolerance–associated MTAs in contrasting wheat genotypes. Heatmap showing allele states at 56 marker–trait associations (MTAs) for cold tolerance in the tolerant genotype SKAU-52 and the susceptible genotype SKAU-4301. Colours represent different allele states as indicated in the legend. Starred MTAs denote seven key loci exhibiting completely divergent alleles between the two genotypes, highlighting a concise set of highly informative genotype-discriminating markers associated with cold tolerance. **Table S1:** List of primers used for qPCR validation of transcripts/genes in the present study. **Table S2:** Distribution of Transcription Families in SKAU_52 (stressed) vs. SKAU_52 (control). **Table S3:** Distribution of Transcription Families in SKAU_4301 (stressed) vs. SKAU_4301(control). **Table S4:** Distribution of Transcription Families in SKAU_52 (control) VS SKAU_4301 (control). **Table S5:** Distribution of Transcription Families in SKAU_52 (stressed) vs. SKAU_4301 (stressed). **Table S6:** KEGG analysis of SKAU_52 (stressed) VS SKAU_4301(stressed). **Table S7:** KEGG analysis of SKAU_52 (control) VS SKAU_4301 (control). **Table S8:** KEGG analysis of SKAU 52 (stressed) vs. SKAU_52 (control). **Table S9:** KEGG analysis of SKAU_4301 (stressed) vs. SKAU_4301 (control). **Table S10:** Differentially expressed genes (DEGs) between SKAU 52 under stress and control conditions. **Table S11:** Differentially expressed genes (DEGs) between SKAU_4301 under stress and control conditions. **Table S12:** Differentially expressed genes under cold stress in SKAU_52 (stressed) vs. SKAU_4301(stressed). **Table S13:** List of differentially expressed proteins identified between SKAU_52 and SKAU_4301 under control conditions. **Table S14:** List of differentially expressed proteins identified in SKAU_52 under stress compared with control conditions. **Table S15:** List of differentially expressed proteins identified in SKAU_4301 under stress compared with control conditions. **Table S16:** List of differentially expressed proteins under cold stress in SKAU_ 52 (Stressed) VS 4301 (stressed). **Table S17:** List of differentially expressed metabolites identified in SKAU_52 under stress compared with control conditions. **Table S18:** List of differentially expressed metabolites identified in SKAU_52 (stressed) vs. SKAU_4301 (stressed). **Table S19:** List of differentially expressed metabolites identified in SKAU_4301 under stress compared with control conditions. **Table S20:** List of differentially expressed metabolites identified between SKAU_52 and SKAU_4301 under control conditions.