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Cold stress impairs nitrogen uptake and enhances translocation through *AMT1* and *NRT2* gene regulation in tomato

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ABSTRACT

Nitrogen is a vital nutrient for plant growth, playing a crucial role in various physiological processes. Cold stress significantly impacts plant physiology, including nitrogen uptake and translocation. This study investigates the effects of cold stress on nitrogen dynamics in tomato plants by examining the expression of ammonium (*AMT1*) and nitrate (*NRT2*) transporter genes. Under normal conditions, *AMT1* and *NRT2* genes are predominantly expressed in the roots, with varying levels of expression in other tissues. However, following exposure to cold stress, a significant downregulation of most *AMT1* and *NRT2* genes in the roots was observed, indicating a reduced capacity for nitrogen uptake and assimilation. Conversely, there was a notable upregulation of these genes in the leaves, suggesting an enhanced capacity for nitrogen translocation and metabolism under cold conditions. This differential expression between roots and leaves highlights the plant's adaptive mechanisms to cope with environmental stress. It indicates a strategy to conserve energy in the roots while increasing nutrient transport in the leaves to support metabolic adjustments. These insights into the molecular basis of nitrogen management under cold stress can inform strategies to enhance crop resilience and productivity.

1. Introduction

Nitrogen (N) is a crucial macronutrient and an essential component of key cellular molecules, including amino acids, nucleic acids, and chlorophyll (Hawkesford et al. 2012). Plants require nitrogen in larger quantities than any other mineral nutrient for optimal growth. They utilize various sources of nitrogen from the soil, including inorganic forms such as ammonium (NH_4^+) and nitrate (NO_3^-), as well as organic complexes like amino acids (Williams and Miller 2001). Among these, ammonium is often preferred due to its lower energy requirement for assimilation compared to nitrate (Bloom et al. 1992). However, nitrate (NO_3^-) is the predominant form of inorganic nitrogen in soil, therefore its uptake and translocation within plants exert a significant influence on their nitrogen use efficiency (Jin et al. 2015).

Nitrate (NO_3^-) is absorbed through the roots and leaves, and transported within the plant by several nitrate transporters, each with distinct features (Bai et al. 2013; Guan 2017). Nitrate assimilation is energy-intensive, requiring substantial amounts of ATP, reducing equivalents, and carbon (C) skeletons (Nunes-Nesi et al. 2010). It is regulated based on nitrogen availability in the environment and the plant's developmental needs. Environmental factors, such as drought and salinity, also influence the activation and deactivation of these systems (Yao et al. 2008). The first identified eukaryotic *NRT2* gene, *crn*A was isolated from *Aspergillus nidulans*, a filamentous fungus, approximately 35 years ago (Johnstone et al. 1990; Unkles et al. 1991). Based on their sequence homologies with *crn*A, a number of barley (Trueman et al. 1996), tobacco (Quesada et al. 1994), soybean (Amarasinghe et al. 1998) and tomato (Ono et al. 2000) *NRT2* genes have since been identified and functionally characterized.

The nitrogen assimilation process starts with the reduction of nitrate to ammonium, which is then incorporated into amino acids (Masclaux-Daubresse et al. 2010). Specific transporters in the plasma membrane are essential for the uptake of these ions by root cells (Shelden et al. 2001). To avoid toxicity, the uptake and metabolism of ammonium are tightly regulated (Sonoda et al. 2003a). The *AMT1* gene family, the first known ammonium transporter family, comprises high-affinity NH_4^+ transporters in Arabidopsis (Ninnemann et al. 1994). The expression of the *AMT1;1* gene in Arabidopsis roots was found to increase approximately four-fold under nitrogen deprivation (Shelden et al. 2001). It was reported that *OsAMT1;1* is the most consistently expressed *AMT1* gene in both roots and shoots, while *OsAMT1;2* and *OsAMT1;3* are primarily expressed in roots (Ninnemann et al. 1994). The transcription factor OsDOF18 regulates ammonium transport and nitrogen distribution by modulating the *OsAMT1;1*, *OsAMT1;3*, *OsAMT2;1*, and *OsAMT4;1* genes (Wu et al. 2017). In tomatoes, *LmAMT1;1*, *LmAMT1;2*, and *LmAMT1;3* exhibit different expression patterns in leaves and root hairs under nitrogen deficiency, varying CO_2 levels, and different light conditions (von Wiren et al. 2000).

Tomato (*Solanum lycopersicum*, *Sl*) is the second most widely consumed vegetable worldwide, following the potato, and plays a critical role in the food industry (FAO 2022). Due to its extensive cultivation, tomato crops require significant amounts

of nitrogenous fertilizers. However, cold stress can disrupt nitrogen accumulation in tomatoes by impairing their ability to absorb and utilize nitrogen efficiently. This disruption can lead to reduced growth, poor fruit production, and inefficient nitrogen use, ultimately affecting overall plant health and productivity (Bhattacharya 2022; Soualiou et al. 2022). Three *AMT1* and four *NRT2* genes have been recently identified and characterized in the tomato genome, with their expression profiles thoroughly analyzed under conditions of drought and salinity stress (Akbudak et al. 2022; Filiz and Akbudak 2020). The present study aimed to expand on this by examining the expression profiles of these genes under cold stress conditions.

2. Material and Methods

2.1. Plant materials and stress treatment

S. lycopersicum (Istek F1; Istanbul Agriculture Co.) plants were grown in a 3:1 peat-to-perlite mixture at 25°C with 50% humidity under a 16-hour photoperiod in a greenhouse for four weeks. Control plants remained in the greenhouse, while treatment plants were exposed to 4°C in a growth chamber for 24 hours. Following the cold treatment, leaves and roots were harvested for RNA isolation.

2.2. RNA isolation and gene expression analysis

RNA was isolated from leaf and root tissues using the RNA Plant Mini Kit (Qiagen, USA) following the manufacturer's instructions. The RNA samples were then treated with RQ1 RNase-Free DNase (Promega, USA). Gel electrophoresis was used to verify the RNA's integrity and check for DNA contamination. RNA quantities were measured with a Qubit fluorometer (Invitrogen, USA). RT-qPCR was performed on a CFX384 Real Time PCR System (Bio-Rad, USA). Gene expression was quantified using 10 ng of RNA per sample with the Luna Universal One-Step RT-qPCR Kit (NEB, USA). Forward and reverse primers (Table 1) were utilized for the RT-qPCR analysis (Akbudak et al. 2022; Filiz and Akbudak 2020). The *actin isoform B* (*Actin*) gene served as an endogenous reference control (Goupil et al. 2009).

2.3. Chromosomal distribution

The locations of the *SlAMT1* and *SlNRT2* genes on each chromosome were obtained from the tomato genome database (Ensembl Plants), and their chromosomal distribution was illustrated using the Mapgene2chrom 2.1 (MG2C v2.1) online tool (http://mg2c.iask.in/mg2c_v2.1/) (Chao et al. 2015; Chao et al. 2021).

2.4. Digital expression pattern

Data from the Tomato Genome Consortium (2012) was obtained to analyze the expression patterns of *SlAMT1* and *SlNRT2* genes. The expression profiles of these genes were examined across different anatomical parts and developmental stages. The heatmap was drawn using the Heatmap program in TBtools software.

3. Results

3.1. Chromosomal distribution of SlAMT1 and SlNRT2 genes

In *S. lycopersicum*, the ammonium transporter 1 (*AMT1*) gene family consists of three members (Filiz and Akbudak 2020), while the nitrate transporter 2 (*NRT2*) gene family has four members (Akbudak et al. 2022). Fig. 1 shows their chromosomal distribution, revealing that the *SlAMT1* and *SlNRT2* genes are spread across six different chromosomes. Notably, aside from *SlNRT2.2* and *SlNRT2.3*, none of the genes are located on the same chromosome.

Table 1. Primers used in the RT-qPCR analysis of *SlAMT1* and *SlNRT2* genes

Gene	Phytozome ID	Forward Primer (5′➔3′)	Reverse Primer (5′➔3′)
AMT1.1	Solyc09g090730	TCGCTAAAGGGGAGTTTGTG	GATTATATGCGCCCCGAGTA
AMT1.2	Solyc04g050440	CAGCAATCACGTCAGGTTGT	AGCTGCCAATGCGTTAAATC
AMT1.3	Solyc03g045070	CCTGTTGTTGCTCATTGGCT	ATCCCACCAACCAAATGCAC
SlNRT2.1	Solyc02g067790	TGGGCTTGCTAATGGATTCG	ATCGCTGGGAAAATGAACGC
SlNRT2.2	Solyc06g010250	GGCAGAGCAGAAACACTTCC	TCAATTGCGTTAAACCACCA
SlNRT2.3	Solyc06g074990	TAGTGAACGGAACGGCTGCT	CACGATTTCCGTCGGGTAAA
SlNRT2.4	Solyc11g069750	TTTTGCTGCTGCCCCTTTAG	TACTACCGGAAACTGAGGCAAC
SlActin	Solyc03g078400	GGGATGGAGAAGTTTGGTGGTGG	CTTCGACCAAGGGATGGTGTAGC

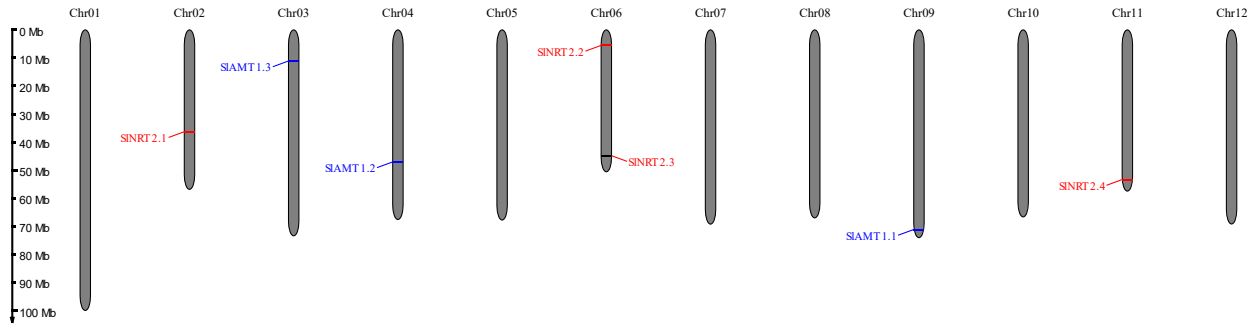

Figure 1. Chromosomal positions of *AMT1* and *NRT2* genes in the tomato genome generated in the MG2C tool. The chromosome number is shown at the top of each chromosome. The genome scale in megabases (Mb) is given on the left.

3.2. Digital expression profile of SlAMT1 and SlNRT2 genes

The expression data of *SlAMT1* and *SlNRT2* genes were retrieved using the RNASeq Expression Browser. A heat map of the gene FPKM values was then constructed using TBtools software. The expression data indicates that all *SlAMT1* and *SlNRT2* genes are predominantly expressed in roots under regular conditions, except for *SlAMT1-3* and *SlNRT2-1* (Fig. 2). Among the *SlAMT1* and *SlNRT2* genes, *SlNRT2-3* exhibits the highest expression, followed by *SlAMT1-1*. In most tissues other than roots, the expression of *SlAMT1* and *SlNRT2* genes is either barely detectable or absent. However, *AMT1-3* is predominantly and robustly expressed in leaf tissues. *SlNRT2-1* shows relatively consistent low expression across tissues and stages, with the highest expression observed in buds. *SlNRT2-2* has a higher expression in buds and roots. *NRT2-3* stands out with extremely high expression in roots. *SlNRT2-4* generally displays very low or zero expression across most conditions, suggesting minimal activity.

For the *SlAMT1* genes, *SlAMT1-1* exhibits high expression in flowers and roots, indicating significant involvement in these tissues. *SlAMT1-2* shows higher expression in buds and roots. *SlAMT1-3* shows significantly high expression in leaves compared to other tissues, indicating a potential key role in leaves. Fig. 2 highlights that, except for *SlNRT2-3* in roots, *SlNRT2* genes are generally less active across all organs and developmental stages compared to *SlAMT1* genes in the tomato genome. The figure also highlights the specific tissues in which certain genes are highly or minimally expressed, with *SlNRT2-3* having dominant expression in roots, *SlAMT1-1* showing notable expression in flowers and roots, and *SlAMT1-3* having high expression in leaves. *SlNRT2-4* generally exhibits low expression.

3.3. Expression profiles of AMT1 and NRT2 genes in tomato under cold stress

Under cold stress, the expression patterns of *AMT1* and *NRT2* genes in tomato root and leaf tissues show significant variations, highlighting their unique physiological responses to the cold stress (Fig. 3). In root tissues, the expression of *SlAMT1.1* shows a modest upregulation with a fold difference of 0.61. In contrast, the other SlAMT family genes, specifically *SlAMT1.2* and *SlAMT1.3*, are downregulated with fold differences of -0.45 and -2.39, respectively. This pattern suggests that cold stress may selectively inhibit certain ammonium transporters in roots. The SlNRT family genes also exhibit pronounced downregulation, with *SlNRT2.1* showing the most significant decrease at -3.92-fold difference. *SlNRT2.2*, *SlNRT2.3*, and *SlNRT2.4* follow this trend with fold differences of -0.93, -0.50, and -0.28, respectively. This widespread downregulation in roots indicates a potential reduction in nitrate transport and assimilation capacity under cold stress.

Conversely, in leaf tissues, the response to cold stress differs. *SlAMT1.1* shows slight upregulation with a fold difference of 0.98, and *SlAMT1.2* is significantly upregulated with a 4.02-fold difference, suggesting an increased demand for ammonium

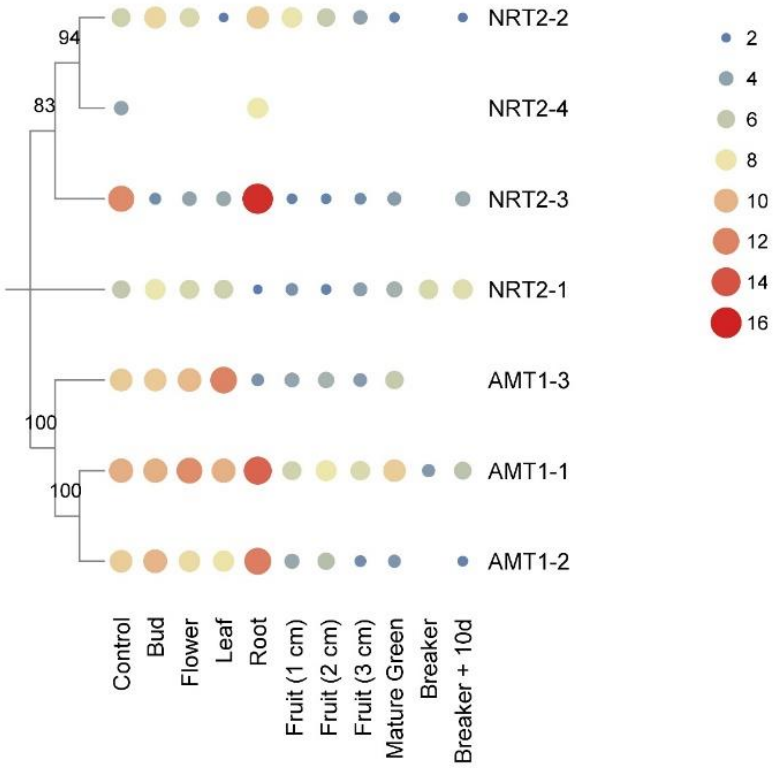

Figure 2. Heatmap of the expression profiles of *AMT1* and *NRT2* genes across various organs and developmental stages in tomato. Hierarchical clustering was employed to generate the heatmap, which was visualized using TBtools software. Expression values were log2-transformed and normalized. In the heatmap, blue elements indicate low relative expression levels, while red elements indicate high relative expression levels.

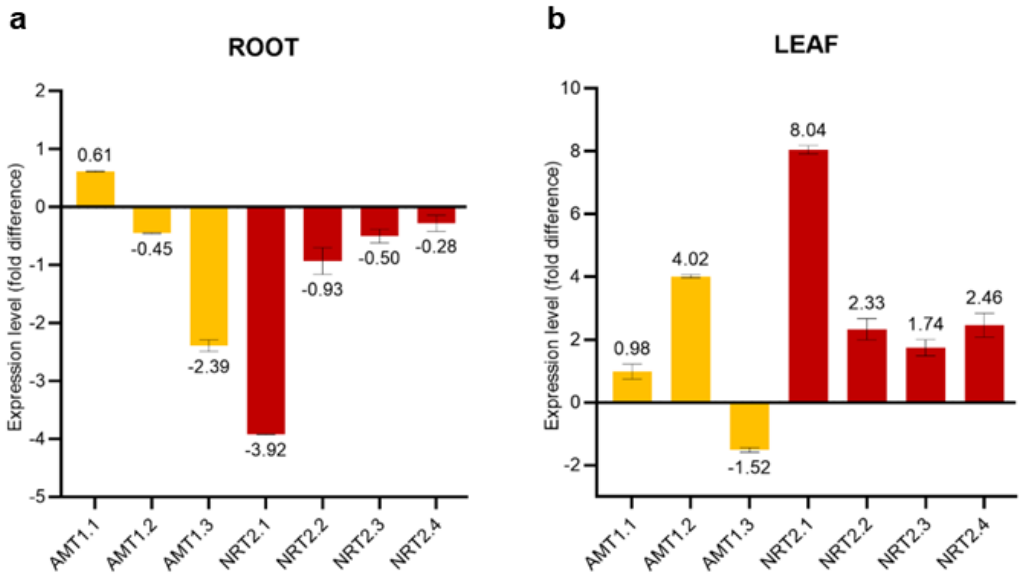

Figure 3. Expression profiling of *AMT1* (a) and *NRT2* (b) genes in tomato leaves and roots subjected to cold treatment, measured by RT-qPCR. Bars above the x-axis indicate upregulation, while bars below the x-axis represent downregulation. Gene expression values are shown on a log2 scale to effectively display the magnitude of both upregulated and downregulated genes. Error bars represent the standard deviation of the mean (SDOM; n= 3).

transport in leaves under cold conditions. *SlAMT1.3*, however, is downregulated with a fold difference of -1.52. The SlNRT family genes in leaves exhibit strong upregulation under cold stress, with *SlNRT2.1* showing a dramatic increase at 8.04-fold difference. *SlNRT2.2*, *SlNRT2.3*, and *SlNRT2.4* are also upregulated, with fold differences of 2.33, 1.74, and 2.46, respectively. This upregulation suggests an enhanced capacity for nitrate transport in leaves, likely to support metabolic adjustments and stress responses under cold conditions.

4. Discussion

The ability of plants to adapt to abiotic stresses, such as cold, is crucial for their survival and productivity. Among the various genes involved in stress responses, AMT1 and NRT2 are essential for nutrient uptake and assimilation (Goel and Singh 2015). Investigating their expression profiles under cold stress can shed light on the adaptive mechanisms of plants and potentially guide agricultural practices to enhance crop resilience.

Several plant species have been found to contain multiple ammonium transporters (AMTs) and nitrate transporters (NRTs). Specifically, Arabidopsis has six AMTs (Gazzarrini et al. 1999), rice has 10 (Sonoda et al. 2003b), soybean has 16 (Kobae et al. 2010), and poplar also has 16 (Wu et al. 2015). In the genome of *Saccharum spontaneum*, researchers have identified 178 *NRT1*, 20 *NRT2*, and six *NRT3* genes distributed across all eight chromosomes (Wang et al. 2019). Furthermore, in wild soybean (*Glycine soja*), 120 *NRT1* and five *NRT2* genes have been discovered (You et al. 2020). In the potato genome, there are 33 *NRT1*, four *NRT2*, and two *NRT3* genes, which show a closer similarity to Arabidopsis *NRT* genes than to those of rice (Zhang et al. 2021). These genes, along with their protein motifs, are conserved in both genomic and peptide sequences, playing essential roles in plant growth, development, and stress adaptation.

AMT gene expression is tightly regulated by the plant's nitrogen status. Under nitrogen-deficient conditions, *AMT* genes are upregulated to boost ammonium uptake (Loqué et al. 2006). Conversely, the availability of ammonium or nitrate can differentially influence *AMT* gene expression, with some genes responding more strongly to ammonium than to nitrate (Dechorgnat et al. 2019). This regulation is crucial for maintaining nitrogen homeostasis and supporting plant growth across varying nutrient conditions.

AMTs are integral to ammonium uptake, translocation, and overall nitrogen management in plants. Beyond their primary role in ammonium uptake, AMTs also play a role in plant responses to abiotic stresses. For instance, overexpression of certain AMTs in Arabidopsis has been shown to enhance root growth under salt stress, indicating that these transporters may mitigate ammonium toxicity under stress (Yi et al. 2020). Similarly, *AMT* genes are upregulated in response to drought stress in species like *Populus simonii* and *Malus prunifolia*, suggesting their involvement in improving nitrogen uptake and metabolism under adverse conditions (Huang et al. 2018). In rice, a key staple crop cultivated in flooded conditions, there are at least 12 *AMT* genes categorized into four subfamilies: *OsAMT1*, *OsAMT2*, *OsAMT3*, and *OsAMT4* (Al-Tawaha et al. 2020). *OsAMT1* subfamily members primarily function as high-affinity transporters, while the other subfamilies mostly consist of low-affinity transporters. Research indicates that knockout of certain *OsAMT1* genes significantly reduces ammonium uptake in rice, highlighting their critical role in nitrogen acquisition (Li et al. 2016). These genes are expressed in various tissues, including the root stele, vascular bundles, and mesophyll cells, and are involved in translocating ammonium from roots to shoots.

Ammonium is considered a superior nitrogen source for plants because its absorption and utilization require less energy compared to nitrate (Li et al. 2013). Filiz and Akbudak (2020) found that all *SlAMT1* genes were mainly downregulated (up to 6-fold) in leaf and root tissues under drought and salt stresses. In this study, under cold stress, the expression of all *NRT* genes, as well as *SlAMT1.2* and *SlAMT1.3*, was downregulated, while *SlAMT1.1* was the only gene upregulated, showing a 0.61-fold increase. This result aligns with literature indicating that under

cold stress, tomato plants reduce nitrogen accumulation, especially in nitrate form, to conserve energy.

Understanding AMTs' roles and regulation in different crops, particularly under stress conditions, can offer valuable insights for improving crop productivity and stress resilience. Comprehensive characterization of *AMT* genes in crops presents potential strategies for enhancing nitrogen use efficiency and developing varieties with better tolerance to nutrient deficiencies and environmental stresses. Abiotic stresses such as salinity and drought significantly alter plant transcriptomes. Previous research has demonstrated that *SlNRT* genes exhibit gene- and tissue-specific responses under salt and drought conditions (Pu et al. 2023). However, these genes follow similar expression patterns in response to cold stress. Although their expression levels vary, all *SlNRT* genes are consistently downregulated in roots and upregulated in leaves. Diverse expression patterns of *NRT2* genes have also been observed in other plants such as potato, cassava, rapeseed, wild sugarcane, and apple, often showing tissue-specific and stress-responsive regulation. Pu et al. (2023) analyzed the expression profile of seven cotton *NRT2* genes under salt, drought, cold, and heat stresses and found no significant differences in the regulation of five genes, while two were downregulated compared to the control.

Overall, the expression results from this study reveal a clear divergence in gene regulation between roots and leaves under cold stress. Roots generally downregulate both ammonium and nitrate transport genes, possibly to conserve energy and resources under adverse conditions. In contrast, leaves upregulate these genes, likely to enhance nutrient transport and support cold stress mitigation processes. This differential expression underscores the adaptive strategies of plants, where distinct tissues modulate their genetic responses to optimize survival and function under environmental stress.

5. Conclusion

The analysis of gene expression under cold stress in tomato root and leaf tissues reveals several key insights into how plants adapt to environmental challenges. There is a clear difference in how genes are regulated in roots compared to leaves under cold stress. Roots tend to downregulate the expression of most ammonium and nitrate transporter genes, while leaves show an upregulation of these genes. This suggests that different parts of the plant prioritize different strategies to cope with cold stress.

The downregulation of both *SlAMT* and *SlNRT* genes in roots implies a strategy focused on conserving energy. Since nutrient uptake and transport require significant metabolic energy, reducing the expression of these transporters could help the plant minimize energy expenditure in roots during stressful conditions. This energy conservation could be critical for maintaining root viability when overall metabolic activity is compromised due to cold stress. On the other hand, the upregulation of *SlAMT* and *SlNRT* genes in leaves indicates a response aimed at enhancing nutrient transport. Leaves, being the site of photosynthesis and other metabolic activities, may require increased nutrient uptake to sustain these processes and to support the synthesis of stress-related proteins and metabolites. By boosting the expression of these transporters, leaves can maintain their metabolic functions and potentially improve cold tolerance.

The contrasting expression patterns underscore the importance of tissue-specific responses to environmental stress. While roots focus on reducing metabolic load, leaves ramp up their nutrient transport capabilities. This division of labor highlights the complex and coordinated nature of plant responses to stress, ensuring that different tissues contribute optimally to the overall survival strategy. The differential expression of genes also points to the adaptive significance of such regulatory mechanisms. By selectively modulating gene expression, plants can fine-tune their physiological responses to meet the demands of different tissues under stress. This ability to differentially regulate gene activity is crucial for plants to thrive in varying environmental conditions.

In summary, the research suggests that under cold stress, plants employ a nuanced approach where roots conserve energy by downregulating nutrient transporters, while leaves enhance their capacity to transport nutrients, thereby supporting essential metabolic activities. This adaptive strategy reflects the plant's need to balance resource allocation and metabolic demands across different tissues to optimize survival and function under adverse conditions.

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Authors' Contribution

DC: Investigation and Data curation, MAA: Conceptualization, Writing- Original Draft, Writing- Review & Editing, Supervision.

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