RESEARCH ARTICLE Legume Research- An International Journal, Volume 48 Issue 8: 1295-1306 (August 2025)

Metabolic Response of *Trifolium ambiguum* to Low-temperature Stress based on Widely Targeted Metabolomics

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10.18805/LRF-863

ABSTRACT

Background: Low-temperature stress is a major abiotic factor that significantly impacts plant growth and productivity, especially in cold climatic conditions. *Trifolium ambiguum* M. Bieb. (Caucasian clover), a perennial legume known for its cold tolerance and it is an ideal model for studying cold adaptation mechanisms. However, the metabolic pathways that underpin its cold tolerance are not well understood. This study used widely targeted metabolomics to examine the metabolic reprogramming of *T. ambiguum* under low-temperature stress.

Methods: *Trifolium ambiguum* seedlings were grown under controlled conditions and 14-day-old plants were exposed to two treatments: low-temperature stress (4°C) and control conditions (25°C). Samples were collected two hours after treatment. Widely targeted metabolomics profiling was performed to analyze metabolic changes induced by low-temperature stress. Key pathways and metabolites involved in the low-temperature stress response were identified through kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment and metabolic network analysis.

Result: Low-temperature stress induced significant metabolic reprogramming in *Trifolium ambiguum*, with notable alterations observed in fatty acid metabolism. This enhancement improved cell membrane fluidity and stability, thereby strengthening the plant's towards cold tolerance. Furthermore, the significant accumulation of proline and glutamate underscores their critical roles in osmotic regulation and antioxidative defense mechanisms. Additionally, the upregulation of secondary metabolites like (+)-Forbesione and flavonoids plays a key role in scavenging reactive oxygen species (ROS) and regulating stress-related signaling. KEGG pathway enrichment analysis revealed that C5-Branched dibasic acid metabolism and alpha-Linolenic acid metabolism play central roles in cold adaptation. C5-Branched dibasic acid metabolism provided essential carbon skeletons and energy substrates, while alpha-linolenic acid metabolism contributed to maintaining membrane stability and supporting antioxidative responses. The enrichment of the TCA cycle and glycerophospholipid metabolism further ensured energy supply and membrane integrity under stress conditions.

Key words: Fatty acid metabolism, Low-temperature stress, *Trifolium ambiguum*, Widely targeted metabolomics.

INTRODUCTION

Low-temperature stress is a major abiotic factor that negatively impacts plant growth, development and agricultural productivity (Ritonga *et al.,* 2020). It damages the plasma membrane and causes intracellular water loss, disrupting cellular structures and inhibiting key physiological processes such as photosynthesis and respiration (Manasa *et al.,* 2022). These disruptions often lead to metabolic dysregulation and excessive production of reactive oxygen species (ROS), causing oxidative damage and, in severe cases, cell death (Jiang *et al.,*2023; Wang *et al.,* 2023). To adapt low-temperature stress, plants have developed strategies such as osmotic regulation (*e.g.*, accumulation of proline and soluble sugars), activation of antioxidant defenses (*e.g.*, enhanced catalase and superoxide dismutase activity) and expression of cold-induced proteins (*e.g.*, antifreeze proteins and LEA proteins), all of which are crucial for improving cold tolerance (Ding *et al.,* 2019; Ding and Yang, 2022).

In recent years, metabolomics has become a powerful tool for uncovering the metabolic mechanisms underlying plant responses to low-temperature stress (Anandan *et al.,* 2023). This approach enables systematic profiling of dynamic metabolic changes, offering insights into key pathways and potential biomarkers involved in stress adaptation (Yang *et al.,* 2023). Studies have shown that low-temperature stress typically induces significant changes in amino acid metabolism (*e.g.*, proline and glutamate), carbon metabolism (*e.g.*, sugars and organic acids) and secondary metabolite accumulation (*e.g.*, flavonoids) (Tarfeen *et al.,* 2022). For instance, metabolomics analyses in alfalfa (*Medicago sativa*) revealed that low-temperature stress significantly increased proline and soluble sugar levels, which enhance

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How to cite this article: Zheng, L., Cao, K. and Wang, M. (2025). Metabolic Response of *Trifolium ambiguum* to Low-temperature Stress based on Widely Targeted Metabolomics. *Legume Research*. **48(8):** 1295-1306. doi: 10.18805/LRF-863.

Submitted: 06-03-2025 **Accepted:** 12-06-2025 **Online:** 19-08-2025

osmotic regulation and provide carbon skeletons for stress adaptation (Sheng *et al.,* 2022). Similarly, in rice (*Oryza sativa*), low temperatures increased levels of organic acids such as pyruvate, malate and citrate, which support energy supply and metabolic stability (Ritonga *et al.,* 2020). Additionally, flavonoid accumulation, such as quercetin, under low-temperature stress has been confirmed in barrel medic (*Medicago truncatula*), where these compounds scavenge ROS and reduce oxidative damage, improving cold tolerance (Zhang *et al.,* 2022).

As a perennial legume forage crop, *Trifolium ambiguum* (Caucasian clover) has garnered significant attention for its exceptional cold tolerance and ecological adaptability (Abberton *et al.,* 2003; Hay *et al.,* 2010). Its unique morphological traits, such as a well-developed rhizome system and deep roots, enable it to thrive under harsh climatic conditions, making it an ideal model for studying the metabolic basis of cold tolerance (Gierus *et al.,* 2012; Kim *et al.,* 2017). Previous studies have shown that the cold tolerance of *Trifolium ambiguum* is not only governed by its genetic makeup but also closely linked to its complex metabolic networks (Andrzejewska *et al.,* 2016). For example, the development of its rhizome system is associated with the accumulation of carbohydrates, amino acids and polyphenols, which play pivotal roles in cold adaptation (Abberton, 2007; Sölter *et al.,* 2007).

Despite these findings, the metabolic responses of *Trifolium ambiguum* to low-temperature stress remain insufficiently studied, particularly regarding the regulation of its key metabolic pathways. To address this gap, this study systematically analyzed the metabolic changes in *Trifolium ambiguum* under low-temperature stress using widely targeted metabolomics. The findings aim to elucidate key metabolic pathways and identify potential biomarkers related to cold tolerance. This study deepens our understanding of plant cold adaptation mechanisms and offers both theoretical insights and practical guidance for the genetic improvement of cold-tolerant crops and sustainable agriculture in cold regions.

MATERIALS AND METHODS

Experimental materials and seed treatment

In this study, *Trifolium ambiguum* seeds were used as experimental materials, provided by inner mongolia agricultural university, China (registration number: N010). To ensure uniform germination and reproducibility, the seeds underwent the following pre-treatments:

Seed cleaning

Seeds of *T. ambiguum* were thoroughly rinsed with distilled water to remove surface contaminants and ensure sterility for subsequent treatments.

Imbibition treatment

Cleaned seeds were placed in moist filter paper within Petri dishes and incubated in the dark at 25°C for 12 hours. This step ensured uniform hydration and facilitated consistent germination.

Germination culture

After imbibition, the seeds were transferred onto a medium containing 1/2 Hoagland nutrient solution and placed in a Percival growth chamber. Germination was conducted at a constant temperature of 4°C under a 16-hour light (70% light intensity) and 8-hour dark photoperiod for seven days.

Seedling transplantation

After seven days of germination, the seedlings were transplanted into 10 cm × 10 cm cultivation containers. The substrate was a 1:1 mixture of vermiculite and Danish Pindstrup soil. The seedlings were then cultivated under the same light cycle and controlled conditions for an additional seven days to promote further growth.

Metabolites extraction

The sample extracts were analyzed using an UPLC-ESI-MS/MS system (UPLC, Waters Acquity I-Class PLUS; MS, Applied Biosystems QTRAP 6500+). The analytical conditions were as follows, UPLC: column, Waters HSS-T3 (1.8 µm, 2.1 mm * 100 mm); The mobile phase comprised solvent A (ultrapure water containing 0.1% formic acid and 5 mM ammonium acetate) and solvent B (acetonitrile with 0.1% formic acid). Sample measurements were performed with a gradient program that employed the starting conditions of 98% A, 2% B and kept for 1.5 min. Within 5.0 min, a linear gradient to 50% A, 50% B was programmed, within 9.0 min, a linear gradient to 2% A, 98% B was programmed and a composition of 2% A, 98% B was kept for 1 min. Subsequently, a composition of 98% A, 2% B was adjusted within 1 min and kept for 3 min. The flow velocity was set as 0.35 mL per minute; The column oven was set to 50°C; The injection volume was 4 µL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

LC-MS/MS analysis

The ESI source operation parameters were as follows: source temperature 550°C; ion spray voltage (IS) 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II(GSII), curtain gas (CUR) was set at 50, 55 and 35 psi, respectively; the collision-activated dissociation(CAD) were medium. Instrument tuning and mass calibration were performed with 10 and 100 ìmol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to medium. DP (declustering potential) and CE(collision energy) for individual MRM transitions was done with further DP and CE optimization. A specific set of MRM transitions were monitored for each period according to the metabolites eluted within this period.

Data analysis

After normalizing the original peak area information with the total peak area, the follow-up analysis was performed. Principal component analysis and Spearman correlation analysis were used to judge the repeatability of the samples within group and the quality control samples. The identified compounds are searched for classification and pathway information in KEGG, human metabolome database (HMDB) and lipid maps databases. According to the grouping information, calculate and compare the difference multiples, T test was used to calculate the difference significance p-value of each compound. The R language package ropls was used to perform OPLS-DA modeling and 200 permutation tests were performed to verify the reliability of the model. The Variable Importance in Projection (VIP) value of the model was calculated using multiple cross-validation. The method of combining the difference multiple, the P value and the VIP value of the OPLS-DA model was adopted to screen the differential metabolites. The screening criteria are FC>1, P value<0.05 and VIP>1. The difference metabolites of KEGG pathway enrichment significance were calculated using hypergeometric distribution test.

RESULTS AND DISCUSSION

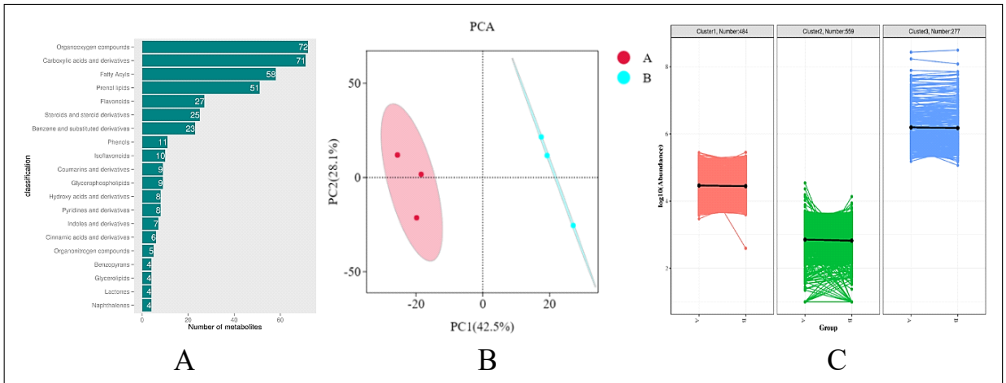
Analysis of the effects of low-temperature stress on metabolites in *trifolium ambiguum*

Widely targeted metabolomics were used to profile the metabolites of *Trifolium ambiguum* under low-temperature stress (treatment group) and normal temperature conditions (control group). In total, 72 organo oxygen compounds and 71 carboxylic acids and derivatives were identified, representing a significant proportion of the metabolites detected. This indicates that these categories play A key roles as metabolic regulators in the plant's response to low-temperature stress. Additionally, 58 fatty acyls and 51 prenol lipids were identified, suggesting that lipid metabolism may play a role in regulating membrane stability under low-temperature stress. Among secondary metabolites, 27 flavonoids and 25 steroids and steroid derivatives were detected, suggesting their potential role in low-temperature stress resistance mechanisms. Furthermore, the presence of 23 benzene derivatives and 11 phenols suggests their antioxidative properties may mitigate oxidative damage during low-temperature stress. A smaller number of 10 isoflavonoids and 9 coumarins and derivatives were also detected, highlighting the diverse roles of secondary metabolites in plant stress adaptation (Fig 1A).

PCA revealed pronounced metabolic differences between the control and treatment groups. The first principal component (PC1), explaining 42.5% of the variance, distinctly separated the metabolic profiles under low-temperature stress, while the second principal component (PC2), accounting for 28.1% of the variance, likely reflected secondary effects. The distinct clustering of the treatment group from the control group further validated the profound impact of low-temperature stress on the plant's metabolic composition (Fig 1B).

K-means clustering analysis further categorized the metabolites into three clusters: Cluster 1 (484 metabolites), cluster 2 (559 metabolites) and cluster 3 (277 metabolites). Among these, cluster 1 and cluster 3 metabolites displayed the most significant differences in abundance between the control and treatment groups. Notably, cluster 3 metabolites exhibited higher abundance in the treatment group, suggesting their specific involvement in the plant's response to low-temperature stress. In contrast, cluster 2 metabolites showed relatively minor changes in abundance, likely representing basal metabolic processes essential for maintaining fundamental cellular functions (Fig 1C).

The identified metabolites, particularly those in lipid and secondary metabolism, reflect the plant's strategic shift towards protecting cellular integrity under low-temperature stress. The higher abundance of fatty acyls and prenol lipids in the treatment group suggests that lipid metabolism plays a key role in maintaining membrane fluidity and stability, which are essential for cellular function during cold stress. This finding aligns with previous studies



A. Metabolite classification statistics; B. Principal component analysis (PCA); C. K-Means clustering analysis.

Fig 1: Effects of low-temperature stress on the metabolome of *Trifolium ambiguum*.

in cold-adapted plants, where lipid composition is dynamically regulated to withstand membrane disruption caused by freezing temperatures.

Additionally, the increased levels of flavonoids, steroids and phenolic compounds under low-temperature stress point to the plant's enhanced antioxidative defense mechanisms. These secondary metabolites likely mitigate oxidative damage by scavenging reactive oxygen species (ROS), which are generated during stress conditions. The detection of benzene derivatives and isoflavonoids further supports this hypothesis, as these compounds are known for their antioxidative properties.

The PCA and clustering analysis further highlight the distinct metabolic re-programming that occurs under low-temperature stress. The separation of the treatment group from the control group underscores the profound impact of cold stress on the plant's overall metabolic profile. The clustering of metabolites into different groups suggests a coordinated shift in metabolic processes, with certain metabolites (cluster 3) being specifically upregulated to respond to the cold stress, while others (cluster 2) maintain baseline functions necessary for survival. This dynamic reorganization of metabolism is essential for optimizing resource allocation and ensuring cellular survival under adverse environmental conditions (Xue *et al.,* 2023).

Partial least squares-discriminant analysis (PLS-DA) of *Trifolium ambiguum* under low-temperature stress

The PLS-DA score plot illustrates the metabolic distinctions between the control group (A) and the treatment group (B). Separation was achieved along the first principal component (t1, 42%) and the second principal component (t2, 25%), highlighting significant differences in metabolic features between the two groups. These results indicate that low-temperature stress has a profound impact on the metabolome of *Trifolium ambiguum*.

From the model parameters, an R^2X value of 0.672 suggests that the model effectively explains the variance within the dataset. The R^2Y value of 0.999 indicates excellent model fitting for group differences, while a Q^2Y value of 0.915 demonstrates strong predictive performance. Additionally, a low RMSEE value of 0.021 further confirms the model's stability and accuracy.

The score plot reveals that the samples from the control group (A1, A2, A3) are tightly clustered, reflecting consistent metabolic characteristics within the group. In contrast, the treatment group (B1, B2, B3) shows a more dispersed distribution, indicating distinct metabolic changes induced by low-temperature stress. This clear separation between the two groups underscores the significant metabolic reprogramming triggered by low-temperature conditions, with group-specific differences in metabolic responses (Fig 2).

The PLS-DA results further support the profound metabolic shifts induced by low-temperature stress in *Trifolium ambiguum*. The clear separation between the control and treatment groups along the first and second principal components (t1 and t2) emphasizes the significant metabolic reprogramming that occurs in response to cold stress. The tight clustering of control group samples indicates a stable, consistent metabolic profile under normal temperature conditions, while the dispersed distribution of treatment group samples suggests a more varied and adaptive metabolic response to cold stress. The strong model fit, demonstrated by the high R^2Y value of 0.999 and the high predictive performance (Q^2Y = 0.915), further validates the robustness of the metabolic differences between the two groups. The low RMSEE value also confirms the accuracy and stability of the model, ensuring that the observed metabolic changes are reliable and not due to model overfitting (Fürtauer *et al.,* 2019).

This metabolic divergence between the control and treatment groups suggests that *Trifolium ambiguum* undergoes a series of adaptive responses to cope with low-temperature stress. The findings align with the hypothesis that cold stress triggers a complex reprogramming of metabolic networks, involving key pathways like lipid metabolism, antioxidative defense and secondary metabolism. Future studies xplore the specific biochemical mechanisms behind these metabolic shifts, as well as the regulatory networks governing the plant's cold stress response.

Analysis of differential metabolites in *Trifolium ambiguum* under low-temperature stress

Metabolomic analysis of the control group (A) and treatment group (B) revealed significant regulatory effects of low-temperature stress on the metabolites of *Trifolium ambiguum*. The volcano plot identified a total of 223 significantly down regulated metabolites (marked in green) and 150 significantly upregulated metabolites (marked in red), while 947 metabolites (marked in gray) showed no

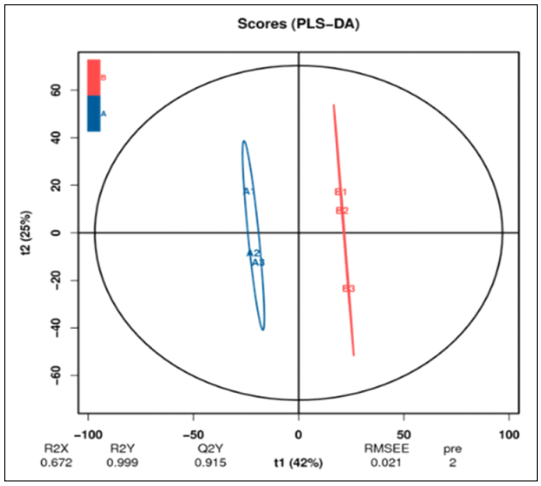


Fig 2: Partial least squares-discriminant analysis (PLS-DA) of metabolites in trifolium ambiguum under low-temperature stress.

significant changes (Fig 3A). This indicates that although most metabolites remained stable under low-temperature stress, the significantly altered metabolites may act as key regulatory factors in the plant's stress response.

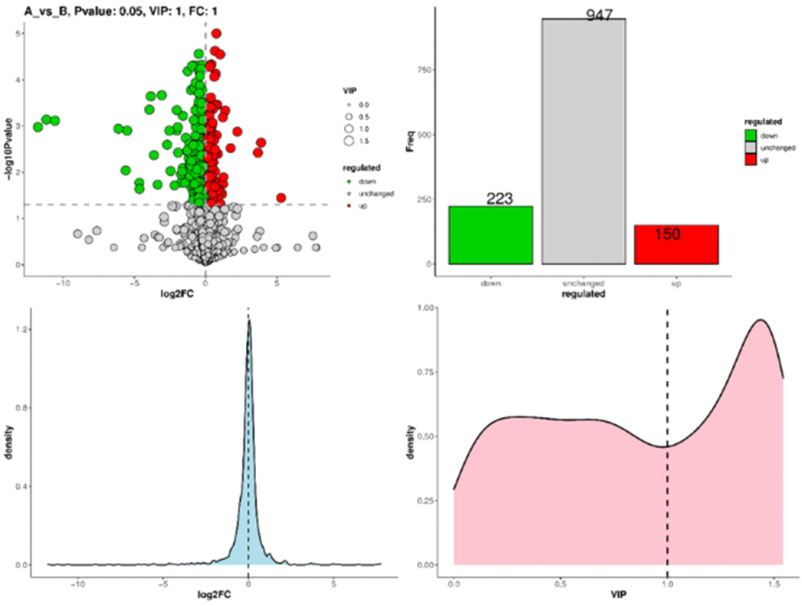
Metabolites with significant upregulation were distributed in the positive log2FC region, indicating their accumulation under low-temperature conditions. Certain secondary metabolites in this category likely play critical roles in stress adaptation by contributing to antioxidative defense or maintaining membrane stability. Conversely, significantly down regulated metabolites were located in the negative log2FC region, suggesting suppression of specific metabolic pathways. For instance, the reduced abundance of metabolites associated with energy metabolism may reflect the plant's reallocation of resources under stress conditions.

The bar plot further quantified the distribution of differential metabolites, showing that the total number of significantly altered metabolites (373, including both up regulated and down regulated) was smaller compared to the number of unchanged metabolites (947) (Fig 3B). This suggests that although low-temperature stress affects the overall metabolic profile, its influence is concentrated on a limited subset of critical metabolites.

The log2FC density plot demonstrated that the majority of metabolites exhibited changes close to a log2FC of 0, with distinct peaks on both the positive and negative sides. This observation underscores the significant regulation of specific metabolites by low-temperature stress (Fig 3C). Additionally, the VIP density plot revealed that a certain proportion of metabolites had VIP values greater than 1, indicating their strong contribution to group differences (Fig 3D). These high-VIP metabolites warrant further investigation to elucidate their functional roles in stress response mechanisms.

This study, using metabolomic analysis, revealed the significant impacts of low-temperature stress on the metabolome of *Trifolium ambiguum*. The results showed that low-temperature stress markedly altered the distribution and abundance of metabolites, particularly those involved in fatty acid metabolism, amino acid metabolism and secondary metabolism. These dynamic changes reflect the metabolic reprogramming processes in plants under stress conditions, highlighting the balance between primary and antioxidative metabolism, which plays a critical role in supporting plant adaptation to low-temperature environments (Song *et al.,* 2023).

The volcano plot and subsequent analysis clearly demonstrate that low-temperature stress induces a selective regulation of metabolites in *Trifolium ambiguum*, with a significant proportion of metabolites either upregulated or downregulated. The upregulation of certain secondary metabolites suggests their critical role in coping with oxidative stress and maintaining membrane integrity, which are essential for plant survival under cold stress. These metabolites likely contribute to the plant's



A. Volcano plot of differential metabolites in *Trifolium ambiguum* under low-temperature stress; B. Histogram of differential metabolites in *Trifolium ambiguum* under low-temperature stress; C. Log2FC density distribution plot of differential metabolites in *Trifolium ambiguum* under low-temperature stress; D. VIP score density distribution plot of key differential metabolites in *Trifolium ambiguum* under low-temperature stress.

Fig 3: Differential metabolites snalysis of *Trifolium ambiguum* under low-temperature stress.

antoxidative defense mechanisms, helping to mitigate the damage caused by reactive oxygen species (ROS) generated during cold stress (Sun *et al.,* 2024).

On the other hand, the downregulation of metabolites related to energy metabolism reflects the plant's strategic reallocation of resources under low-temperature stress. This might indicate a shift towards more energy-efficient processes or the prioritization of stress responses over normal metabolic functions, as plants often adjust their metabolic pathways to conserve energy during periods of environmental stress. The reduced abundance of energy-related metabolites could also suggest a slowdown in growth or cell division, typical of plants under stress conditions. The fact that a majority of metabolites exhibited only small changes (log2FC close to 0) further reinforces the idea that while low-temperature stress triggers significant regulatory shifts in key pathways, the overall metabolic system remains relatively stable, with most metabolites unaffected. The VIP density plot highlights the importance of specific metabolites in distinguishing between the control and treatment groups, providing valuable insights into the most influential metabolites that could serve as biomarkers for cold stress tolerance.

Overall, these findings emphasize the complex metabolic reprogramming that occurs in *Trifolium ambiguum* under low-temperature stress. By selectively regulating key metabolites, the plant appears to enhance its capacity to cope with adverse conditions, maintaining cellular integrity and oxidative balance while reallocating resources for stress adaptation. These results provide a deeper understanding of the molecular mechanisms underlying cold tolerance and offer potential targets for improving stress resilience in plants.

Radar plot analysis of differential metabolites in *Trifolium ambiguum* under low-temperature stress

The radar plot illustrates the top 10 differential metabolites identified by their VIP scores between the control group (A) and treatment group (B). Key metabolites include (+)-forbesione, ziyuglycoside II, wedelolactone, prunetin and N-((-)-Jasmonoyl)-S-Isoleucine. Additionally, Lividic acid, Ledebouriellol, Kaempferide, Arglabin and Angeloylgomisin Q were identified as significantly altered metabolites under low-temperature stress. The radar chart uses radial lengths to represent the relative abundance of these metabolites, providing a clear visual representation of their significant changes under low-temperature stress (Fig 4).

The analysis revealed that (+)-Forbesione and ziyuglycoside II exhibited significantly higher abundance in the treatment group compared to the control group, suggesting their potential roles in the adaptive response to low-temperature stress. In particular, (+)-Forbesione, a secondary metabolite, may play a crucial role under stress conditions through its antioxidative properties or involvement in signal transduction pathways.

Conversely, metabolites such as N-((-)-Jasmonoyl)-S-Isoleucine and Lividic acid showed significantly reduced abundance under low-temperature stress. This downregulation may indicate the suppression of specific metabolic pathways, particularly those associated with plant growth and energy metabolism.

In addition, changes in flavonoids such as Kaempferide and Prunetin highlight the critical role of secondary metabolism in plant adaptation to low-temperature conditions. These compounds likely contribute to enhanced antioxidative defenses or modulation of signaling molecules, thereby improving the plant's tolerance to low-temperature stress.

The radar plot further emphasizes the differential regulation of metabolites under low-temperature stress, showcasing the specific compounds that are most significantly altered. The higher abundance of (+)-Forbesione and Ziyuglycoside II in the treatment group supports their role in stress adaptation. (+)-Forbesione, in particular, stands out due to its potential antioxidative effects, which could help neutralize ROS and protect cells from oxidative damage. This aligns with previous studies that have highlighted the importance of secondary metabolites in maintaining cellular integrity under stress conditions. The reduced levels of metabolites like N-((-)-Jasmonoyl)-S-Isoleucine and Lividic acid further suggest that cold stress may inhibit certain metabolic pathways, particularly those associated with growth and energy production. This could be a mechanism for the plant to conserve energy and prioritize stress resilience over normal metabolic functions. Similarly, the downregulation of jasmonic acid-related metabolites may indicate a shift in hormonal signaling, which is known to be involved in plant stress responses. Flavonoids like Kaempferide and Prunetin, which were altered under cold stress, are known for their roles in antioxidative defense and may also be involved in modulating plant hormone signaling. Their accumulation under low-temperature stress highlights the importance of secondary metabolites in protecting the plant

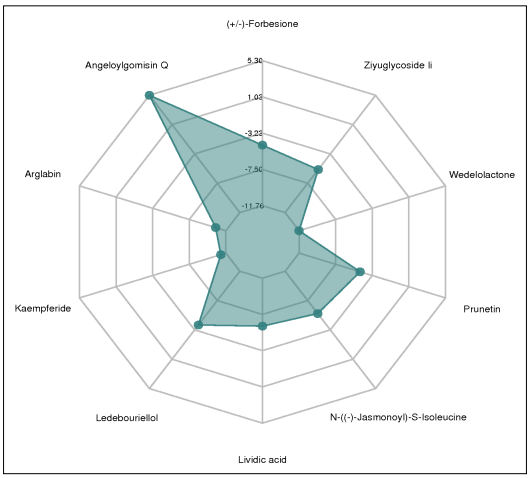


Fig 4: Radar plot of differential metabolite distribution in *Trifolium ambiguum* under low-temperature stress.

from environmental damage and enhancing its overall stress tolerance (Li *et al.,* 2021).

In summary, the differential regulation of these key metabolites under low-temperature stress reflects a complex network of metabolic adaptations that help *Trifolium ambiguum* cope with the challenges of cold environments. These changes underscore the importance of both primary and secondary metabolic pathways in facilitating plant survival and resilience under adverse conditions. Future studies could focus on further elucidating the precise mechanisms by which these metabolites contribute to cold tolerance and their potential applications in improving stress resilience in other crops (Liu *et al.,* 2024).

KEGG enrichment analysis of differential metabolites in *Trifolium ambiguum* under low-temperature stress

KEGG pathway enrichment analysis of the differential metabolites revealed significant impacts of low-temperature stress on the metabolic pathways of *Trifolium ambiguum*. The enrichment results highlighted key pathways with notable changes in rich factor and statistical significance (p-value), providing insights into their functional roles in low-temperature stress adaptation. The distribution of upregulated and downregulated metabolites further emphasized the functional characteristics of these pathways under stress conditions (Qin *et al.,* 2024).

Among the significantly enriched pathways, alpha-Linolenic acid metabolism and C5-Branched dibasic acid metabolism exhibited high enrichment factors and strong statistical significance ($p<0.1$). These fatty acid-related metabolic pathways are likely critical in low-temperature stress responses, possibly through regulating membrane lipid composition and generating signaling molecules. Additionally, the significant enrichment of the Isoflavonoid biosynthesis pathway suggests that isoflavonoids contribute to antioxidative defense and stress-resistance under low-temperature conditions (Fig 5).

Energy metabolism pathways, including the Citrate cycle (TCA cycle), Propanoate metabolism and Glycerophospholipid metabolism, were also significantly enriched. Changes in these pathways indicate the plant's metabolic adjustments to cope with unfavorable environmental conditions. For instance, enhanced TCA cycle activity could sustain energy production, while modifications in glycerophospholipid metabolism might contribute to membrane stability and signal transduction under low temperatures (Zhu *et al.,* 2024).

Amino acid metabolism pathways were prominently enriched as well, including Arginine and proline metabolism, Valine, leucine and isoleucine degradation and D-Amino acid metabolism. The enrichment of proline metabolism, in particular, highlights its importance in osmotic adjustment and antioxidative defense. Meanwhile, the regulation of branched-chain amino acid metabolism may provide energy and carbon skeletons necessary for metabolic adaptation to stress (Jin *et al.,* 2017).

The KEGG pathway enrichment analysis further underscores the significant metabolic reprogramming occurring in *Trifolium ambiguum* under low-temperature stress. The enrichment of fatty acid-related pathways, such as alpha-linolenic acid metabolism, highlights the importance of lipid metabolism in cold stress responses. This pathway's involvement in regulating membrane composition and generating signaling molecules is critical for maintaining cell membrane integrity and fluidity under freezing temperatures, which is essential for sustaining cellular functions. Similarly, the enrichment of isoflavonoid biosynthesis points to the role of secondary metabolites in antioxidative defense, further supporting the idea that these compounds help mitigate oxidative damage induced by cold stress (Jian *et al.,* 2020).

The significant enrichment of energy metabolism pathways, particularly the TCA cycle, suggests that *Trifolium ambiguum* enhances energy production to support its metabolic processes during stress. This is essential for maintaining cellular functions and ensuring that energy reserves are mobilized when external resources are limited. The alterations in glycerophospholipid metabolism also point to the importance of maintaining membrane stability and facilitating cell signaling under low-temperature conditions, further emphasizing the plant's adaptive mechanisms at the molecular level. Amino acid metabolism pathways, particularly proline metabolism, were notably enriched. Proline is well-documented for its role in osmotic adjustment and antioxidative defense under stress and its accumulation likely helps the plant balance cellular osmotic pressure while neutralizing reactive oxygen species. Additionally, the regulation of branched-chain amino acid metabolism, including valine, leucine and isoleucine degradation, suggests that these amino acids are involved in energy production and carbon skeleton supply, which are crucial for sustaining metabolic adaptation under cold stress.

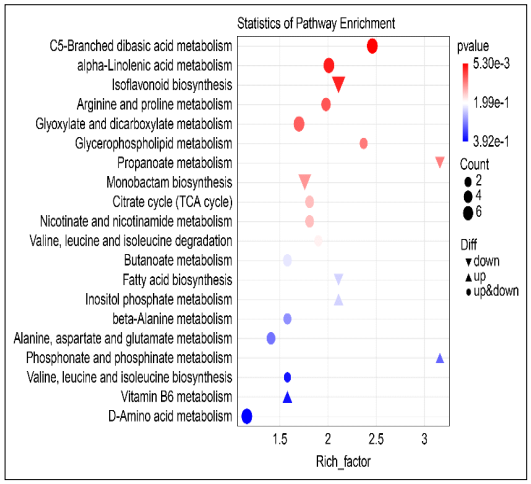


Fig 5: KEGG enrichment plot of differential metabolites in *Trifolium ambiguum* under low-temperature stress.

Chord diagram analysis of differentially enriched metabolic pathways in *Trifolium ambiguum* under low-temperature stress

The chord diagram analysis comprehensively illustrates the effects of low-temperature stress on the metabolic pathways of *Trifolium ambiguum*, with a particular focus on C5-Branched dibasic acid metabolism, alpha-Linolenic acid metabolism and Isoflavonoid biosynthesis.

Among these pathways, C5-Branched dibasic acid metabolism exhibited the highest number of metabolite connections, with key metabolites such as NEG_q55 and NEG_q301 predominantly showing positive log2FC values. This indicates that low-temperature stress may activate this pathway. The involvement of branched dibasic acids in this pathway suggests its role in providing carbon skeletons for energy metabolism and maintaining cellular balance, which are critical for plant adaptation to low-temperature stress (Fig 6).

In the alpha-Linolenic acid metabolism pathway, metabolites such as NEG_t47 and NEG_t349 were significantly upregulated under low-temperature stress. This indicates that fatty acid metabolism plays a pivotal role in the stress response. This pathway is likely associated with key processes such as regulating membrane fluidity, producing antioxidative molecules and generating signaling compounds, highlighting its importance in low-temperature stress adaptation mechanisms.

Isoflavonoid biosynthesis, such as NEG_q51 and NEG_q303, exhibited significantly negative log2FC values, suggesting a certain degree of metabolic reconfiguration in this secondary metabolic pathway. This result may reflect a resource conservation strategy in which the plant reduces the synthesis of secondary metabolites to prioritize essential survival functions under stress conditions (Dong *et al.,* 2022). The reduction in isoflavonoid metabolites may also be related to resource redistribution, as the plant focuses on maintaining core metabolic functions rather than producing non-essential compounds under adverse environmental conditions (Li *et al.,* 2024).

Overall, the increased activity in C5-Branched dibasic acid metabolism and alpha-Linolenic acid metabolism underscores the metabolic adaptability of *Trifolium ambiguum* to low-temperature stress, while the suppression of Isoflavonoid biosynthesis highlights the plant's optimization of resource allocation during cold adaptation.

The chord diagram analysis further deepens our understanding of the metabolic shifts in *Trifolium ambiguum* under low-temperature stress, emphasizing the intricate balance the plant strikes between different metabolic pathways. The activation of C5-Branched dibasic acid metabolism, reflected in the upregulation of metabolites such as NEG_q55 and NEG_q301, highlights the importance of providing energy precursors and maintaining cellular balance during cold stress. This pathway's involvement in energy metabolism and carbon skeleton production plays a vital role in sustaining essential physiological processes when external energy sources may be limited.

In contrast, the upregulation of metabolites in alpha-Linolenic acid metabolism (*e.g.*, NEG_t47 and NEG_t349) further supports the crucial role of lipid metabolism under low-temperature stress. Fatty acids, especially those involved in membrane composition and the production of signaling molecules, are essential for maintaining membrane integrity and promoting stress signaling. The activation of this pathway could be key to the plant's ability to manage oxidative stress and ensure membrane stability, critical for survival in cold environments (Sun *et al.,* 2021). The downregulation of isoflavonoid biosynthesis, as indicated by the negative log2FC values of metabolites such as NEG_q51 and NEG_q303, represents an adaptive shift in the plant's metabolic strategy. By limiting the synthesis of secondary metabolites like isoflavonoids, *Trifolium ambiguum* may be conserving resources and redirecting them toward more immediate survival functions, such as maintaining energy balance and repairing damaged cellular components. This resource allocation strategy is a common response in plants under stress, where energy-intensive processes like secondary metabolite synthesis are often downregulated in favor of essential metabolic activities.

The findings from the chord diagram analysis further underscore the plant's strategic metabolic reprogramming in response to low-temperature stress. By prioritizing the activation of energy-related pathways like C5-Branched dibasic acid and alpha-Linolenic acid metabolism, *Trifolium ambiguum* effectively enhances its ability to

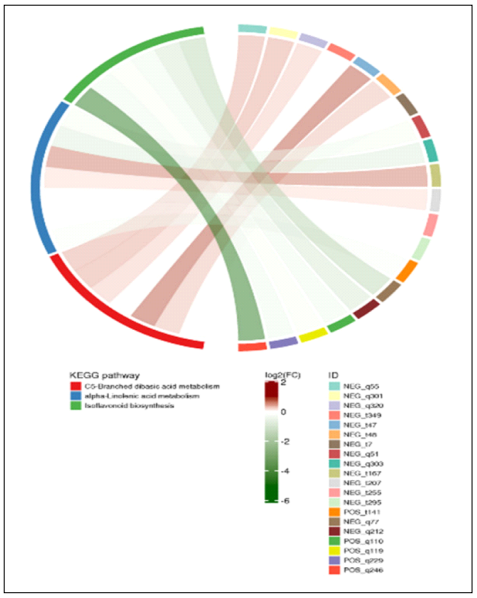


Fig 6: KEGG enrichment chord diagram of differential metabolites in *Trifolium ambiguum* under low-temperature stress.

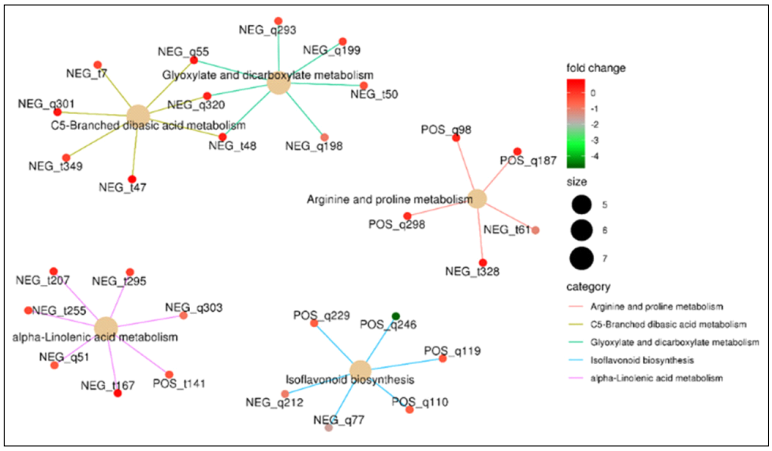


Fig 7: KEGG enrichment network of differential metabolites in *Trifolium ambiguum* under low-temperature stress.

maintain cellular functions crucial for survival under cold conditions. These pathways not only support membrane stability and energy production but also provide important signaling molecules that help the plant cope with oxidative damage and environmental stress (Jin *et al.,* 2017).

The downregulation of Isoflavonoid biosynthesis, on the other hand, suggests a shift in metabolic priorities (Xu *et al.,* 2023). While isoflavonoids typically serve important roles in stress defense, their reduced synthesis under cold stress likely reflects a broader strategy of resource optimization. By limiting energy-expensive processes like secondary metabolite production, the plant allocates its limited resources toward essential survival functions such as energy metabolism, antioxidative defenses and cell repair mechanisms (Zhao *et al.,* 2019).

KEGG enrichment network analysis of differential metabolites in *Trifolium ambiguum* under low-temperature stress

C5-Branched dibasic acid metabolismNEG_q55, NEG_q301 and NEG_t349 showing significant upregulation (marked in red). This suggests that this pathway plays a crucial role in providing carbon skeletons and energy to support plant adaptation to low-temperature environments (Fig 7).

Similarly, alpha-Linolenic acid metabolism exhibited significant upregulation of metabolites, including NEG_t167 and NEG_t295, highlighting the essential role of fatty acid metabolism in regulating membrane fluidity and generating antioxidative signaling molecules. This pathway provides dual support by contributing both structural stability and signal transduction mechanisms under low-temperature stress.

In contrast, some metabolites within the Isoflavonoid biosynthesis pathway, such as POS_q229 and POS_q246, were downregulated (marked in green). This downregulation likely reflects the plant's resource allocation strategy under low-temperature conditions, where the synthesis of non-essential secondary metabolites is reduced to prioritize fundamental metabolic needs (Fu *et al.,* 2023).

The Arginine and proline metabolism pathway showed significant upregulation of metabolites such as POS_q98 and NEG_t328, with proline accumulation playing a pivotal role in enhancing antioxidative capacity and osmotic adjustment, thereby improving plant cold tolerance. Additionally, the Glyoxylate and dicarboxylate metabolism pathway exhibited upregulation of metabolites like NEG_q293 and NEG_q199, which likely contribute to replenishing intermediates of the TCA cycle, supporting energy metabolism and maintaining physiological activity under low-temperature conditions (Xie *et al.,* 2022).

The synergistic interaction between C5-Branched dibasic acid metabolism and alpha-Linolenic acid metabolism is particularly noteworthy. The former provides essential carbon skeletons for the later, while the latter facilitates the synthesis of membrane lipids and antioxidative molecules through fatty acid metabolism. Together, these pathways form a robust adaptive regulatory network that enables *Trifolium ambiguum* to cope effectively with low-temperature stress.

The synergistic relationship between C5-Branched dibasic acid metabolism and alpha-Linolenic acid metabolism highlights the plant's integrated approach to cold stress management (Yang *et al.,* 2023). C5-Branched dibasic acids, by providing carbon skeletons and intermediates, not only fuel essential metabolic processes but also support the biosynthesis of fatty acids (Wu *et al.,* 2024). In turn, the upregulation of alpha-Linolenic acid metabolism ensures the synthesis of membrane lipids and antioxidative molecules, critical for maintaining membrane fluidity and mitigating oxidative stress. Together, these interconnected pathways work in tandem to reinforce the plant's structural integrity and stress signaling capacity under low-temperature conditions (Wang *et al.,* 2021).

Additionally, the upregulation of proline metabolism, along with its role in osmotic adjustment and antioxidative defense, provides a clear indication of the plant's strategy to preserve cellular functions under stress. Proline acts as a compatible solute, protecting cellular structures from

damage caused by dehydration and cold-induced oxidative stress. This, along with the enhanced Glyoxylate and dicarboxylate metabolism, which replenishes TCA cycle intermediates and supports energy production, further emphasizes the plant's focus on maintaining energy balance and metabolic homeostasis during environmental stress.

The downregulation of Isoflavonoid biosynthesis reflects an important adaptive response, where the plant conserves energy by limiting the synthesis of non-essential secondary metabolites. This resource allocation strategy prioritizes critical metabolic processes that ensure the plant's survival under adverse conditions (Zhou *et al.,* 2023). By downregulating energy-intensive pathways like Isoflavonoid biosynthesis, *Trifolium ambiguum* optimizes its metabolism to focus on core functions such as energy production, membrane stability and stress signaling (Lu *et al.,* 2023).

CONCLUSION

This study unveiled extensive metabolic reprogramming in *Trifolium ambiguum* under low-temperature stress, characterized by enhanced primary metabolic pathways and the adaptive regulation of secondary metabolites. Fatty acid and amino acid metabolism played fundamental roles in improving membrane stability, boosting antioxidative capacity and providing energy support, forming the foundation of cold tolerance. Secondary metabolites, such as (+)-Forbesione and flavonoids, offered additional protection through signaling and antioxidative functions.

KEGG enrichment and network analysis further emphasized the pivotal roles of C5-Branched dibasic acid metabolism and alpha-linolenic acid metabolism, which collectively supported cold adaptation through carbon skeleton supply, energy metabolism and signal regulation. The upregulation of the TCA cycle and glycerophospholipid metabolism provided essential energy and structural support, highlighting the complexity of metabolic network regulation under low-temperature stress.

This study revealed the metabolic mechanisms underpinning low-temperature stress adaptation in *Trifolium ambiguum*, providing new insights into plant metabolic regulation under abiotic stress. These findings lay a foundation for the development of cold-tolerant crops and emphasize the potential of metabolic engineering in sustainable agriculture. These findings offer a theoretical foundation and practical guidance for enhancing crop cold tolerance through metabolic regulation. The methods and conclusions of this study can be applied to other plant species, providing a reference framework for future molecular breeding and metabolic engineering efforts.

ACKNOWLEDGEMENT

This work was supported by the national natural science foundation of China (Grant No. 32160334) and the science and technology program of inner mongolia autonomous region (Project No. 2023KJHZ0031), titled "collection and precise identification of germplasm resources of gramineae plants".

Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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