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Dynamic transcriptomic and metabolomic adaptation mechanisms of *Trifolium ambiguum* under different durations of cold stress

Kefan Cao[1,3], Sijing Wang[2], Qian Wu[3] & Fan Huang[1]✉

Cold stress is one of the major environmental challenges faced by plants, severely affecting their growth, development, and yield. With the intensification of global climate change, the impact of cold stress on plants is becoming increasingly significant. Plants respond to cold stress through complex metabolic regulatory networks, including the accumulation of protective metabolites, modulation of antioxidant defenses, and activation of secondary metabolic pathways. This study aims to explore the dynamic metabolic and transcriptomic responses of *T. ambiguum* under long-term cold stress, and to reveal its adaptive mechanisms. By integrating metabolomics and transcriptomics, this study provides an indepth analysis of gene expression and metabolic responses of *T. ambiguum* under different cold stress treatments (2 h, 6 h, 12 h), focusing on key metabolic pathways and signal transduction mechanisms involved in cold stress adaptation. To further investigate the relationship between genes and metabolites, weighted gene co-expression network analysis (WGCNA) was applied to construct the gene-metabolite coexpression network under cold stress. Several functional modules that play significant roles in cold response were identified. Notably, the pink module was found to be associated with lipid metabolism, sugar metabolism, and signal transduction pathways, while the black module was closely linked to plant hormone signaling and antioxidant responses. Additionally, KEGG enrichment analysis revealed that key pathways such as glycerophospholipid metabolism, proline metabolism, and plant hormone signal transduction work synergistically in cold stress adaptation, regulating cellular homeostasis and maintaining energy supply under prolonged cold stress. The results indicate that *T. ambiguum* enhances its cold tolerance through dynamic coordination between its transcriptomic and metabolic responses. This study provides new molecular biological evidence for the cold adaptation mechanisms of *T. ambiguum* and offers theoretical support for improving cold tolerance in related crops.

Trifolium ambiguum M.Bieb., a perennial leguminous forage plant native to the Caucasus region, is renowned for its exceptional cold tolerance and environmental adaptability[1,2]. Apart from its importance as a forage crop, *T. ambiguum* is valued for its high-protein leaves, which provide high-quality feed for livestock[3]. Additionally, it plays a vital role in ecological restoration and soil improvement, making it a crucial species for sustainable agriculture in cold regions[4].

Cold stress significantly impacts agricultural production worldwide, particularly in temperate and cold regions. Plants exposed to low temperatures undergo a series of physiological and molecular responses that allow them to adapt to harsh conditions. These responses include changes in gene expression, metabolic reprogramming, and the accumulation of compatible solutes, which collectively help mitigate cold-induced damage[5–7]. Understanding these mechanisms is essential for developing strategies to enhance crop resilience to abiotic stresses, thereby ensuring food security in the face of climate change.

[1]Institute of Grassland Research, Chinese Academy of Agricultural Science, 120 Wulanchabu East Street, Saihan District, Hohhot 010010, China. [2]Yinshanbeilu Grassland Eco-hydrology National Observation and Research Station, Institute of Water Resources and Hydropower Research, Beijing 100038, China. [3]College of Grassland Science/Key Laboratory of Grassland Resources of Ministry of Education, Inner Mongolia Agricultural University, Hohhot 010018, China. ✉email: huangfan@caas.cn

The production of specialized metabolites, such as phenolics, terpenoids, and flavonoids, is strongly induced by cold stress. These metabolites play a key role in protecting plants from oxidative damage and enhancing their tolerance to abiotic stresses[8,9]. Among these, the phenylpropanoid pathway stands out as a central metabolic route responsible for the biosynthesis of flavonoids, including anthocyanins and flavonols[10]. These metabolites not only contribute to cold stress adaptation but also play significant roles in modulating plant responses to both biotic and abiotic stresses[11–13].

Recent advancements in omics technologies have facilitated the comprehensive elucidation of flavonoid biosynthesis pathways in various plant species, including black poplar (*Populus trichocarpa*), sorghum (*Sorghum bicolor*), sweet chestnut (*Cyclocarya paliurus*), and apple (*Malus domestica*)[14–17]. These studies have demonstrated that flavonoids—such as flavones, isoflavones, anthocyanins, and flavonols—are integral to plant cold tolerance and stress responses. Furthermore, transcription factors, such as MYB proteins, have been identified as key regulators of flavonoid biosynthesis, significantly influencing plant adaptation to environmental stresses[18–20].

Despite extensive research on cold stress response mechanisms in plants, studies focusing on the molecular responses of *T. ambiguum* remain scarce. In particular, there is a lack of a systematic understanding of how specialized metabolites are regulated and how they contribute to cold tolerance. This study integrates metabolomics and transcriptomics analyses to investigate the cold stress response in *T. ambiguum*, aiming to uncover its molecular mechanisms of cold adaptation.

The specific objectives of this research are: (1) to identify key metabolic changes in *T. ambiguum* under cold stress; (2) to elucidate the regulatory mechanisms of flavonoid biosynthesis pathways and the roles of associated genes; and (3) to provide theoretical insights for improving cold tolerance in leguminous crops through molecular breeding.

By unraveling the molecular mechanisms underlying cold tolerance in *T. ambiguum*, this study not only contributes to our understanding of plant adaptation to abiotic stresses but also offers new perspectives for crop improvement and sustainable agriculture in cold regions.

Results

Quality assessment of RNA-seq data

To ensure the reliability of the transcriptomic data, the sequencing quality of 12 *T. ambiguum* samples under different cold stress durations was assessed. As summarized in Table 1, each sample yielded over 20 million clean reads after quality filtering, with the number of clean bases ranging from 6.05 to 7.11 Gb. The average GC content of all samples ranged from 41.25% to 41.75%, reflecting stable base composition across conditions. Importantly, all samples exhibited high sequencing accuracy, with Q30 scores exceeding 96.9%, indicating that more than 96.9% of bases had a Phred quality score greater than 30.

These results confirm the overall high quality of the RNA-seq data, providing a solid foundation for downstream analyses such as differential gene expression, pathway enrichment, and co-expression network construction. The consistency in read length and GC content across biological replicates further supports the robustness and reproducibility of the sequencing results.

Transcriptional response to cold stress at different time points in *T. ambiguum*

Heatmap analysis demonstrated the gene expression patterns under different cold stress durations (2, 6, and 12 h). In the E group (control), there were minimal differences in gene expression, and the clustering between biological replicates was tight, indicating that the transcriptome of the plant remained relatively stable under normal growth conditions. With the extension of cold stress, from the F group (2 h of cold stress) to the G group (6 h of cold stress), and then to the H group (12 h of cold stress), gene expression underwent gradual changes. Notably, as the cold stress duration increased, the differences between samples became more pronounced, particularly in the H group (12 h of cold stress), where the expression patterns became more diverse. This shift reflects the plant's gradual adaptation and response mechanisms to cold stress at different time points. Cluster analysis showed that the E group samples (E1, E2, E3) clustered tightly together, indicating high consistency in gene expression under normal conditions. In contrast, after cold stress treatment, especially at 12 h (H group),

Samples	Clean reads	Clean bases	GC Content	%≥Q30
E1	20,746,290	6,204,824,065	41.51%	97.58%
E2	20,231,514	6,052,561,578	41.59%	97.63%
E3	20,930,728	6,256,318,163	41.75%	97.67%
F1	20,771,380	6,215,570,622	41.42%	96.92%
F2	22,757,057	6,814,439,431	41.28%	97.06%
F3	23,764,240	7,112,116,922	41.28%	96.98%
G1	20,440,499	6,105,161,806	41.62%	97.91%
G2	21,391,950	6,401,109,360	41.36%	97.26%
G3	22,113,866	6,619,241,828	41.25%	97.31%
H1	20,654,180	6,177,963,064	41.26%	97.48%
H2	21,054,352	6,298,751,520	41.30%	97.63%
H3	20,512,721	6,137,774,317	41.39%	97.45%

Table 1. Summary of RNA-seq data quality for *T. ambiguum* samples under cold stress.

the clustering of samples became more dispersed, revealing the plant's gradual adaptation to cold stress. Samples from the F and G groups displayed intermediate expression patterns, reflecting the activation of more complex transcriptional responses as the cold stress duration increased. This clustering analysis suggests that cold stress not only gradually regulates plant gene expression over time, but also, with prolonged exposure, activates more stress resistance responses and mechanisms (Fig. 1a).

The Venn diagram illustrated the overlap and specificity of differentially expressed genes (DEGs) in *T. ambiguum* under cold stress at different durations (2, 6, and 12 h). In the E vs. F comparison (control vs. 2-hour cold stress), a total of 13,291 DEGs were unique to this comparison, among which 2,701 genes overlapped with F vs. G, and 3,169 genes overlapped with G vs. H. In the F vs. G comparison (2 vs. 6 h), 3,996 DEGs were specific, and 1,488 overlapped with G vs. H. The G vs. H comparison (6 vs. 12 h) had 4,879 unique DEGs. Additionally, 1,510 DEGs were shared among all three pairwise comparisons, indicating the presence of a core set of cold-responsive genes that persist throughout the cold stress treatment. These data demonstrate dynamic but partially overlapping gene expression responses across time points (Fig. 1b).

Differential expression gene (DEG) analysis revealed significant effects of cold stress at different time points on gene expression in *T. ambiguum*. In the E vs. F comparison (control vs. 2 h of cold stress), the number of DEGs was much higher compared to the other two comparisons, with a total of 18,271 DEGs identified, including 11,616 upregulated genes and 9,055 downregulated genes. This suggests that short-term cold stress (2 h) significantly activated the plant's gene response, with most genes showing upregulation. Upregulated genes are likely involved in early cold responses, such as antioxidant reactions, cell membrane stability regulation,

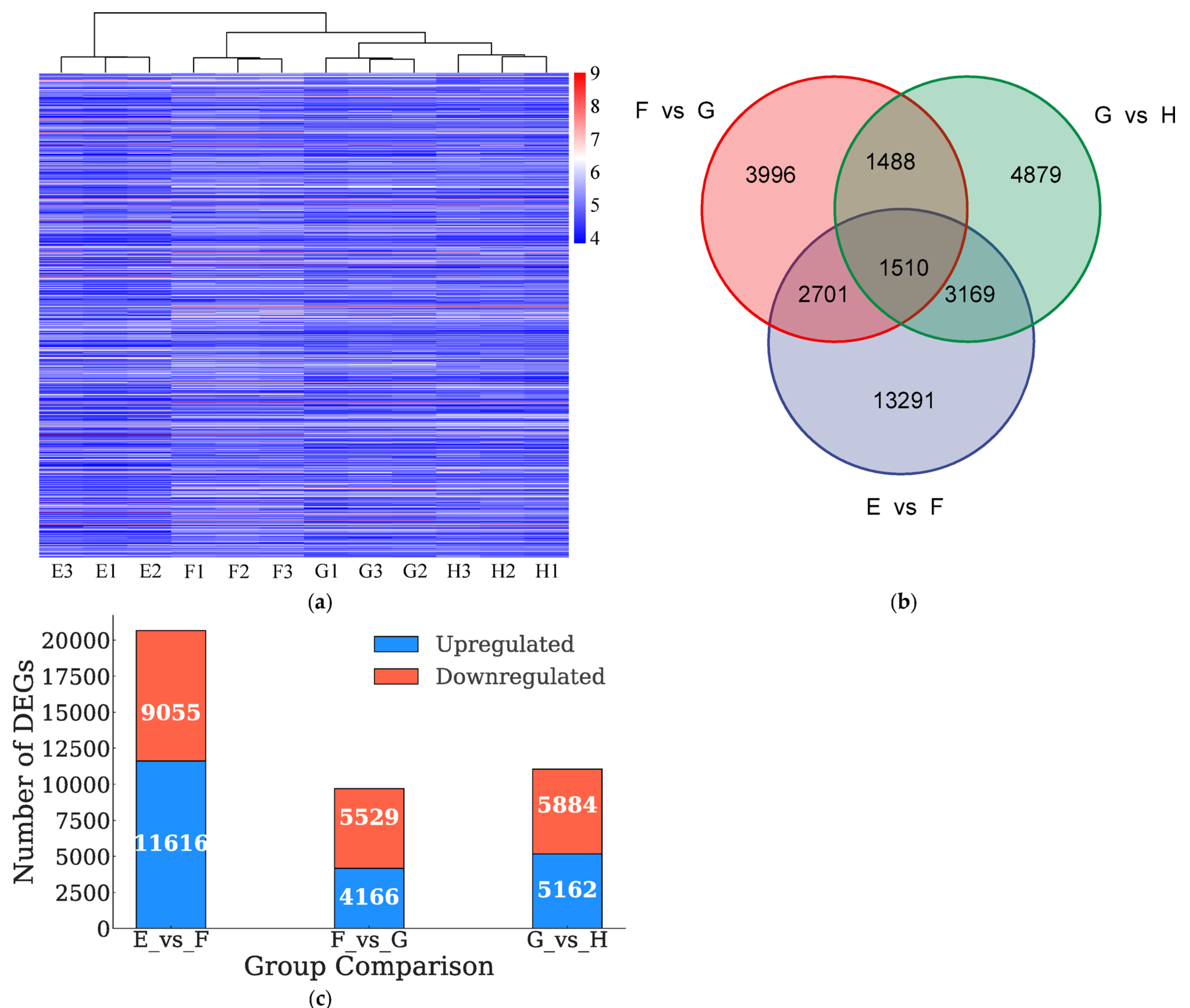

Fig. 1. Transcriptional response of *T. ambiguum* under cold stress at different time points: (**a**) Heatmap analysis of gene expression patterns across different groups (E vs. F, F vs. G, G vs. H) under cold stress; (**b**) Venn diagram showing the overlap of differentially expressed genes (DEGs) between different treatment groups; (**c**) Bar graph displaying the number of upregulated and downregulated DEGs in the comparisons of E vs. F, F vs. G, and G vs. H.

and heat shock protein synthesis, all of which help the plant maintain cellular function and metabolic stability under cold stress. In the F vs. G comparison (2 h vs. 6 h of cold stress), 9,695 DEGs were identified, with 5,529 upregulated genes and 4,166 downregulated genes. Compared to the 2-hour cold stress treatment, 6 h of cold stress led to further changes in gene expression, particularly in the upregulated genes. After 6 h of cold stress, more genes were upregulated, likely related to the activation of more complex regulatory mechanisms in the plant, such as secondary metabolite synthesis, lipid metabolism changes, and endogenous hormone regulation. These changes reflect the plant's adaptive adjustments to stress. In the G vs. H comparison (6 h vs. 12 h of cold stress), the number of DEGs further increased, with a total of 10,046 DEGs identified, including 5,884 upregulated genes and 5,162 downregulated genes. After 12 h of cold stress, significant changes in gene expression were observed, especially with the continued increase in the number of upregulated genes, indicating that the plant's adaptive responses to cold stress gradually strengthened, particularly in genes related to stress resistance, cell membrane protection, fatty acid metabolism, and hormone regulation (Fig. 1c).

KEGG pathway enrichment analysis reveals gene expression responses to cold stress at different time points in *T. ambiguum*

KEGG enrichment analysis revealed the profound effects of cold stress on gene expression at different time points (2 h, 6 h, and 12 h) in *T. ambiguum*. In the comparison of E vs. F (control group vs. 2 h of cold stress), significantly enriched pathways included Photosynthesis - antenna proteins and Photosynthesis, indicating that early in cold stress, plants adapt to low-temperature challenges by enhancing gene expression related to the photosynthetic process. The enrichment of the photosynthesis pathway suggests that plants attempt to improve the efficiency of light energy utilization to compensate for metabolic suppression caused by low temperatures. Furthermore, the enrichment of Carbon fixation in photosynthetic organisms and Carbon metabolism pathways indicates that cold stress stimulates plants to strengthen carbon metabolic processes, maintaining energy supply to ensure proper cellular function. The related enrichment of Glutathione metabolism suggests that plants initiate antioxidant systems early during cold stress to counteract oxidative stress induced by low temperatures (Fig. 2a).

With the extension of cold stress, in the comparison of F vs. G (2 h vs. 6 h of cold stress), continued enrichment of pathways included Photosynthesis, Carbon metabolism, as well as Biosynthesis of amino acids and Glutathione metabolism. Notably, the enrichment of Biosynthesis of amino acids indicates that, with prolonged cold stress, plants regulate osmotic pressure by synthesizing specific amino acids (such as arginine and proline) and enhance cellular stress resistance. Meanwhile, further enrichment of Glutathione metabolism suggests that plants continue to activate antioxidant systems, reducing cellular damage caused by reactive oxygen species (ROS) (Fig. 2b).

In the comparison of G vs. H (6 h vs. 12 h of cold stress), in addition to the continued enrichment of pathways related to Photosynthesis and Carbon metabolism, the enrichment of Circadian rhythm - plant and Glucuronide metabolism became key features. The enrichment of Circadian rhythm - plant suggests that plants adjust their biological rhythms under prolonged cold stress to adapt to the physiological pressures induced by low temperatures. By regulating their biological clock, plants can optimize metabolic processes, improve energy efficiency, and control stress resistance mechanisms (Fig. 2c). Furthermore, the enrichment of Glucuronide metabolism indicates that plants adjust secondary metabolic pathways to enhance cold tolerance and regulate defensive responses within cells. The enrichment of pathways such as Fatty acid metabolism, Indole acetic acid metabolism, and Glucuronide metabolism further emphasizes the multifaceted impact of cold stress on plant lipid metabolism and hormone regulation, suggesting that plants maintain cell membrane stability by increasing fatty acid synthesis and metabolism under cold stress, while also regulating growth and development through hormone control (such as auxin regulation), ultimately improving cold adaptation.

Validation by quantitative Real-Time PCR (qRT-PCR)

To validate the reliability of the RNA-seq data, 15 differentially expressed genes (DEGs) were randomly selected for qRT-PCR analysis. These genes represent key biological processes involved in cold stress response, such as antioxidant defense (e.g., *evm.TU.ctg9333.22* in glutathione metabolism), lipid metabolism (*evm.TU.ctg2798.293*), hormone signaling (*evm.TU.ctg3912.106* in ABA pathway), and secondary metabolism (*evm.TU.ctg9348.24*, involved in flavonoid biosynthesis), based on KEGG and GO annotations.

The expression patterns of these genes under four cold stress conditions (control [E], 2 h [F], 6 h [G], and 12 h [H]) were analyzed using the $2^{-\Delta\Delta Ct}$ method. The qRT-PCR results showed expression trends that were highly consistent with the RNA-seq data. Linear regression analysis revealed a strong positive correlation between the two datasets, with the equation $y = 0.5721x + 3.3259$ and an R^2 value of 0.8569 (Figure S1), indicating a high degree of reliability in transcriptome sequencing (Fig. 3).

These results confirm that the RNA-seq data accurately reflect the gene expression dynamics of *T. ambiguum* under different durations of cold stress.

Metabolomic analysis of *T. ambiguum* under cold stress at different time points

Principal component analysis (PCA) of metabolite data revealed significant metabolic differences between the control group (E) and the cold stress groups (F, G, H). In the PC1 (26.2%) and PC2 (20.9%) dimensions, the control group (E) samples clustered closely together, indicating minimal biological variation. In contrast, the cold stress groups (F, G, H) showed a gradual dispersion of sample points with the extension of cold stress duration. Notably, the 12-hour cold stress group (H) exhibited a significant increase in sample dispersion, suggesting that the duration of cold stress had a substantial impact on the plant's metabolic response, and that prolonged stress may enhance the plant's metabolic response (Fig. 4a).

Correlation analysis further indicated high consistency among biological replicates within each treatment group, with correlation coefficients approaching 1 for all groups, especially in the 12-hour cold stress group (H),

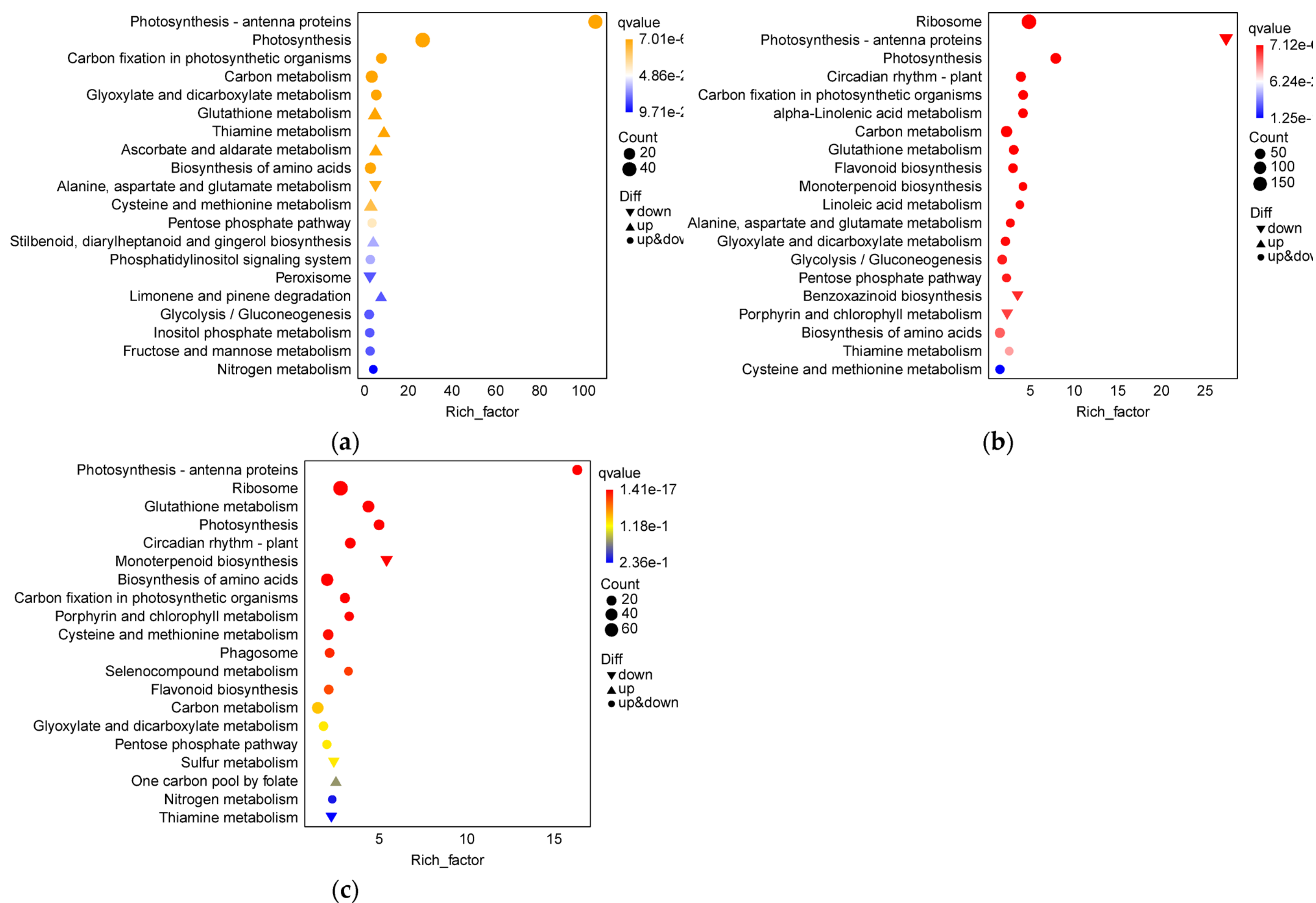

Fig. 2. KEGG pathway enrichment analysis of *T. ambiguum* under cold stress at different time points: (**a**) KEGG pathway enrichment analysis for the comparison of E vs. F (control group vs. 2 h of cold stress); (**b**) KEGG pathway enrichment analysis for the comparison of F vs. G (2 h vs. 6 h of cold stress); (**c**) KEGG pathway enrichment analysis for the comparison of G vs. H (6 h vs. 12 h of cold stress).

where the coefficients between samples exceeded 0.99. This suggests that the metabolic responses of plants under cold stress remained highly consistent across different samples (Fig. 4b).

Heatmap analysis supported this finding, showing significant differences in gene expression patterns across the treatment groups, with the most pronounced changes observed in the 12-hour cold stress group (H). As the cold stress duration increased, the impact on plant gene expression became progressively deeper, indicating that low temperatures may induce adaptive responses such as antioxidant reactions and cellular protection mechanisms to cope with prolonged cold stress (Fig. 4c).

Thus, the combined results from PCA, correlation analysis, and heatmap analysis demonstrate that cold stress not only significantly alters the metabolic responses of plants, but also induces persistent changes in gene expression as the duration of the stress treatment increases.

Metabolite analysis of *T. ambiguum* under cold stress

K-means clustering analysis of metabolite responses to cold stress in T. ambiguum

To investigate the effects of cold stress on the metabolites of *T. ambiguum*, we performed K-means clustering analysis and divided the metabolites into three clusters (Cluster1, Cluster2, Cluster3). The results showed significant differences in the response of metabolites to cold stress among the clusters, reflecting the diverse regulation of plant metabolic pathways.

Metabolites in Cluster1 exhibited large fluctuations in abundance under cold stress, particularly in the cold stress groups (F, G, H). Specifically, after 12 h of cold stress (H group), there were significant changes in metabolite abundance, indicating that these metabolites had a strong responsiveness to cold stress. This change may be closely associated with the plant's antioxidant mechanisms, cellular protection functions, and osmotic regulation processes. As the stress duration increased, particularly in the 12-hour cold stress group (H), the fluctuations in metabolite abundance intensified, suggesting that these metabolites play an important role under cold stress. Notably, after 12 h of treatment, the metabolite abundance fluctuations became more pronounced, possibly indicating that the plant had activated additional physiological response mechanisms to cope with the prolonged cold stress.

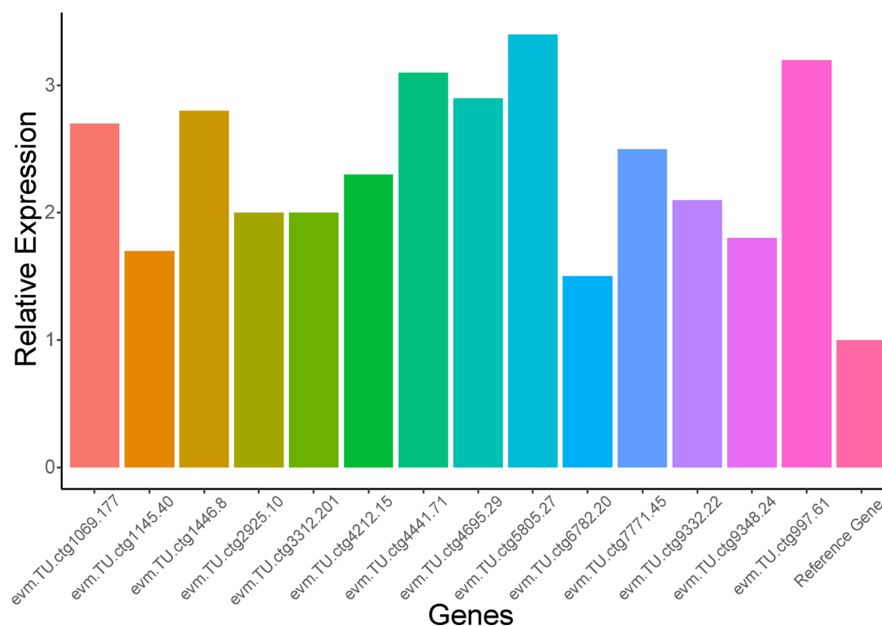

Fig. 3. Relative expression levels of 15 selected DEGs and one reference gene under cold stress conditions, as measured by qRT-PCR.

In contrast to the dramatic changes in Cluster1, metabolites in Cluster2 showed relatively small fluctuations in abundance, exhibiting a more stable trend across different cold stress groups (F, G, H). These metabolites appeared to respond more slowly or weakly to cold stress and may represent basic metabolic intermediates or structural metabolites that maintain relatively stable abundance under cold stress. The stable expression of these metabolites likely helps maintain the plant's normal physiological functions, ensuring basic metabolic stability in stressful environments. Cold stress did not significantly affect the synthesis or degradation processes of these metabolites, leading to more moderate changes in their abundance.

Metabolites in Cluster3 exhibited relatively uniform abundance distribution across the groups, with no significant differences between groups. These metabolites did not undergo significant changes under cold stress, suggesting that they are essential components in the plant's metabolism, which may not be directly involved in the cold stress response. Instead, they are likely involved in maintaining basic metabolic activities. The stability of these metabolites suggests that they continue to function normally under cold stress, ensuring the plant's regular physiological operation (Fig. 5).

Through K-means clustering analysis, we found that cold stress impacts metabolites in a differentiated manner. Some metabolites (such as those in Cluster1) responded significantly to cold stress, with abundance fluctuations helping the plant cope with the physiological challenges posed by low temperatures. Meanwhile, other metabolites (such as those in Cluster2 and Cluster3) maintained stable abundance, supporting the plant in maintaining basic physiological stability under stress. The distinct response characteristics of metabolites in different clusters suggest that plants may adapt to cold stress by modulating specific metabolic pathways while maintaining the stability of other basic metabolic processes, thereby optimizing survival and growth.

Radar plot analysis of metabolite changes in T. ambiguum under cold stress at different time points

The radar plot analysis of metabolites revealed the effects of cold stress at different time points (E vs. F, F vs. G, G vs. H) on the metabolites of *T. ambiguum*. First, in the comparison of E vs. F (control group vs. 2 h of cold stress), several metabolites showed significant changes after 2 h of cold stress. For example, the abundance of *(3Z)−3-[(4-hydroxyphenyl)methylene]−2-methyl-1,6,7,8,8a-tetrahydropyrrolo[1,2-a]pyrazine-14-dione* significantly increased in the F group, suggesting that cold stress rapidly activated certain metabolic pathways, potentially related to the plant's stress response and enhanced antioxidant capacity. Additionally, the abundance of *1D-Myo-Inositol* also increased significantly. As an osmotic regulator in plant cells, the increase in this metabolite may indicate a mechanism for the plant to cope with changes in cellular osmotic pressure induced by cold stress. This early-stage metabolic response is relatively rapid, suggesting that the plant is quickly adapting to the environmental changes (Fig. 6a).

In the comparison of F vs. G (2 h vs. 6 h of cold stress), with the extension of cold stress duration, further significant changes in the abundance of several metabolites were observed. Notably, the abundance of *Dihydrobaicalein* and *Gastrodin* increased significantly in the G group. This indicates that after 6 h of cold stress, the plant may have initiated stronger physiological regulatory mechanisms, especially in terms of antioxidant responses and secondary metabolite synthesis. For example, *Dihydrobaicalein*, a flavonoid compound with antioxidant activity, increased in abundance, suggesting that the plant might enhance its antioxidant reactions to cope with the increased levels of free radicals and reactive oxygen species induced by cold stress. *Gastrodin*, a phenylethanoid compound, showed increased abundance, which may be associated with the protection of

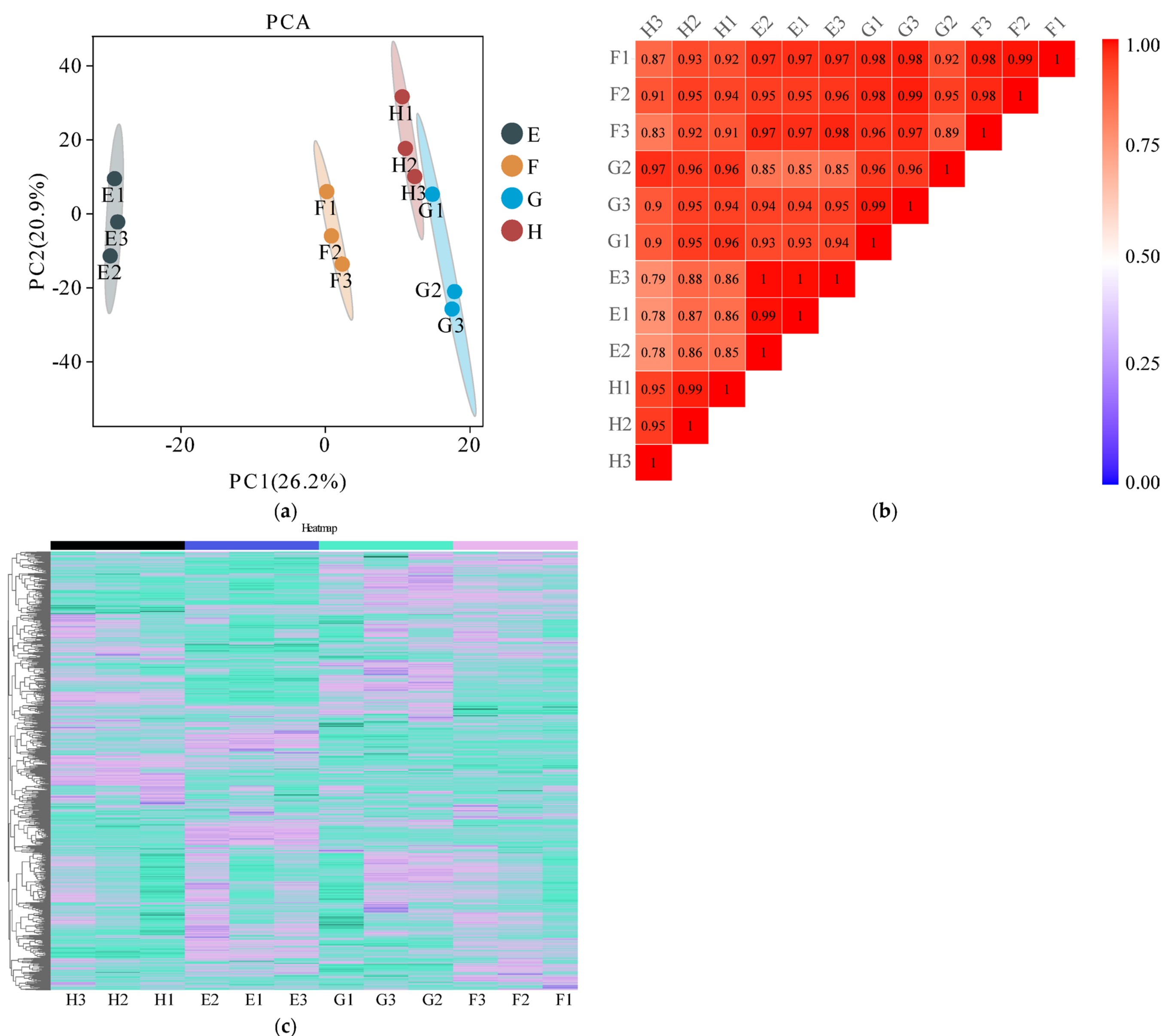

Fig. 4. Metabolite analysis of *T. ambiguum* under cold stress at different time points: (**a**) Principal component analysis (PCA) score plot for 12 samples under cold stress treatment; (**b**) Correlation analysis heatmap showing the similarity between biological replicates in each group (E, F, G, H); (**c**) Heatmap analysis of metabolic responses across different treatment groups (E, F, G, H) under cold stress.

plant cell membranes, enhanced antioxidant function, and responses to environmental stress. Therefore, after 6 h of cold stress, the metabolic regulation of the plant showed a marked enhancement, particularly in terms of antioxidant and osmotic protection mechanisms (Fig. 6b).

In the comparison of G vs. H (6 h vs. 12 h of cold stress), the changes in metabolite abundance intensified further, especially the significant changes in *15,16-Epoxy-15-ethoxy-6beta,13-dihydroxyalbd-8-en-7-one and 2-Oxokovalool*. These changes suggest that as the cold stress duration increased, the plant's metabolic pathways underwent more profound regulation. Specifically, *15,16-Epoxy-15-ethoxy-6beta,13-dihydroxyalbd-8-en-7-one*, a compound associated with plant stress responses, showed increased abundance in the H group, indicating that the plant had entered an adaptive state to cope with prolonged cold stress. Similarly, changes were observed in *2-Oxokovalool*, which may play an important role in lipid metabolism and stress resistance in the plant. As the duration of cold stress increased, the plant progressively enhanced its adaptation to low temperatures through the regulation of secondary metabolites, alterations in lipid metabolism, and improved antioxidant capacity (Fig. 6c).

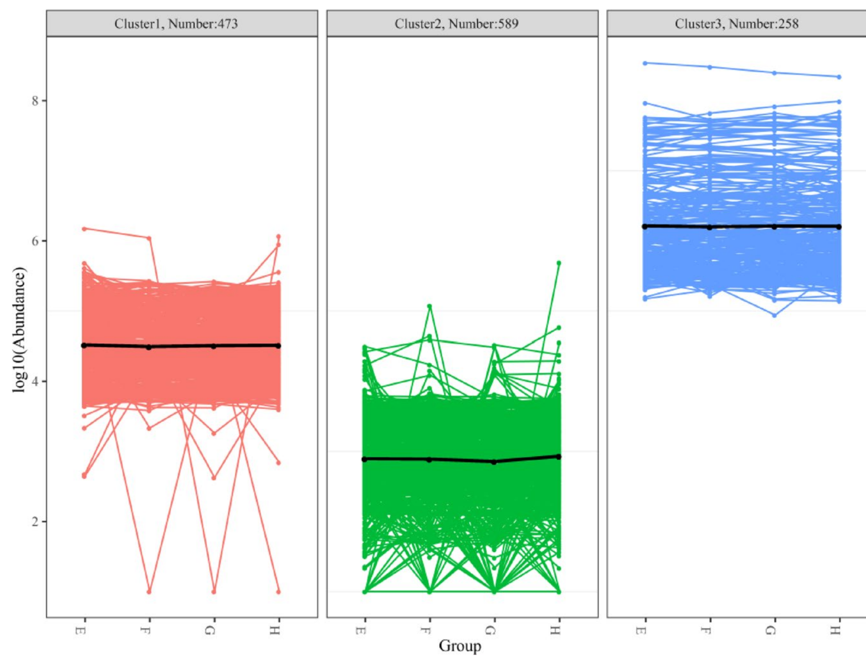

Fig. 5. Metabolite analysis of *T. ambiguum* under cold stress: Boxplot analysis of metabolite abundance in three clusters (Cluster 1, Cluster 2, Cluster 3) across different cold stress groups (E, F, G, H).

KEGG pathway enrichment analysis reveals multiple regulatory effects of cold stress on metabolic pathways in T. ambiguum at different time points

KEGG enrichment analysis revealed the multiple regulatory effects of cold stress on metabolic pathways at different time points in *T. ambiguum*. In the comparison of E vs. F (control group vs. 2 h of cold stress), significantly enriched pathways included Glycerophospholipid metabolism, alpha-Linolenic acid metabolism, and Fatty acid degradation. The enrichment of these pathways suggests that early in cold stress (2 h), plants regulate lipid metabolism to maintain the fluidity and stability of cell membranes, preventing membrane damage caused by low temperatures. The upregulation of glycerophospholipid metabolism and fatty acid degradation pathways may help plants enhance their cold tolerance by adjusting membrane lipid composition and regulating energy metabolism. The enrichment of alpha-Linolenic acid metabolism further emphasizes how plants use fatty acid metabolism to regulate membrane antioxidant capacity and protection, thereby alleviating oxidative damage caused by cold stress (Fig. 7a).

In the comparison of F vs. G (2 h vs. 6 h of cold stress), as the duration of cold stress extended, more metabolic pathways were activated, including the Phosphatidylinositol signaling system, Plant hormone signal transduction, and Arginine and proline metabolism. The enrichment of these pathways reflects the gradual initiation of more complex physiological regulatory mechanisms in plants after 6 h of cold stress. The Phosphatidylinositol signaling system, as a crucial cell signaling pathway, likely promotes rapid plant responses to environmental changes by regulating membrane structural changes and signaling networks under cold stress. The upregulation of Plant hormone signal transduction suggests that plants activate hormone signaling pathways (such as ABA and JA) to regulate growth, development, and stress responses. The enrichment of Arginine and proline metabolism indicates that plants synthesize these amino acids to adjust cell osmotic pressure, mitigating the effects of cold stress on cellular hydration and enhancing cold tolerance (Fig. 7b).

Further analysis in the comparison of G vs. H (6 h vs. 12 h of cold stress) revealed significant enrichment of metabolic pathways, including Arachidonic acid metabolism, Glucosinolates biosynthesis, and Aminoacyl-tRNA biosynthesis. The enrichment of Arachidonic acid metabolism suggests that after 12 h of cold stress, plants enhance antioxidant responses through fatty acid metabolism pathways and may synthesize antioxidant-active compounds to counteract oxidative stress caused by low temperatures (Fig. 7c). Additionally, the enrichment of Glucosinolates biosynthesis indicates that plants strengthen their cold adaptation mechanisms at this stage by adjusting secondary metabolic pathways, particularly in antioxidant, antifreeze, and osmotic regulation. The regulation of Aminoacyl-tRNA biosynthesis reflects the plant's regulation of protein synthesis under cold stress, which may help enhance cellular cold tolerance and maintain protein stability and function.

Integrated KEGG pathway enrichment analysis of transcriptomics and metabolomics

Metabolic and gene expression dynamics in T. ambiguum under prolonged cold stress In the comparison E vs. F (control vs. 2 h of cold stress), the scatter plot shows minimal changes in metabolite abundance, with only a few metabolites exhibiting significant shifts, while gene expression undergoes more substantial changes, reflecting the plant's initial response to cold stress. This indicates that in the early stages of stress (2 h), *T. ambiguum* activates transcriptional responses, especially in pathways related to oxidative stress and membrane stabilization (Fig. 8a). In F vs. G (2 h vs. 6 h of cold stress), with an extended exposure to low temperatures, both gene ex-

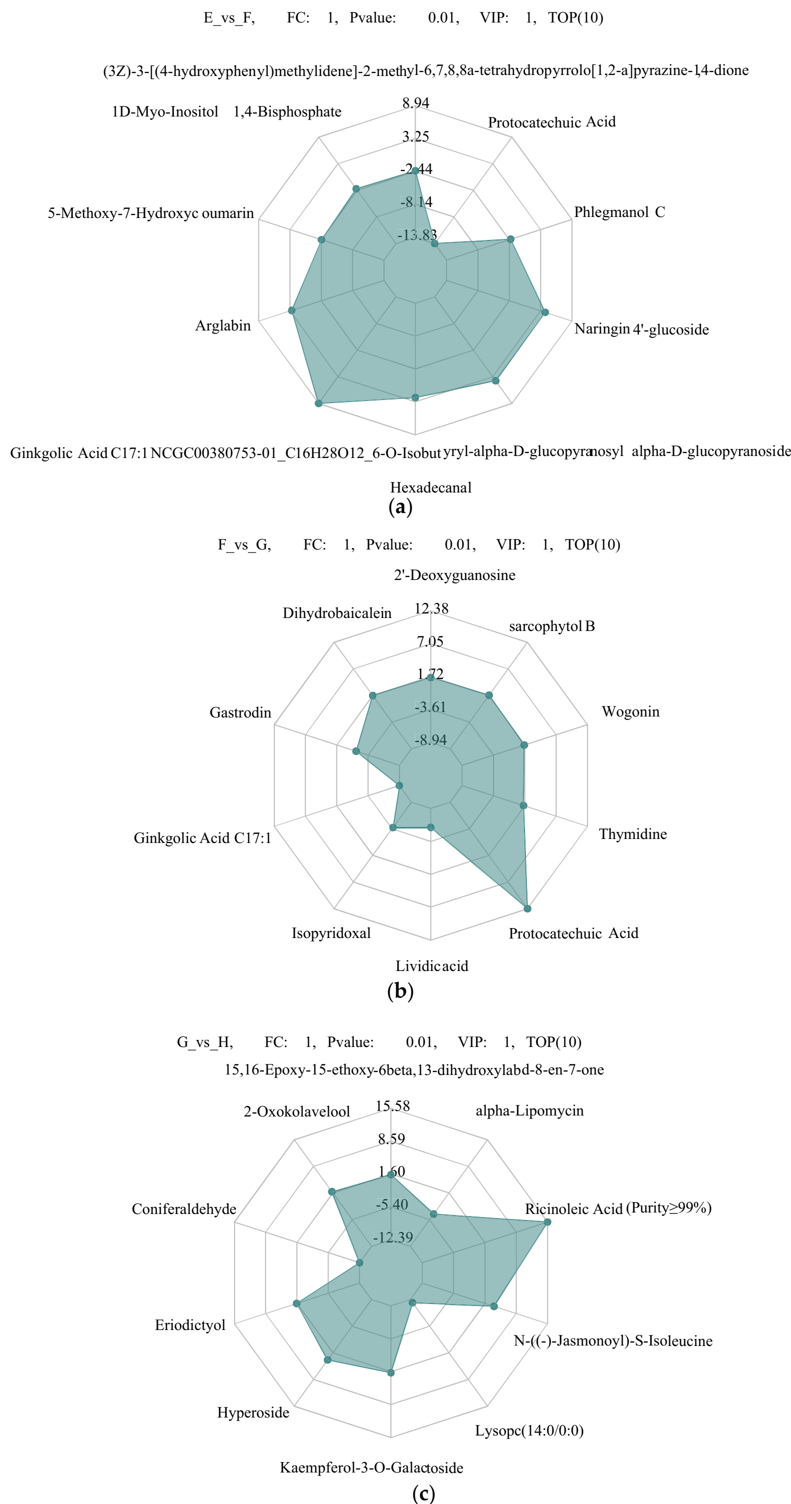

Fig. 6. Metabolite analysis of *T. ambiguum* under cold stress: (**a**) Radar plot analysis of metabolites in the comparison of E vs. F (control group vs. 2 h of cold stress); (**b**) Radar plot analysis of metabolites in the comparison of F vs. G (2 h vs. 6 h of cold stress); (**c**) Radar plot analysis of metabolites in the comparison of G vs. H (6 h vs. 12 h of cold stress).

pression and metabolite profiles undergo more pronounced changes. Notably, metabolites associated with lipid metabolism and proline metabolism show considerable upregulation, indicating that the plant is intensifying its response to maintain membrane integrity and regulate osmotic pressure as cold stress prolongs. The activation of phosphatidylinositol signaling and plant hormone signal transduction reflects a more complex regulatory mechanism, enhancing the plant's ability to adapt to prolonged stress (Fig. 8b). In G vs. H (6 h vs. 12 h of cold stress),

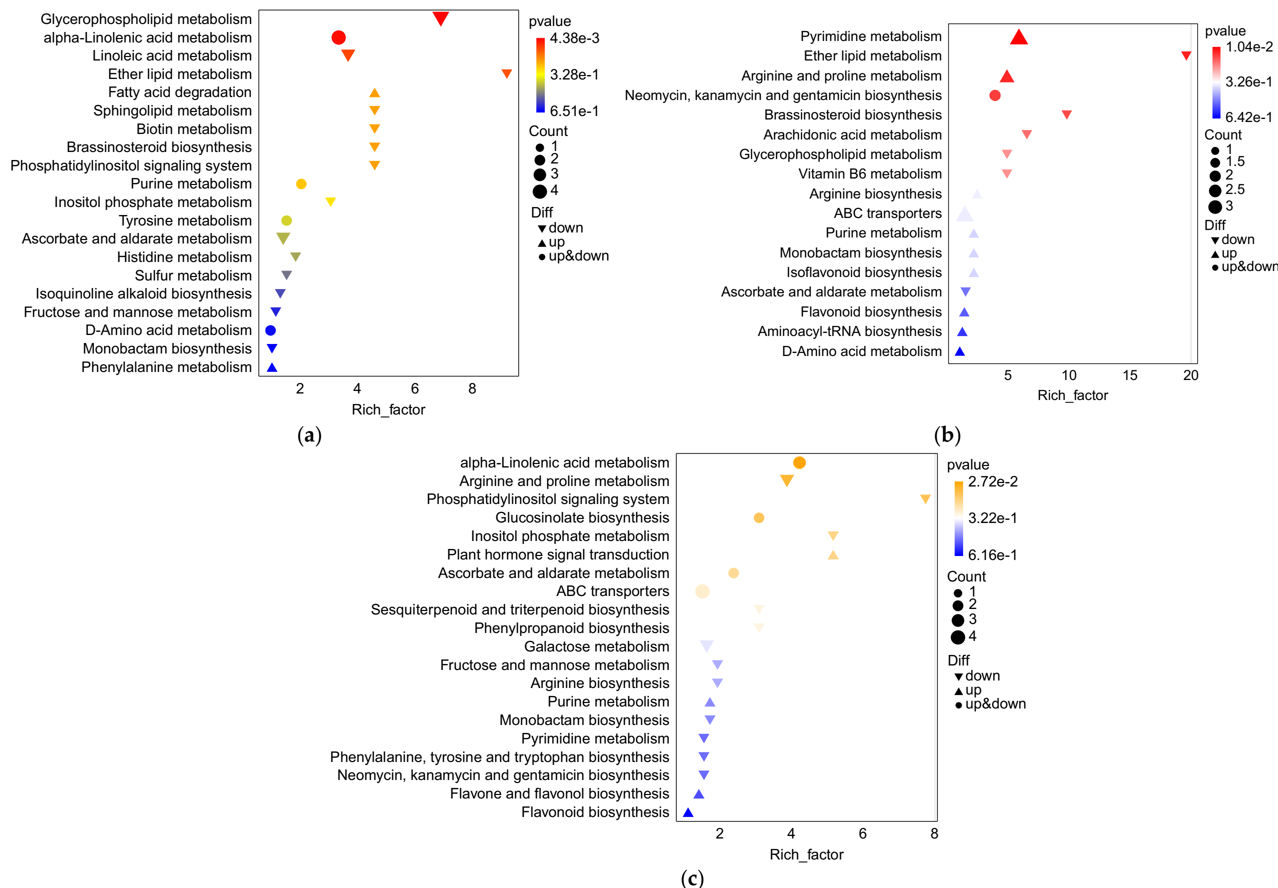

Fig. 7. KEGG pathway enrichment analysis of *T. ambiguum* under cold stress: (**a**) Enrichment analysis of metabolic pathways in the comparison of E vs. F (control group vs. 2 h of cold stress); (**b**) Enrichment analysis of metabolic pathways in the comparison of F vs. G (2 h vs. 6 h of cold stress); (**c**) Enrichment analysis of metabolic pathways in the comparison of G vs. H (6 h vs. 12 h of cold stress).

the plant's response continues to escalate, with significant upregulation of metabolites involved in arachidonic acid metabolism, glutathione biosynthesis, and proline metabolism (Fig. 8c). These changes indicate a robust adaptation process, where the plant amplifies its antioxidant defenses and further regulates osmotic pressure, thus ensuring cellular protection and stability under prolonged cold stress. The increasing log2FC values in both genes and metabolites across these comparisons highlight *T. ambiguum*'s dynamic and time-dependent response to cold stress, showcasing its capacity for metabolic and transcriptional reprogramming to adapt to and mitigate the adverse effects of prolonged low temperatures.

KEGG enrichment analysis of metabolic and transcriptomic responses to cold stress in T. ambiguum

The KEGG enrichment analysis results provide insights into the combined metabolic and transcriptomic responses of *T. ambiguum* to cold stress over time. In the E vs. F (control vs. 2 h of cold stress) comparison, pathways like Glycerophospholipid metabolism, alpha-Linolenic acid metabolism, and Fatty acid degradation are significantly enriched, demonstrating an early response to cold stress at both the transcriptional and metabolic levels. The combined increase in Glycerophospholipid metabolism and Fatty acid degradation indicates that, in response to cold stress, the plant adjusts membrane lipid compositions and regulates energy metabolism to stabilize cell membranes. Concurrently, alpha-Linolenic acid metabolism plays a crucial role in reducing oxidative damage, suggesting a coordinated effort between gene expression and metabolite accumulation to combat the stress (Fig. 9a).

In the F vs. G (2 h vs. 6 h of cold stress) comparison, there is a noticeable extension of the plant's response, with the activation of additional pathways like Phosphatidylinositol signaling, Plant hormone signal transduction, and Arginine and proline metabolism. These pathways reflect a complex interaction between the transcriptome and metabolome, where genes involved in signal transduction are upregulated to orchestrate cellular responses to cold stress. Phosphatidylinositol signaling enhances the plant's ability to adjust membrane integrity and signal transduction in response to prolonged stress. Additionally, the upregulation of genes related to Plant hormone signal transduction emphasizes the role of phytohormones (such as ABA and JA) in regulating plant growth and stress tolerance. Proline metabolism, important for osmotic regulation, is also enhanced, indicating that both

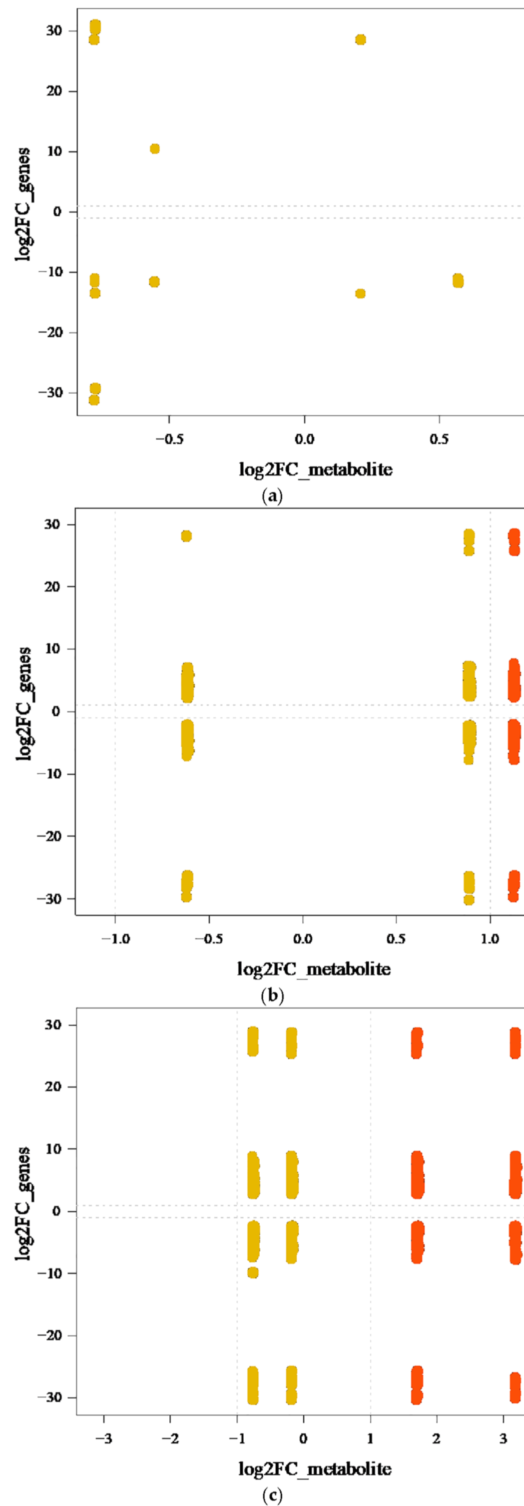

Fig. 8. Metabolite and gene expression dynamics of *T. ambiguum* under cold stress: (**a**) scatter plot comparing log2 fold change (log2FC) of genes and metabolites for E vs. F (control vs. 2 h of cold stress); (**b**) scatter plot for F vs. G (2 h vs. 6 h of cold stress); (**c**) scatter plot for G vs. H (6 h vs. 12 h of cold stress).

the metabolic and gene expression profiles work together to manage cellular hydration and reduce the effects of low temperatures (Fig. 9b).

In the G vs. H (6 h vs. 12 h of cold stress) comparison, further intensification of the plant's response is observed with significant enrichment in Arachidonic acid metabolism, Glutathione biosynthesis, and Proline metabolism. These pathways are key for long-term adaptation, as they involve both transcriptional activation

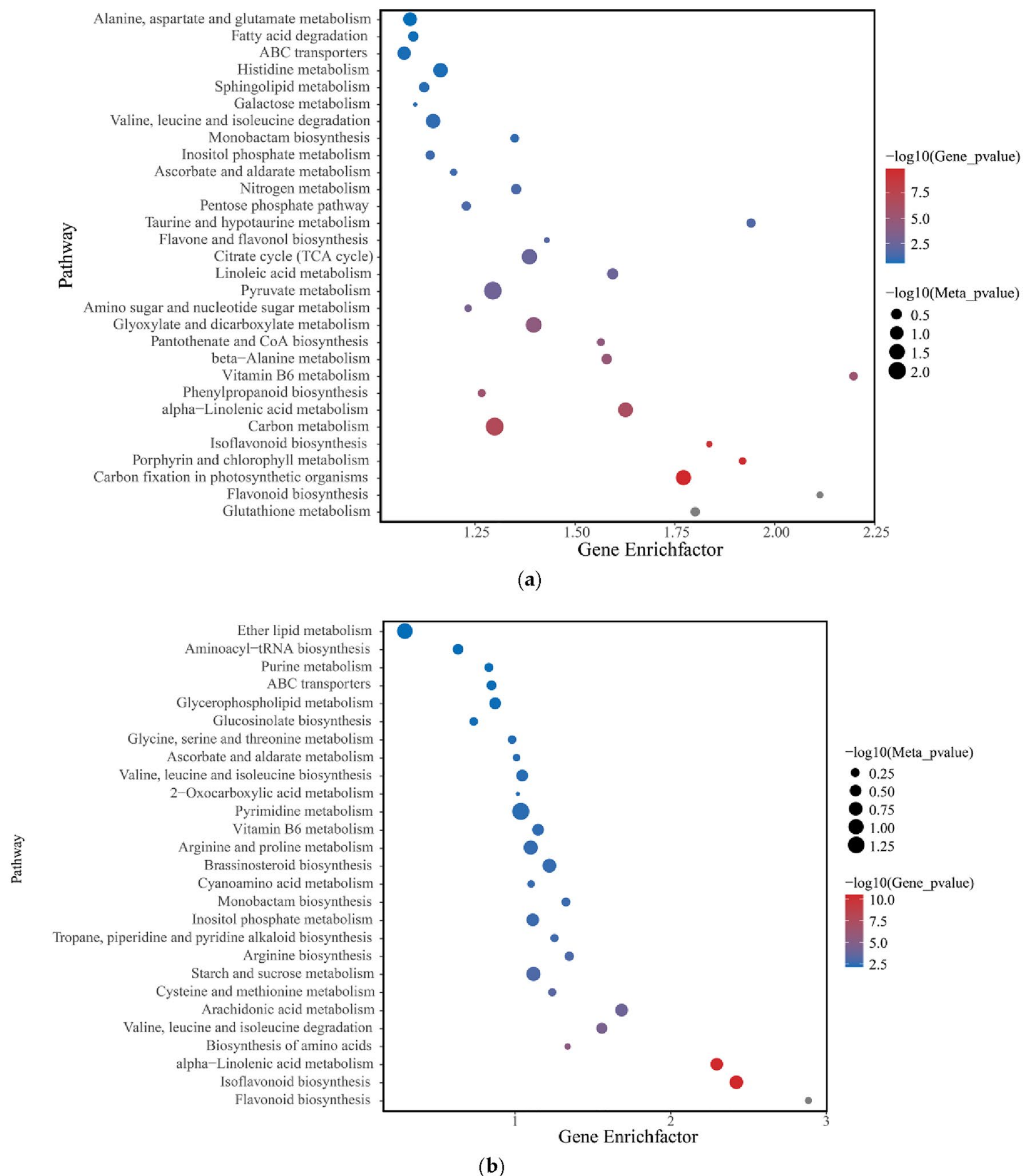

Fig. 9. Metabolite and gene expression dynamics of *T. ambiguum* under cold stress: (**a**) KEGG pathway enrichment analysis for E vs. F (control vs. 2 h of cold stress); (**b**) KEGG pathway enrichment analysis for F vs. G (2 h vs. 6 h of cold stress); (**c**) KEGG pathway enrichment analysis for G vs. H (6 h vs. 12 h of cold stress).

and changes in metabolite levels to strengthen antioxidant defenses and osmotic regulation. Arachidonic acid metabolism plays a critical role in reinforcing the antioxidant systems, while Glutathione biosynthesis enhances cellular detoxification processes (Fig. 9c). The sustained enrichment of Proline metabolism in this extended period signifies the plant's strategic response to osmotic stress, ensuring cellular stability under prolonged cold exposure. The coordinated activation of these metabolic and transcriptomic pathways highlights the integrated nature of the plant's stress response mechanisms, with gene expression and metabolite production working in synergy to improve resilience to cold stress over time. These results underscore *T. ambiguum*'s adaptive capacity through the combined efforts of the transcriptome and metabolome to mitigate the detrimental effects of cold stress.

Modular hierarchical clustering of isoflavonoid metabolites under cold stress

To further explore the regulatory patterns of isoflavonoid metabolites in *T. ambiguum* under cold stress, this study employed metabolomics to identify all isoflavonoid metabolites and utilized hierarchical clustering to

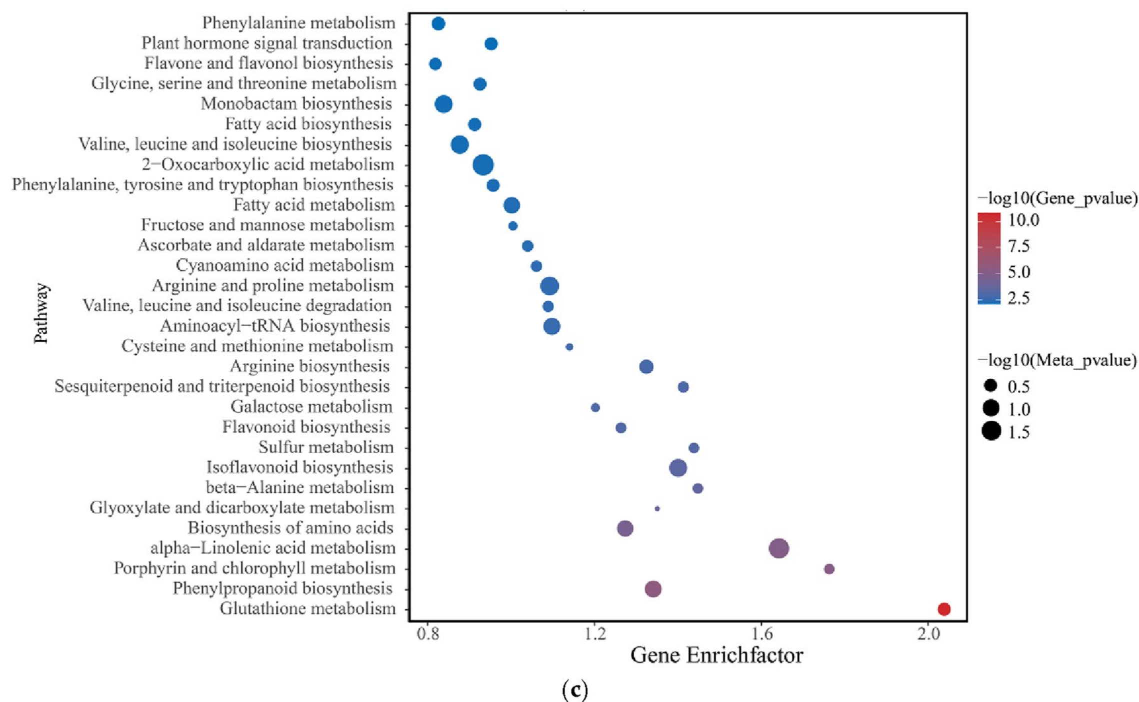

Fig. 9. (continued)

analyze their distribution. The clustering dendrogram provided insights into the relationships among metabolites across different modules, offering a novel perspective on the dynamic regulation of metabolites in response to cold stress.

In the modular hierarchical clustering of metabolites, different-colored modules represented functional relatedness or similarities in expression patterns among metabolites. Notably, the blue and cyan modules contained significantly enriched metabolites, including key isoflavonoid compounds such as Biochanin A, Pratensein, and Formononetin. These metabolites are likely involved in critical processes such as antioxidant defense, regulation of secondary metabolism, and adaptation to cold stress. The hierarchical organization of these modules reflects synergistic expression patterns, indicating that the plant finetunes metabolic pathways to fulfill physiological requirements under cold stress conditions (Fig. 10a).

The heatmap further illustrated the expression levels and trends of various isoflavonoid metabolites within each module. For example, Biochanin A and 5,7-Dihydroxyisoflavone exhibited significant upregulation in the red module, suggesting their prominent protective roles under cold stress. Conversely, Glycitin and Prunetin showed relatively stable expression in the blue module, indicating their potential roles in maintaining basic metabolic processes. The differential expression of metabolites across modules highlighted the plant's dynamic regulatory strategy for secondary metabolism in response to cold stress (Fig. 10b).

In summary, the distribution and expression trends of isoflavonoid metabolites across different modules suggest that *T. ambiguum* achieves precise regulation of isoflavonoid metabolism under cold stress through modular control. These findings provide valuable insights into the plant's secondary metabolic adjustments and adaptive strategies in cold stress conditions.

Correlation analysis of isoflavonoid metabolites with Module-Specific relationships

To further investigate the potential relationships between isoflavonoid metabolites and functional modules, this study performed a correlation analysis between metabolites and module eigengenes (MEs) using Weighted Gene Co-expression Network Analysis (WGCNA). Significant associations were visualized through a heatmap, providing insights into the functional roles of isoflavonoid metabolites under cold stress.

The heatmap revealed that various isoflavonoid metabolites exhibit significant correlations with multiple modules. Notably, Glycitin showed a strong positive correlation with the MEblack module ($r=0.91$, $p=4e\text{-}05$), suggesting that this metabolite is closely associated with the core functions of the MEblack module and may play a critical role in the cold stress response. In contrast, Vestitol and Biochanin A exhibited significant negative correlations with the MEplum1 module ($r=-0.90$, $p=6e\text{-}05$ and $r=-0.88$, $p=2e\text{-}04$, respectively), indicating that these metabolites may have inhibitory or balancing roles within this module.

Additionally, some metabolites, such as Glycitin and Biochanin A, demonstrated significant correlations with multiple modules, suggesting diverse functional roles. Glycitin, for example, showed a strong positive correlation with the MEblack module ($r=0.91$, $p=4e\text{-}05$) and a moderate positive correlation with the MEpink module ($r=0.66$, $p=0.02$). Similarly, Biochanin A exhibited a strong positive correlation with the MEpink module ($r=0.79$, $p=0.002$) and the MEturquoise module ($r=0.78$, $p=0.003$). These findings imply that these metabolites are involved in multiple biological processes across different functional modules (Fig. 11).

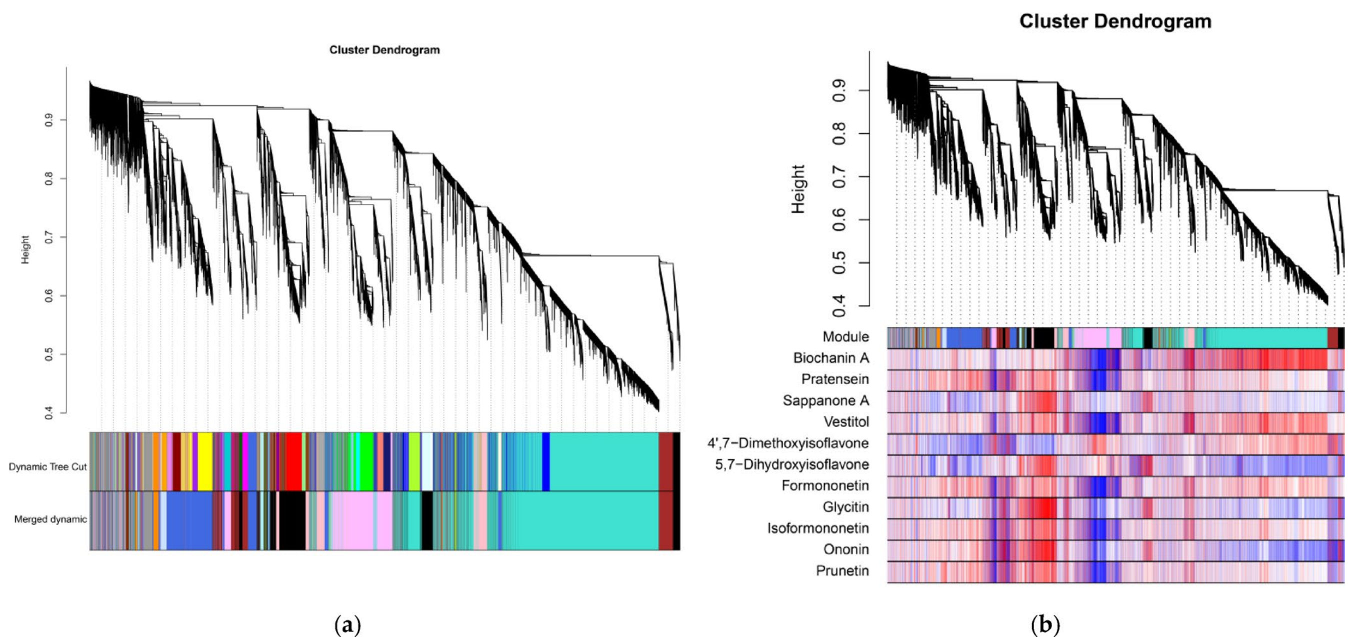

Fig. 10. Hierarchical clustering dendrogram and module-trait relationship analysis in *T. ambiguum*. (**a**) Hierarchical clustering dendrogram of genes based on dynamic tree cutting. Modules are represented by different colors, with merged dynamic modules shown below. (**b**) Hierarchical clustering dendrogram with annotated metabolite modules. The heatmap below the dendrogram shows the expression levels of key metabolites associated with each module, with red indicating higher expression and blue indicating lower expression.

Based on these correlation patterns, the study focused on the MEpink and MEblack modules for further investigation. These two modules were prioritized due to their significant involvement in metabolite regulation and their potential links to key biological processes. Metabolites such as Biochanin A and Vestitol in the MEpink module are likely to regulate specific biological functions under stress conditions, while the MEblack module, which is positively correlated with several isoflavonoid metabolites, appears to function as a core regulatory module involved in metabolic responses to cold stress.

This targeted analysis of the MEpink and MEblack modules lays the foundation for subsequent construction of metabolite networks and functional validation studies. These findings provide important insights into the molecular mechanisms underlying the adaptive responses of *T. ambiguum* to cold stress, highlighting the dynamic regulatory roles of isoflavonoid metabolites.

Functional enrichment and core gene identification in key modules

KEGG functional enrichment analysis of genes in the pink module revealed significant enrichment in several key metabolic pathways and biological processes under cold stress (Fig. 12a). Notably, the "phosphatidylinositol signaling system" and "glycolysis/gluconeogenesis" pathways were strongly enriched, indicating that genes in the pink module play an essential role in regulating signal transduction and carbon metabolism during cold stress. Furthermore, pathways such as "amino sugar and nucleotide sugar metabolism" and "plant-pathogen interaction" were also enriched, suggesting that these genes are closely involved in the plant's stress response and defense mechanisms.

To identify core functional genes within the pink module, a gene co-expression network was constructed using WGCNA (Fig. 12b). Key genes were selected based on their node degree, with *evm.TU.ctg2798.293* identified as the central gene. Its high connectivity within the network suggests that it plays a pivotal role in metabolic regulation and signal transduction. Heatmap analysis further illustrated the expression pattern of this core gene across different treatments (Fig. 12c). The results showed that *evm.TU.ctg2798.293* exhibited the highest expression in the control group (CK), the lowest expression during the mid-phase of cold stress (G), and intermediate expression levels at the G and H stages. This dynamic expression pattern indicates that the gene is involved in adaptive regulation during both the early and late stages of cold stress, with its suppression during the mid-phase possibly reflecting energy conservation or resource reallocation.

KEGG enrichment analysis of differentially expressed genes in the black module (Fig. 12d) revealed significant enrichment in several critical metabolic and signaling pathways. Among these, the "circadian rhythm - plant" pathway was significantly enriched, highlighting the importance of time-regulation mechanisms in modulating gene expression during cold stress. Additionally, the enrichment of pathways such as "plant hormone signal transduction," "flavonoid biosynthesis," and "glutathione metabolism" suggests that genes in the black module enhance plant stress tolerance by regulating antioxidant capacity and secondary metabolite production.

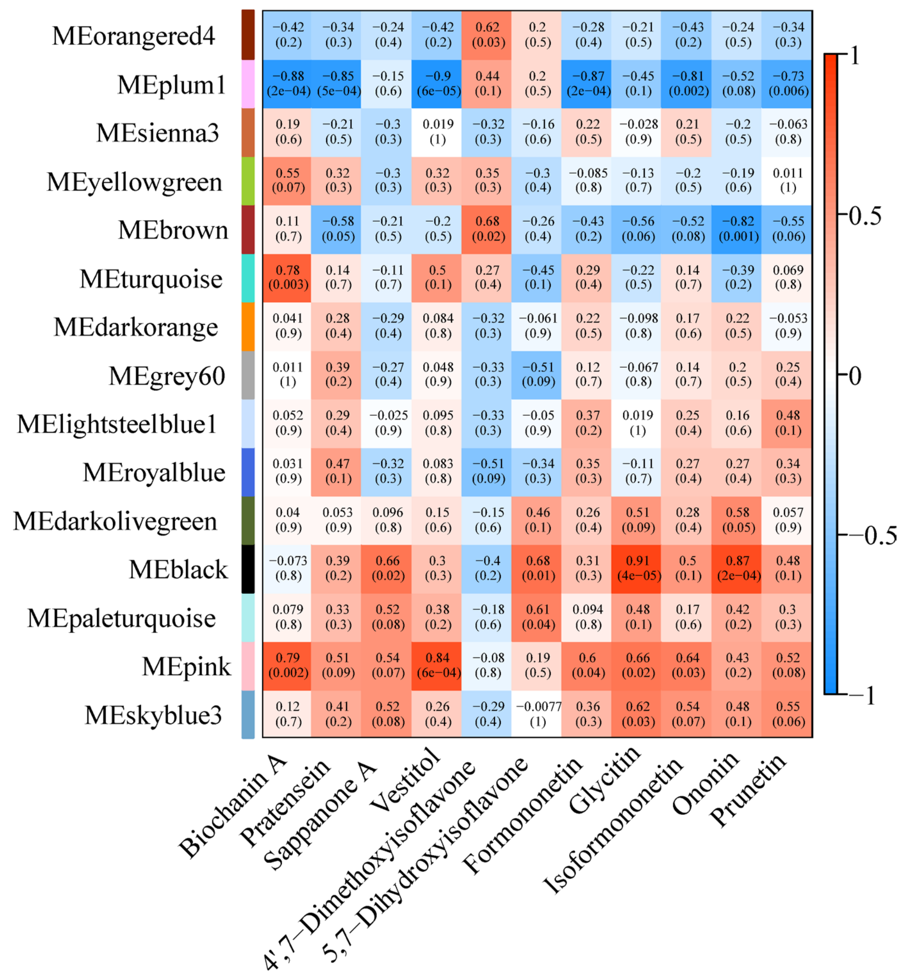

Fig. 11. Correlation heatmap between WGCNA modules and isoflavonoid metabolites in *T. ambiguum*.

Network analysis (Fig. 12e) identified several core genes within the black module, including *evm.TU.ctg3912.106* and *evm.TU.ctg2138.58*, which occupy critical positions in the network and likely play pivotal roles in regulating module functions and stress responses. Heatmap analysis (Fig. 12f) revealed the dynamic expression profiles of these core genes under various treatment conditions. *evm.TU.ctg3912.106* exhibited the highest expression during the G treatment and the lowest expression in the CK treatment, indicating significant activation during the mid-phase of cold stress, with low expression during the early phase and control conditions. *evm.TU.ctg2138.58* displayed a progressive induction throughout the cold stress process, with the highest expression observed in the H treatment, followed by G and H, and the lowest expression in the CK treatment. This suggests that the gene's activity increases with the duration of cold stress, peaking during the late stage. Additionally, *evm.TU.ctg4243.97* exhibited extremely low expression in the CK treatment but high expression in the F, G, and H treatments, indicating continuous activation under cold stress conditions, likely contributing to stress adaptation.

In conclusion, functional enrichment analysis and core gene identification of differentially expressed genes in the pink and black modules reveal their significant roles in cold stress response. Genes in the pink module primarily regulate signal transduction and carbon metabolism, while genes in the black module are more involved in time regulation, hormone signaling, and antioxidant metabolism. These findings provide an important theoretical foundation for further research into the molecular mechanisms underlying cold stress responses.

Transcriptomic and metabolomic adaptation of *T. ambiguum* to cold stress

The transcriptomic and metabolomic responses of *T. ambiguum* under cold stress exhibit a highly coordinated regulatory network. Cold stress significantly induced calcium influx, triggering the Ca^{2+}/CaM/CDPKs signaling cascade, which further upregulated ICE1, CBF1, CBF2, and CBF3 genes, thereby enhancing the expression of downstream cold-regulated (COR) genes and improving plant cold tolerance. WGCNA identified two critical gene modules: Pink Module and Black Module. The Pink Module (*evm.TU.ctg2798.293*) was enriched in genes involved in glycerophospholipid metabolism, glycolysis, and gluconeogenesis, highlighting the role of membrane lipid remodeling and energy metabolism in cold adaptation. The Black Module (*evm.TU.ctg3912.106*, *evm.TU.ctg2138.58*) was associated with plant hormone signaling (ABA, JA, ethylene) and flavonoid metabolism

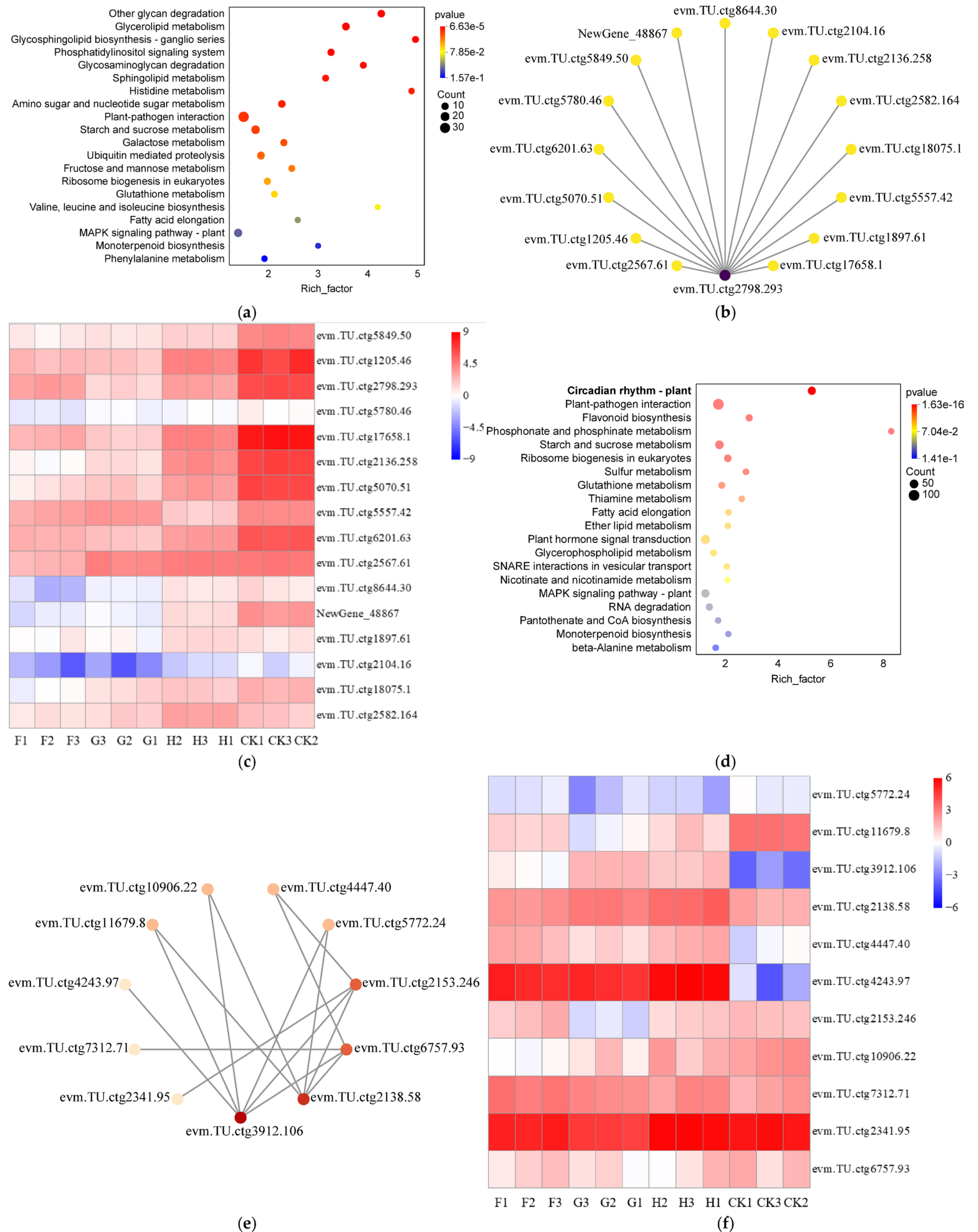

Fig. 12. KEGG pathway enrichment and hub gene analysis for pink and black modules identified by WGCNA in *T. ambiguum*. (**a**–**c**) represent KEGG pathway enrichment, hub gene network, and heatmap of hub gene expression for the pink module, respectively. (**d**–**f**) represent KEGG pathway enrichment, hub gene network, and heatmap of hub gene expression for the black module, respectively. The bubble plot (**a**, **d**) highlights enriched pathways with their significance (*p*-value), while hub genes (**b**, **e**) are visualized in a network diagram. Heatmaps (**c**, **f**) display the expression patterns of hub genes across different cold stress conditions and time points, with red indicating higher expression and blue indicating lower expression.

(Biochanin A, Glycitin), indicating that plants mitigate cold-induced oxidative stress through hormone regulation and flavonoid accumulation. Transcriptomic analysis further revealed that glycerophospholipid and fatty acid metabolism pathways were significantly activated, reinforcing the importance of membrane lipid remodeling in maintaining cellular integrity under cold stress. Moreover, α-linolenic acid metabolism was significantly upregulated, leading to enhanced jasmonic acid (JA) biosynthesis, a key signaling molecule known to modulate cold stress responses. Metabolomic analysis showed a significant increase in antioxidant enzyme activities (SOD, POD, CAT) and an upregulation of glutathione metabolism, suggesting a robust oxidative stress mitigation mechanism. Simultaneously, the accumulation of osmoprotectants (proline, soluble sugars, betaine) contributed to cellular osmotic balance and dehydration protection. Collectively, this study proposes an integrated model of *T.ambiguum* cold adaptation (Fig. 13), elucidating a comprehensive regulatory network from signal perception (Ca^{2+} signaling) to transcriptional regulation (ICE1-CBF-COR pathway) and metabolic adaptation (membrane remodeling, hormone signaling, antioxidant response, and osmoprotection). These findings provide novel insights into the molecular basis of cold tolerance in leguminous plants and offer potential targets for future crop improvement strategies.

Discussion

In the context of global climate change, Cold Stress has emerged as a significant abiotic stressor affecting plant growth, development, and yield[21,22]. To adapt to Cold Stress, plants utilize complex metabolic regulatory networks that involve the accumulation of protective metabolites, modulation of antioxidant defenses, and activation of secondary metabolic pathways[24,25]. *T. ambiguum*, a crucial forage resource, demonstrates metabolic response characteristics under Cold Stress that are vital for understanding its adaptability[23]. This knowledge also provides a theoretical foundation for exploring the molecular mechanisms of Cold Stress tolerance in plants and informs related breeding strategies. However, research on the dynamic metabolic response of *T. ambiguum* to Cold Stress remains relatively limited, highlighting a valuable opportunity for the current investigation.

Gene Expression Dynamics of *T. ambiguum* Under Cold Stress at Different Time Points.

This study systematically analyzed the dynamic gene expression changes in *T. ambiguum* under cold stress at different time points (2 h, 6 h, and 12 h), revealing that as the duration of cold stress increased, the plant progressively activated more complex regulatory gene networks. Heatmap analysis indicated that under normal

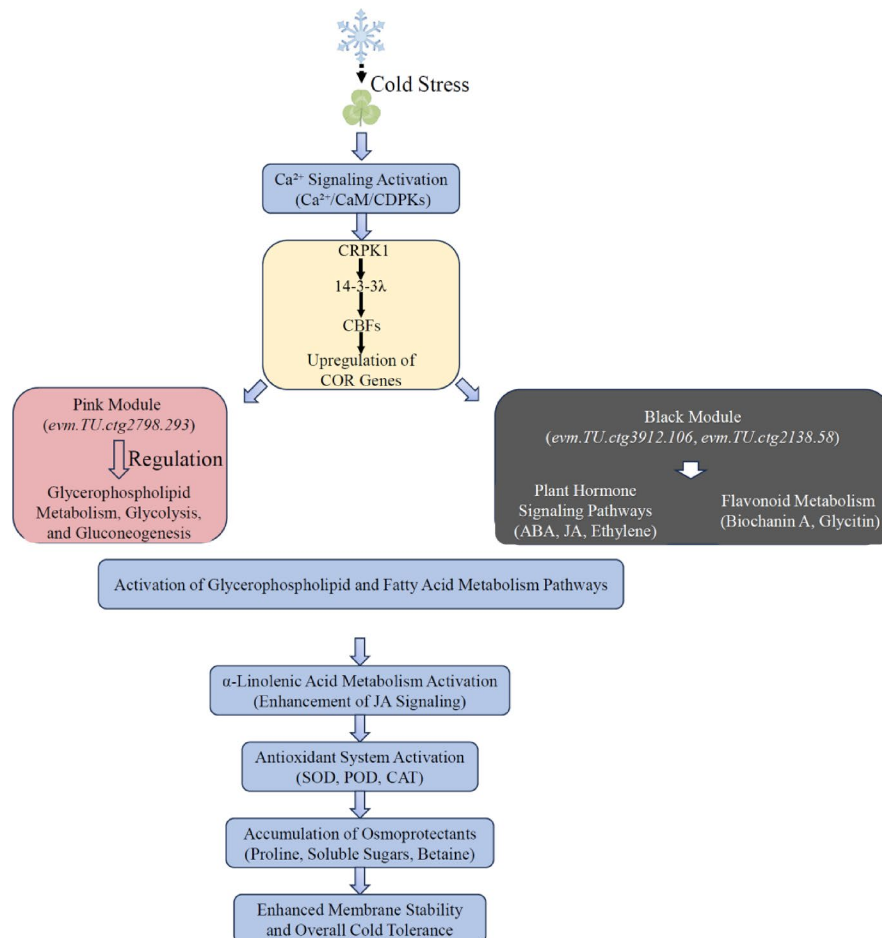

Fig. 13. A proposed model illustrating the transcriptomic and metabolomic adaptation of *T. ambiguum* under cold stress.

growth conditions, the plant's transcriptome was relatively stable, but after cold treatment, gene expression gradually changed, especially after 12 h of cold stress, where gene expression diversity significantly increased[24–26]. Additionally, Venn diagram and differentially expressed genes (DEGs) analysis showed that a large number of genes were regulated within 2 h of cold stress, while gene expression stabilized after 6 h, and more stress-resistance genes were further activated at 12 h. This dynamic process is similar to the transcriptomic changes observed in *Brassica napus*, *Oryza sativa*, and *Triticum aestivum* under cold stress, where the early stage (2 h) mainly involves antioxidant responses and osmotic regulation, while the later stages (6–12 h) activate more complex biological processes such as lipid metabolism and hormone signaling. The ICE1–CBF–COR signaling pathway is a central regulatory cascade involved in plant cold stress responses. Traditionally, ICE1 (Inducer of CBF Expression 1) was considered to function as an upstream regulator of the CBF transcription factors, directly activating their expression during cold exposure. However, recent studies have suggested a revised model where CRPK1 (Cold-Regulated Protein Kinase 1) and 14-3-3λ proteins are positioned upstream of CBFs, modulating their activation during cold stress[27,28].

Specifically, CRPK1 and 14-3-3λ have been proposed to act as key regulators of CBF expression, linking cold stress signaling pathways to the activation of cold-responsive (COR) genes. Kidokoro et al. (2020) and Liu et al. (2017) demonstrated that these upstream components interact with CBFs in a manner that does not necessarily rely on ICE1, suggesting a more complex regulatory network involving multiple proteins for cold adaptation[29]. In *Arabidopsis thaliana*[30], *Medicago sativa*[31], and *Trifolium pratense*[32], the ICE1–CBF–COR pathway has been widely studied, where ICE1 plays a role in activating CBFs in response to cold stress. However, recent findings suggest that CRPK1 and 14-3-3λ proteins may act as upstream activators of CBFs, independent of ICE1, highlighting the complexity of cold stress signaling networks in these species[33]. In *T. ambiguum*, our findings indicate that several CBF-like transcription factors and COR genes such as *COR15B* and *COR47* were induced under cold stress, supporting the involvement of the ICE1–CBF–COR pathway in cold adaptation. However, in light of the recent advancements in cold-responsive signaling, it is possible that the CRPK1–14-3-3λ–CBF cascade plays a more prominent role in regulating cold stress responses than previously thought. Further research will be necessary to clarify the exact roles of these proteins in *T. ambiguum* and their potential cross-talk with the ICE1–CBF–COR pathway. KEGG enrichment analysis further revealed the dynamic changes in key metabolic pathways. In the early cold stress (2 h), pathways such as photo-synthesis-antenna proteins, photosynthesis, carbon fixation, and glutathione metabolism were significantly enriched, indicating that the plant initially enhanced photo-synthesis and energy metabolism to cope with the cold stress challenge[24,34]. This phenomenon is consistent with the strategy observed in *Oryza sativa* and *Brassica napus* during early cold stress, where enhanced photosynthesis helps to maintain energy supply. Furthermore, the enrichment of glutathione metabolism suggests that the plant rapidly activates its antioxidant system to eliminate reactive oxygen species (ROS) induced by cold stress, a similar mechanism reported in *Medicago sativa* and *Actinidia chinensis* under cold stress[35,36]. As the stress duration extended (6 h), the plant's metabolic regulatory mechanisms were further adjusted. The continued enrichment of photosynthesis and carbon metabolism pathways suggests that the plant optimized carbon fixation and photosynthetic efficiency to maintain energy supply[37]. Additionally, the significant enrichment of amino acid biosynthesis, particularly the synthesis of arginine and proline, indicates that the plant enhanced osmotic regulation during the mid-phase (6 h) to maintain cellular homeostasis and reduce water loss and osmotic stress caused by low temperatures. This phenomenon is consistent with the mechanisms of proline accumulation in *Capsicum annuum* under low-temperature conditions, which enhance cold tolerance[38]. Moreover, the continued enrichment of glutathione metabolism further corroborates the crucial role of maintaining antioxidant capacity during cold adaptation, suggesting that as the stress duration increases, the plant continuously removes ROS to prevent oxidative damage. This trend has also been observed in *Cucurbita maxima* and *Glycine max* under cold stress[39,40]. After 12 h of cold stress, the plant entered a deeper level of adaptive regulation, showing significant enrichment in plant circadian rhythm and glucuronic acid metabolism. The significant enrichment of circadian rhythm suggests that the plant may adjust its circadian-related genes to adapt to the cold environment, optimize energy metabolism, and coordinate stress resistance mechanisms. This phenomenon aligns with the circadian regulation mechanisms observed in *Nicotiana tabacum* and *Cucumis sativus* under cold stress[41,42]. Additionally, the enrichment of glucuronic acid metabolism implies that the plant may enhance its cold tolerance by adjusting the synthesis of secondary metabolites, such as flavonoids and phenolic compounds, a phenomenon also reported in *Arachis hypogaea* and *Chrysanthemum morifolium* under low-temperature stress[43,44]. Further analysis revealed the enrichment of fatty acid metabolism, indole acetic acid metabolism (IAA), and glucuronic acid metabolism, suggesting that cold stress not only affects the stability of the plant cell membrane but also regulates auxin signaling to control growth and development, improving the plant's long-term adaptability[45,46].

Metabolic response of *T. ambiguum* to cold stress at different time points

Cold stress significantly affects the metabolism and physiological processes of plants, and plants adapt to low-temperature environments by regulating a series of metabolic pathways and gene expression. This study further confirms the time-dependent cold tolerance adaptation process of *T. ambiguum* under cold stress through metabolomics analysis, revealing that the plant gradually enhances its cold tolerance by dynamically adjusting metabolic pathways at different stages of cold stress treatment.

First, PCA analysis showed significant differences in the metabolic patterns between the control group (E) and cold stress groups (F, G, H). As the duration of cold stress increased, the composition of metabolites changed significantly, especially after 12 h of cold treatment, where the dispersion of metabolites increased significantly, indicating that the plant's metabolic response gradually became more complex. Similar changes have been confirmed in cold adaptation studies of *Oryza sativa* and *Cucumis sativus*, which suggest that plants initially

accumulate antioxidant metabolites to cope with cold stress, and under prolonged stress, they adjust carbon metabolism, amino acid metabolism, and secondary metabolite synthesis to enhance their adaptability[24,42].

K-means clustering analysis further revealed the different roles of metabolites during cold adaptation. Metabolites in Cluster 1 fluctuated significantly under cold stress, especially after 12 h, when they accumulated significantly. These metabolites may be involved in mechanisms such as reactive oxygen species (ROS) scavenging, osmotic regulation, and membrane stability maintenance. Similar trends have been observed in studies of *Brassica napus* and *Glycine max*, indicating that such metabolites play an important role in the formation of cold tolerance in plants[47,48]. In contrast, metabolites in Cluster 2 showed minimal changes, suggesting that these are basic metabolic products or structural metabolites primarily used to maintain the plant's metabolic steady state, rather than directly involved in the stress response[49,50]. Metabolites in Cluster 3 showed no significant changes across the treatment groups, likely representing part of the plant's core metabolic pathways, maintaining stability under extreme conditions. A similar phenomenon has been reported in studies of *Cocos nucifera* and *Arachis hypogaea* cold adaptation[37,43].

KEGG enrichment analysis further revealed key metabolic pathways induced by cold stress. During the early phase of cold stress (2 h), glycerophospholipid metabolism, alpha-linolenic acid metabolism, and fatty acid degradation pathways were significantly enriched, indicating that the plant adjusts membrane lipid composition and energy metabolism to stabilize cell membranes and reduce damage caused by cold stress. This metabolic adjustment strategy is consistent with cold tolerance mechanisms in *Cucurbita maxima* and *Medicago sativa*[35,39]. As cold stress continued for 6 h, the significant activation of the phosphatidylinositol signaling system, plant hormone signal transduction, and proline metabolism pathways suggested that the plant relies on hormonal signaling and osmotic regulation to enhance stress resistance. This phenomenon is in agreement with cold adaptation studies of N*icotiana tabacum* and *Capsicum annuum*, highlighting the key roles of ABA (abscisic acid), JA (jasmonic acid), and other plant hormones in cold stress signal transduction[11,51]. When cold stress was extended to 12 h, the significant enrichment of arachidonic acid metabolism, glucosinolate biosynthesis, and aminoacyl-tRNA biosynthesis pathways reflected the plant's enhanced adaptation through fatty acid metabolism, secondary metabolite synthesis, and protein homeostasis regulation[19,31,52]. This metabolic adjustment pattern has also been confirmed in cold tolerance studies of wheat, peanut, and *Chrysanthemum morifolium*, indicating that plants improve cold resistance by remodeling metabolic networks during prolonged cold stress[44,53].

In summary, this study reveals the time-dependent metabolic regulation of *T. ambiguum* under cold stress. The plant gradually enhances its adaptation to cold stress by activating multiple pathways, including antioxidant responses, osmotic regulation, lipid metabolism, and secondary metabolite synthesis.

Integrated transcriptomic and metabolomic analysis of *T. ambiguum* under cold stress

This study, through combined metabolomics and transcriptomics analysis, reveals the dynamic changes in the metabolism and gene expression of *T. ambiguum* under prolonged cold stress. In the early phase of cold stress (E vs. F, control vs. 2 h of cold stress), overall metabolite changes were minimal, with only a few metabolites showing significant shifts in abundance, whereas gene expression exhibited notable regulation. This indicates that, in the early stages of stress, plants mainly rely on transcriptional regulation to respond to environmental changes, particularly through the rapid activation of genes related to antioxidant response and membrane stability to reduce oxidative damage and maintain cellular function. This trend is consistent with the short-term cold stress adaptation strategies observed in studies on *Oryza sativa*, *Triticum aestivum*, and *Brassica napus*, where plants primarily rely on gene regulation in response to short-term stress with minimal changes in metabolites[24,34,54]. As the duration of cold stress increased (F vs. G, 2 h vs. 6 h), both metabolic and gene expression changes became more pronounced, with significant upregulation of metabolites related to lipid metabolism and proline metabolism, indicating that plants begin to enhance membrane stability and osmotic regulation to adapt to the cold environment. Additionally, the activation of phosphatidylinositol signaling and plant hormone signaling pathways indicates that cold stress triggers more complex cellular signaling mechanisms to promote long-term adaptation to the stress[18,20]. During the prolonged cold stress phase from 6 h to 12 h (G vs. H), metabolic adaptation further strengthened, with significant upregulation of pathways involved in arachidonic acid metabolism, glutathione biosynthesis, and proline metabolism. This suggests that plants maintain cellular homeostasis and ensure survival under prolonged cold by enhancing anti-oxidant defense and osmotic regulation. This dynamic adaptation model has also been verified in cold stress studies of *Vicia sativa* and *Xanthoceras sorbifolia*, indicating that plants generally undergo three phases of adaptation: short-term stress response (2 h), mid-term metabolic adaptation (6 h), and long-term homeostasis regulation (12 h) to cope with prolonged cold environments[55,56].

KEGG enrichment analysis further reveals the synergistic role of metabolic and transcriptional regulation in cold adaptation. In the early phase of cold stress (E vs. F, 2 h), significant enrichment of pathways such as glycerophospholipid metabolism, alpha-linolenic acid metabolism, and fatty acid degradation suggests that plants rapidly adjust membrane lipid composition and energy metabolism to stabilize the cell membrane and mitigate cold-induced membrane damage. The enhancement of alpha-linolenic acid metabolism reflects that plants improve antioxidant capacity via fatty acid metabolism pathways to reduce cold-induced oxidative damage. This metabolic regulation pattern has also been reported in studies of cold tolerance mechanisms in *Cucurbita maxima* and *Averrhoa carambola*[39,57]. As the cold stress continued to 6 h (F vs. G), the metabolic and transcriptional regulation mechanisms of the plants became further complicated. The enrichment of pathways such as phosphatidylinositol signaling, plant hormone signal transduction, and proline metabolism indicates that plants rely on signal transduction, hormone regulation, and osmotic protection mechanisms to maintain cellular homeostasis. The activation of ABA (abscisic acid) and JA (jasmonic acid) signaling pathways suggests that plants enhance hormone-mediated defense mechanisms under prolonged cold, which aligns with the cold adaptation strategies of *Ulmus pumila* and *Prunus mume*[56,58]. When cold stress extended to 12 h (G

vs. H), significant enrichment in pathways such as arachidonic acid metabolism, glutathione biosynthesis, and aminoacyl-tRNA synthesis indicates that plants enter a deeper phase of metabolic remodeling. The enhancement of arachidonic acid metabolism suggests that plants may strengthen their antioxidant system via fatty acid metabolism pathways, synthesizing metabolites with antioxidant activity to reduce oxidative damage. This metabolic adaptation strategy has been confirmed in cold stress studies of *Chrysanthemum morifolium* and *Glycine max*[40,44]. Furthermore, the continued enrichment of glutathione metabolism further supports the strategy of enhancing antioxidant capacity to maintain cellular stability under cold stress, which is consistent with metabolic regulation patterns observed in *Musa acuminata* and *Pisum sativum* during long-term cold adaptation[48,59].

In summary, this study reveals the dynamic metabolic and transcriptional regulatory mechanisms of *T. ambiguum* under cold stress. In the early phase (2 h), plants primarily rely on membrane lipid metabolism and antioxidant defense to rapidly respond to cold challenges[60,61]. In the mid-phase (6 h), plants activate signal transduction and hormone regulation, enhancing osmotic regulation and membrane stability. In the long-term adaptation phase (12 h), plants reinforce their cold tolerance through fatty acid metabolism, secondary metabolite synthesis, and protein stability regulation. This time-dependent regulatory model is consistent with cold stress studies on *Brassica oleracea*, *Malus domestica*, and *Chenopodium quinoa*, demonstrating that plants typically enhance cold tolerance through the synergistic regulation of transcription and metabolism[62–64].

WGCNA-Based analysis of gene and metabolite Co-Expression in *T. ambiguum* under cold stress

This study, through the application of Weighted Gene Co-expression Network Analysis (WGCNA), systematically explored the synergistic relationship between gene expression and metabolite patterns in *T. ambiguum* under cold stress, revealing the critical regulatory roles of specific modules in the plant's cold response. These findings provide new insights into the complex molecular mechanisms that underlie plant adaptation to cold stress and establish a crucial foundation for identifying and functionally validating cold-tolerant genes. A co-expression network of genes and metabolites under cold stress was constructed through WGCNA, which identified several functional modules. Notably, the pink and black modules showed significant enrichment in metabolic regulation and signal transduction. Hierarchical clustering and specificity analysis of these modules revealed a strong correlation in the expression trends of isoflavonoid metabolites, such as *Biochanin A* and *Glycitin*, across multiple modules. This suggests that the plant dynamically regulates secondary metabolites through a modular expression system in response to cold stress.

KEGG functional enrichment analysis of the pink module revealed significant enrichment in genes related to pathways such as the "phosphatidylinositol signaling system" and "glycolysis/gluconeogenesis." These results suggest that the pink module regulates carbon metabolism reprogramming via signal transduction under cold stress, providing the plant with essential energy and metabolites to adapt to environmental changes[65]. This finding is consistent with cold stress signaling mechanisms observed in T. pratense[32]. For example, the "phosphatidylinositol signaling system" plays a crucial role in regulating membrane stability and signal perception, and the upregulation of related genes indicates that this module facilitates a rapid cold stress response by enhancing signal transduction efficiency[18]. Additionally, the enrichment of the "aminosugar and nucleotide sugar metabolism" pathway suggests that genes in the pink module regulate sugar metabolism balance, promoting cell wall remodeling and metabolite transport under cold stress, thereby enhancing cold tolerance[66].

Within the pink module, the high connectivity of the core gene *evm.TU.ctg2798.293* indicates its pivotal role as a regulator of the module's function. This gene exhibits higher expression levels at both early (F) and late (G) stages of cold stress, with a decrease in expression during the middle (G) stage. Its dynamic expression pattern suggests it plays a central role in the rapid initiation and long-term adaptation to cold stress. This core gene likely influences the expression of other genes by regulating signal transduction pathways, such as the "phosphatidylinositol signaling system," thereby facilitating global adaptation to cold stress through the modulation of metabolic fluxes[65].

In contrast to the pink module, KEGG functional enrichment analysis of the black module revealed significant gene enrichment in pathways such as "plant circadian rhythm," "plant hormone signal transduction," and "flavonoid biosynthesis." Notably, the enrichment of the "plant circadian rhythm" pathway suggests that the black module regulates the timing of gene expression by modulating circadian rhythmrelated genes, allowing the plant to adapt to fluctuations in the cold environment. This temporal regulation mechanism has been validated in studies on *Fagopyrum tataricum* [69], where it is believed to enhance plant adaptability by optimizing the stress response time window. Genes in the black module were also significantly enriched in secondary metabolism-related pathways, such as "glutathione metabolism" and "flavonoid biosynthesis," suggesting that this module improves plant cold tolerance by enhancing antioxidant capacity and accumulating secondary metabolites. For example, the upregulation of glutathione metabolism-related genes likely mitigates oxidative stress by scavenging reactive oxygen species (ROS)[66]. Furthermore, the activation of the flavonoid biosynthesis pathway suggests that the black module enhances cellular protection by accumulating flavonoid antioxidants.

In the black module, the high connectivity of the core genes *evm.TU.ctg3912.106* and *evm.TU.ctg2138.58* indicates their central role in regulating the module's functions. The expression of *evm.TU.ctg3912.106* peaks at the mid-stage (G) of cold stress, suggesting its role in enhancing plant tolerance by activating antioxidant and metabolic regulatory pathways during prolonged stress. Conversely, the expression of *evm.TU.ctg2138.58* peaks at the late stage (H), indicating its importance in long-term adaptation through the dynamic remodeling of metabolic pathways. WGCNA module analysis revealed a strong correlation between the expression trends of isoflavonoid metabolites in the pink and black modules, suggesting that plants fine-tune metabolite regulation through modular control in response to cold stress. Metabolites such as *Biochanin A* and *Glycitin* exhibited

significant correlations across multiple modules, indicating that they regulate antioxidant capacity and secondary metabolic fluxes at distinct stages of cold stress. This finding further supports the concept that plants achieve metabolic remodeling through multi-layered modular regulation to adapt to complex environments[66].

Methods

Seed material and Pre-treatment

In this study, seeds of *Trifolium ambiguum* were used as experimental material, provided by Inner Mongolia Agricultural University (Accession No.: N010). To ensure uniform germination and reproducibility of the experimental results, the following pre-treatments were performed on the seeds: (1) Seed Washing: The seeds were thoroughly washed with distilled water to remove surface contaminants, ensuring no interference from pollutants during the experiment. (2) Seed Disinfection: After washing, the seeds were immersed in a 1% sodium hypochlorite solution for 10 min to eliminate surface microorganisms. Following disinfection, the seeds were rinsed with sterile distilled water at least three times to completely remove any residual disinfectant. (3) Imbibition Treatment: The disinfected seeds were wrapped in moistened filter paper and placed in petri dishes. They were kept moist at 25 °C in the dark for 12 h to promote uniform germination. (4) Germination culture: After imbibition, the seeds were transferred to ½ Hoagland nutrient solution medium and placed in a Percival growth chamber. The culture conditions were set to a constant temperature of 4 °C, with a 16-hour light period (70% light intensity) and an 8-hour dark period for a duration of seven days to allow complete germination. (5) Seedling transplanting: Following germination, the seedlings were transplanted into 10 cm × 10 cm containers filled with a 1:1 mixture of perlite and Danish Pindstrup soil. The seedlings were then cultured under the same light cycle and environmental conditions for an additional seven days to promote further growth and adaptation.

Cold stress treatment

Seven-day-old *T. ambiguum* seedlings were divided into treatment and control groups to investigate the effects of cold stress: (1) Cold Stress Application: Seedlings in the treatment group were transferred to a 4 °C environment for low-temperature exposure. Samples were collected at 2 h, 6 h, and 12 h after treatment. (2) Control group: Seedlings were maintained at 25 °C without low-temperature treatment. Each group was replicated three times biologically. Immediately following treatment, leaf samples were collected, frozen in liquid nitrogen, and stored at − 80 °C for further analysis. Samples were labeled as follows: control group (E), 2 h Cold Stress Treatment (F), 6 h Cold Stress Treatment (G), and 12 h Cold Stress Treatment (H).

The selection of sampling times (2, 6, and 12 h) is based on previous studies of cold stress responses, which used similar time points and considered variations across different stress stages (e.g., early response and adaptation). Preliminary experimental results also confirmed that these time points effectively capture physiological and molecular changes in plants under cold stress, justifying their selection for sampling.

Widely targeted metabolomics analysis and data processing methods

Widely targeted metabolomics analysis was performed using a UPLC-ESI-MS/MS system (UPLC: Waters Acquity I-Class PLUS; MS: Applied Biosystems QTRAP 6500+), employing a Waters HSS-T3 column (1.8 μm, 2.1 mm × 100 mm) with a gradient pro-gram of solvent A (pure water with 0.1% formic acid and 5 mM ammonium acetate) and solvent B (acetonitrile with 0.1% formic acid). The MS parameters included a source temperature of 550 °C, ion spray voltage of 5500 V (positive) and − 4500 V (negative), and optimized DP and CE for individual MRM transitions, with nitrogen as the collision gas. After normalizing the original peak areas, principal component analysis (PCA) and Spearman correlation analysis were used to evaluate sample repeatability and quality control. Identified compounds were annotated using KEGG, HMDB, and Lipidmaps databases. Differential metabolites were screened using fold change ($FC > 1$), T-test p-value (< 0.05), and VIP value (> 1) from OPLS-DA modeling with 200 permutation tests to validate the model, and KEGG pathway enrichment significance was ana-lyzed using a hypergeometric distribution test. This comprehensive workflow ensures the accurate detection, reliable modeling, and robust identification of differential metabolites.

Metabolomic data were standardized using the internal standardization method, normalizing the total peak area across all samples. This method minimized biases be-tween experiments, ensuring consistent and accurate metabolite quantification.

Comprehensive transcriptome sequencing and data analysis workflow

RNA extraction was performed using the RNAprep Pure Plant Kit (Tiangen, Beijing, China) for plants and TRIzol Reagent (Life Technologies, CA, USA) for animals, followed by RNA quantification and integrity assessment using NanoDrop 2000 and the RNA Nano 6000 Assay Kit on the Agilent Bioanalyzer 2100 system. For transcriptome sequencing, 1 μg of RNA per sample was used to prepare sequencing libraries with the Hieff NGS Ultima Dual-mode mRNA Library Prep Kit, involving mRNA purification, cDNA synthesis, end-repair, adaptor ligation, PCR amplification, and quality assessment on the Agilent Bioanalyzer 2100 system. Libraries were sequenced on an Il-lumina NovaSeq platform to generate 150 bp paired-end reads. Raw reads were processed to obtain clean data through quality control steps, including adapter removal and low-quality read filtering, with mapping to the reference genome performed using Hisat2. Gene expression levels were quantified using FPKM, while differential expression analysis was conducted with DESeq2 (for biological replicates) or edgeR (for non-replicates) based on thresholds of adjusted p-value < 0.01 and Fold Change ≥ 2. KEGG pathway enrichment analysis was carried out using KOBAS and clusterProfiler, and alternative splicing events were quantified using rMATS software. This workflow ensured the generation of high-quality transcriptomic data and comprehensive analyses.

After sequencing, low-quality sequences were filtered out to ensure the quality of the aligned reads. Of the filtered sequences, 95% were successfully aligned, while 5% were unaligned.

NO.	Gene ID	Forward primer	Reverse primer
1	*evm.TU.ctg4212.15*	TTTCAATTCACAAATTGGGT	TGGAGCATTCTCAGCTCTTT
2	*evm.TU.ctg1446.8*	ATGAGTTACTATAACCAACA	GCTGCTTGGCTGCTCTTT
3	*evm.TU.ctg4441.71*	TCCATCCACTTGTTTCCAC	GAGGTGAACTCTTCGCCAA
4	*evm.TU.ctg2925.10*	ATGAGTGTAGATCTGAAGAA	TGGTTTATTGTTCTGACGCA
5	*evm.TU.ctg771.45*	ATGTCTTTGACAAATACAATT	GAGTCATTGATGACCATATGG
6	*evm.TU.ctg5805.272*	ATGTTTTGGCATATGATGCAG	CAGGAAGCGCTGCCGCCACC
7	*evm.TU.ctg1446.8*	ATGAGTTACTATAACCAACA	GCTGCTTGGCTGCTCTTT
8	*evm.TU.ctg6782.20*	ATGTCTTTGACAAATACAATT	GACGACTGAATTTTACTTTT
9	*evm.TU.ctg9348.24*	GTGCAAGTCAGCTTCAAAC	TTTGAGGCCAACAGACCAAA
10	*evm.TU.ctg9333.22*	ATGAATATGCATACCGGTCCA	AAGTGGTTGCCTAAAGGGAGTG
11	*evm.TU.ctg11415.40*	ATGTTTTGGCATATGATGCAG	GCAAGGATTCTATATGCAAC
12	*evm.TU.ctg4695.29*	ATGAAAATTCAGTGTGATGTG	TTAACTCGGTTAGTTCTTGAG
13	*evm.TU.ctg997.61*	GCGATATTAATTAAACCAA	CATTGATGGTGGATCTGGA
14	*evm.TU.ctg10694.177*	ATGGGCGATGGAGGTGTCGC	TGCTTCTGGATGGGGAAG
15	*evm.TU.ctg3312.201*	GTTTTCGTGTCAAAATCTCAC	TGTTGGGAGCTATAGTTGACT

Table 2. qRT-PCR primer information.

Combined analysis of transcriptomic and metabolomic data

In the combined analysis of transcriptomic and metabolomic data, differentially expressed genes (DEGs) and differential metabolites (DMs) were identified using statis-tical thresholds ($p<0.05$). DEGs and DMs were mapped to pathways through the KEGG database, with enzyme–gene associations established via EC numbers. Enriched pathways with significant overlaps of DEGs and DMs were identified using hypergeometric tests. Correlations between DEGs and DMs were calculated using Pearson correlation coefficients, retaining interaction pairs with $r>0.90$ ($p<0.01$). A network of these interactions was constructed and visualized using Cytoscape to reveal integrated molecular relationships.

The modules were defined as clusters of genes or metabolites that are co-expressed or correlated, as determined by Weighted Gene Co-expression Network Analysis (WGCNA). These modules represent coordinated patterns of biological processes and are key to understanding stress adaptation mechanisms.

qRT-PCR validation

To validate the results of the differentially expressed genes (DEGs), 15 DEGs were randomly selected for measurement using qRT-PCR. Primers were designed using Primer Premier 5.0 software (Table 2). The qRT-PCR was performed following the instructions provided in the TB Green Premix Ex Taq™ II kit (Takara).

The reaction system included 10 μL of TB Green Premix Ex Taq™ II, 1 μL of forward primer, 1 μL of reverse primer, 1 μL of cDNA, and 7 μL of RNase-Free H_2O, for a total volume of 20 μL. The thermal cycling conditions consisted of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s [70].

The AtActin gene was used as the internal reference to normalize expression levels [71].The relative expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. All gene expression analyses were performed with three biological replicates.

Data availability

The datasets generated for this study can be found in the NCBI sequence reads archive (SRA) database under BioProject No. PRJNA1232370.

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Author contributions

Kefan Cao. was responsible for data collection and analysis, resource provision, drafting the initial ver-sion of the paper, and revising the paper. Sijing Wang. was in charge of experimental methods, resource provision, and drafting the initial version of the paper. Qian Wu. was responsible for data collection and analysis and drafting the initial version of the paper. Fan Huang. was responsible for research design and methods, revising and improving the paper. All authors have agreed to the final version of the manuscript and are willing to take responsibility for the accuracy and authenticity of the entire research work to ensure that any issues related to the accuracy or integrity of any part of the manuscript are appropriately investigated and resolved. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to F.H.

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