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Chen Xi

Guizhou Academy of Agricultural Sciences

Chen Caijun

Guizhou Academy of Agricultural Sciences

Wang Xiaoli

wangxiaolizhenyuan@126.com

Guizhou Academy of Agricultural Sciences

Zhao Degang

Guizhou Academy of Agricultural Sciences

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Effects of Low Nitrogen Stress on the Cell Structure and Chloroplast Proteome of *Festuca arundinacea*

Chen Xi¹, Chen Caijun², Wang Xiaoli^{2,*} and Zhao Degang^{1,*}

¹Ministry of Agriculture and Rural Affairs Key Laboratory of Crop Genetic Resources and Germplasm Innovation in Karst Region, Guizhou Institute of Crop Germplasm Resources, Guizhou Academy of Agricultural Sciences, Guiyang 550006, China; 15761626900@163.com

²Guizhou Institute of pratacultural, Guizhou Academy of Agricultural Sciences, Guiyang 550006, China; 15761626900@163.com

*Correspondence: dgzhao@gzu.edu.cn and wangxiaolizhenyuan@126.com; Tel.: 13885012693, 13595092896

Abstract

Nitrogen is an essential nutrient for plant growth, development, and metabolism, and its deficiency can severely impact physiological functions and overall growth. This study aimed to investigate the effects of low nitrogen on the cell structure and chloroplast protein levels of tall fescue (*Festuca arundinacea*). The seedlings were subjected to low nitrogen stress, and the ultrastructures of their subcellular components were observed via transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The differential expression of chloroplast proteins under low-nitrogen stress was analyzed via quantitative proteomics via iTRAQ technology. The results revealed that the leaf cells presented pronounced protrusions and irregular surfaces, whereas normal mesophyll cell structures and intact chloroplasts were observed in the full-nitrogen group (nonstressed plants) of *Festuca arundinacea*. In contrast, leaf cells under low-nitrogen conditions appeared smoother and lacked protrusions, with mesophyll cells displaying necrosis or structural abnormalities and some membrane structures appearing blurred. Additionally, chloroplasts presented structural abnormalities, including the formation of oversized starch granules. Photosynthesis was inhibited under low-nitrogen conditions. A total of 441 differentially expressed chloroplast proteins were identified, with 177 upregulated and 264 downregulated. Notably, the expression of photosystem II core proteins and metabolic pathway synthases was downregulated, whereas the expression of certain antioxidant proteins was upregulated. These results indicate that low nitrogen stress disrupts the dynamic balance of the chloroplast proteome and inhibits photosynthetic efficiency. This study provides a theoretical foundation for optimizing nitrogen fertilization and improving stress resistance in turfgrass species.

Keywords: *Festuca arundinacea*, Low nitrogen stress, Chloroplast proteomics, Cell ultrastructure

1. Introduction

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be carefully reviewed, and key publications should be cited. Please highlight controversial and diverging hypotheses when necessary. Finally, the main aim of the work is briefly mentioned, and the principal conclusions are highlighted. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets—e.g., [1] or [2,3], or [4–6]. See the end of the document for further details on the references.

Nitrogen is an essential element for plant growth and development; it forms the structural components of plants, participates in the catalysis of physiological metabolic processes, and plays a crucial role in chlorophyll synthesis. Variations in external nitrogen levels directly affect the nitrogen content of leaves, thereby influencing their photosynthetic efficiency (John et al., 1983). Nitrogen deficiency in plants is often characterized by slow growth, yellowing of leaves, reduced tillering, and decreased photosynthetic rates (Nunes-Nesi et al., 2010). It is estimated that nitrogen fertilizer production accounts for 1–2% of global energy consumption; however, crops utilize only 30–50% of the nitrogen input (Smith et al., 2002). While nitrogen fertilization significantly enhances crop yield, excessive application of nitrogen fertilizer in agricultural practices reduces nitrogen efficiency, increases production costs, and contributes to environmental pollution (Ahmed et al., 2017). Therefore, it is crucial to reduce nitrogen fertilizer usage, improve nitrogen use efficiency, and study plant adaptation mechanisms under low nitrogen conditions to protect the ecological environment.

Currently, most research on nitrogen stress has focused on the entire proteome, with studies conducted on crops such as rice (Zhao et al., 2012), maize (Wang et al., 2022), wheat (Yan et al., 2021), and tobacco (Liu et al., 2020). Zhang et al. (2019) examined the wheat transcriptome under nitrogen stress and reported significant alterations in key transcription factor families, including

WRKY, MYB, and NAC. These families are crucial in regulating gene transcription and offer insights into how plants adapt to low-nitrogen conditions. Liu et al. (2020) explored the impact of nitrogen stress on the photosynthetic capacity and nitrogen metabolism enzyme activities of plants and reported a decrease in these parameters under stress. However, they discovered that low-nitrogen-tolerant varieties presented greater leaf and root activity than low-nitrogen-sensitive varieties did, emphasizing the genetic basis for nitrogen stress tolerance. Furthermore, Liao et al. (2024) focused on selecting low-nitrogen-tolerant germplasms in Chinese pumpkin subjected to low-nitrogen treatment. This research has contributed to the identification of valuable germplasms with enhanced nitrogen tolerance, which could serve as a resource for breeding programs aimed at improving nitrogen use efficiency in crops. Collectively, these studies emphasize the complex genetic and physiological responses of plants to nitrogen stress and highlight the potential for selecting and breeding crops with improved tolerance to low-nitrogen conditions. The biomass and nitrogen content of low-nitrogen-tolerant millet are increased, resulting in improved nitrogen use efficiency (Chen et al., 2023). The impact of low nitrogen on plants is characterized primarily by inhibited growth, leaf chlorosis, and reduced photosynthetic efficiency, all of which are closely associated with alterations in plant cell structure. Chloroplasts are the core organelles responsible for photosynthesis. Low nitrogen stress not only compromises the structural integrity of chloroplasts but also induces significant changes in the composition of photosynthesis-related proteins within chloroplasts (Wang et al., 2014), which in turn affects the functionality of photosystem I and photosystem II, thereby impairing the energy capture ability and carbon metabolism capacity of plants. Furthermore, low nitrogen stress may activate a plant's antioxidant enzyme system, thereby helping mitigate damage caused by oxidative stress (Foyer and Noctor, 2011).

Festuca arundinacea (tall fescue) is a perennial herbaceous plant in the grass family that is commonly utilized as a cool-season forage grass or turfgrass. It is widely employed in landscaping and ecological restoration in China. However, its cultivation and seed production are strongly dependent on nitrogen fertilizers, which contributes to increasing environmental pollution due to excessive nitrogen application. Therefore, it is essential to develop new low-nitrogen-tolerant germplasms of tall fescue and elucidate the molecular mechanisms underlying its adaptation to low-nitrogen stress. Nonetheless, research on the changes in the chloroplast proteome of *Festuca arundinacea* under low nitrogen stress and its adaptation mechanisms remains limited. This study investigated the effects of low-nitrogen stress on the cell structure and chloroplast proteome of *Festuca arundinacea*. By employing quantitative proteomics (iTRAQ) technology in conjunction with organelle ultrastructure analysis, this study examined the impact of low-nitrogen stress on the cell and chloroplast proteomes of *Festuca arundinacea*, identifying differentially expressed proteins in the chloroplast proteome as well as stress-specific proteins associated with the nitrogen stress response. This research provides a reference for future studies aimed at enhancing nitrogen use efficiency in forage grasses.

2. Results

2.1. Scanning electron microscopy (SEM) image of the cell structure of *Festuca arundinacea* under nitrogen stress

Scanning electron microscopy (SEM) images revealed that in the full-nitrogen group (nonstressed), the cell structure of *Festuca arundinacea* displayed prominent protrusions and an irregular surface. In contrast, under low-nitrogen treatment, the cell structure appeared smoother and flatter, lacking protrusions. Additionally, there were no significant changes in the stomata of tall fescue under nitrogen stress (Figure 1).

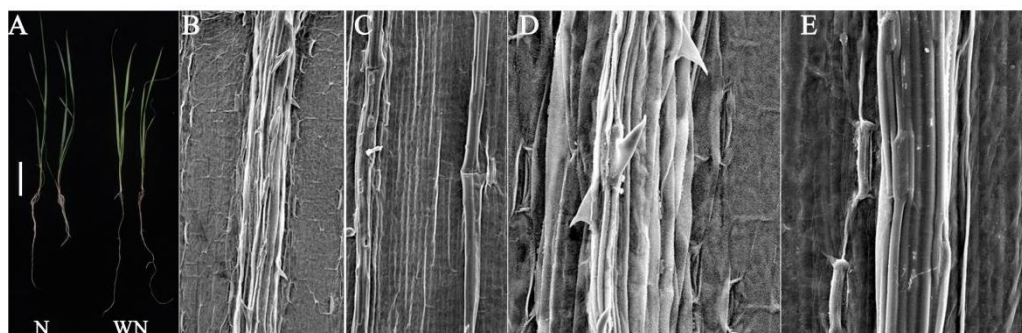


Figure 1. The chloroplast cell structure of *Festuca arundinacea* leaves under low-nitrogen conditions. (A) Low nitrogen stress and total nitrogen treatment of tall fescue seedlings. (B) The cell morphology of the total nitrogen group was 10 times greater. (C) The cell morphology was 10 times greater under low nitrogen stress. (D) The cell morphology of the total nitrogen group was 40 times. (E) The cell morphology under low nitrogen stress was 40 times greater.

2.2. Transmission electron microscopy (TEM) of *Festuca arundinacea* under nitrogen stress

In the full-nitrogen group (nonstressed), the mesophyll cell structure of tall fescue plants appeared normal. The chloroplasts were elongated and oval in shape, exhibiting intact structures and full morphology. The granal thylakoid layers were clearly visible, and the surfaces were appropriately adorned with starch grains and osmiophilic granules. The thylakoid stacks were neatly arranged, forming grana. Conversely, in the low-nitrogen group, the mesophyll cells of tall fescue plants exhibited necrosis and abnormal morphology. Some membrane structures were blurred, the cytoplasm was lost, the cell membranes were discontinuous, and the grana lamellae were disrupted or even disintegrated. The chloroplasts displayed an abnormal morphology characterized by twisted structures, blurry stroma lamellae, increased numbers of osmiophilic granules, swollen thylakoid membranes, and the formation of numerous giant starch granules. The thylakoid membranes were loosely organized (Figure 2).

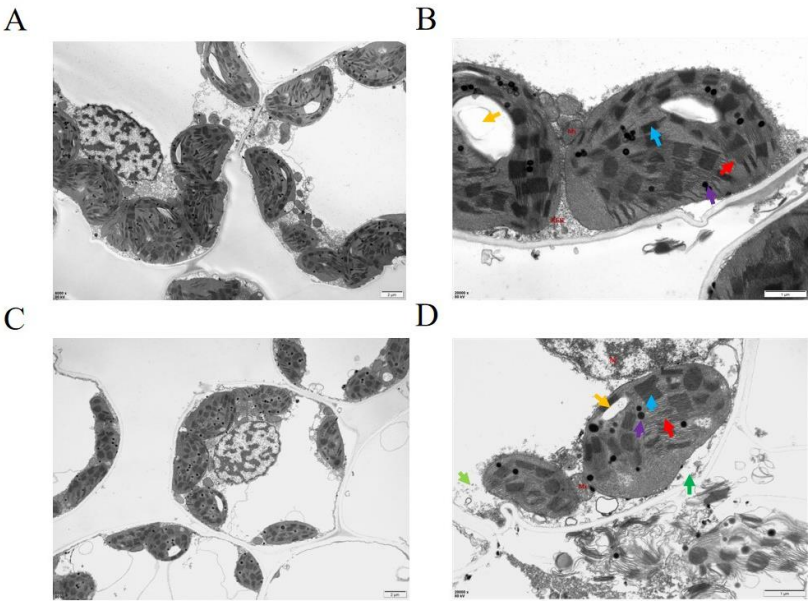


Figure 2. The chloroplast ulTrasTStructure of *Festuca arundinacea* leaves under low-nitrogen conditions.

(A) The mesophyll cells of the total nitrogen groups (×10000). (B) The mesophyll cells of total nitrogen groups (×20000). (C) The mesophyll cells under low nitrogen stress (×10000). (D) The mesophyll cells under low nitrogen stress (×20000).

mitochondria (Mi), Coarse endoplasmic reticulum(RER), Osmium granules(↑), lamellar(↑), stacking (↑), discontinuous cell membrane (↑), starch granules (↑), loss of cytoplasmic contents (↑).

2.3 Chloroplast proteome data of *Festuca arundinacea*

The proteomic changes in the leaves of *Festuca arundinacea* under low-nitrogen stress were analyzed via iTRAQ technology. Protein expression was considered significantly altered if the adjusted P value (adj. P value) was ≤ 0.05, and the fold change was either ≥ 2 (indicating upregulated expression) or ≤ 0.50 (indicating downregulated expression) in the comparison groups. A total of 441 differentially expressed chloroplast proteins were identified, comprising 177 upregulated and 264 downregulated proteins (see Table 1). Gene Ontology (GO) functional clustering was performed on all nitrogen stress-induced differentially expressed genes via the Gene Ontology database (<http://geneontology.org>). The results indicated that the majority of the proteins were localized in the cytoplasm (77), subcellular organelles (61), and plasma membrane (25) according to the results of the cellular component clustering analysis. In the biological process clustering, the most prominent processes included metabolism (338), cellular processes (332), component organization (153), and response to stimuli (142). Genes associated with antioxidant activity (215) and catalytic activity (207) were predominant in the molecular function clustering.

Table 1. Count of differential proteins extracted under lower nitrogen.

Compared name	Total quant	Upregulated	Downregulated
WN/N	2267	177	264

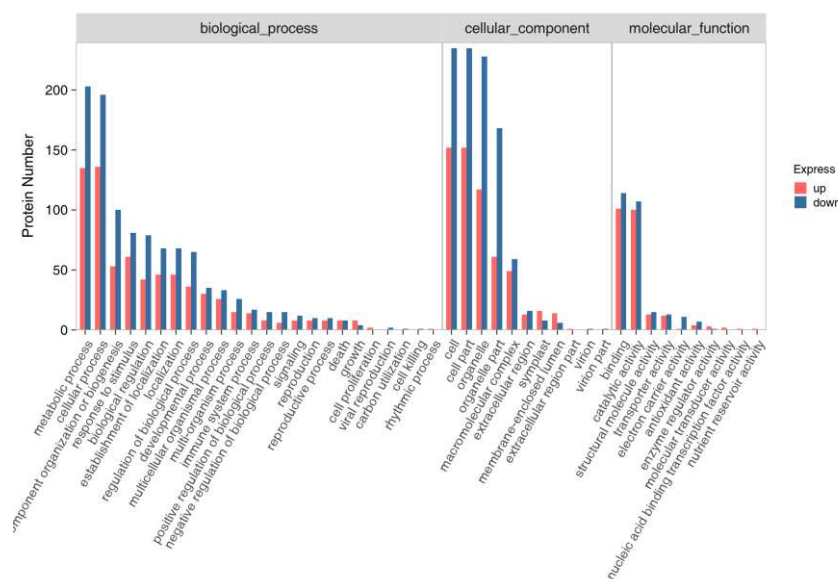


Figure 3. GO classification annotation of differentially expressed proteins.

2.4 Chloroplast differential protein metabolic pathway of *Festuca arundinacea*

A total of ten chloroplast proteins (Unigene73547.p1, Unigene7563.p1, Unigene17980.p1, Unigene73680.p1, Unigene129909.p1, Unigene18656.p1, Unigene56125.p1, Unigene1820.p1, and Unigene59680.p1) were downregulated, whereas only Unigene58649.p1 was upregulated (Table 2). These genes are implicated in various processes, including nitrogen absorption, nitrogen fixation, nitrogen assimilation, and nitrogen transport.

Table 2 Changes in the expression of redox genes under different N levels

Gene name	MW[kDa]	Fold change	Up/down
Unigene73547.p1	52177.8	0.30	down
Unigene7563.p1	44563.4	0.16	down
Unigene17980.p1	49966	0.43	down
Unigene73680.p1	39917.4	0.12	down
Unigene129909.p1	51280.8	0.26	down
Unigene18656.p1	27308.4	0.19	down
Unigene56125.p1	21014.2	0.14	down
Unigene1820.p1	40985.3	0.22	down
Unigene59680.p1	130266.2	3.08	Up
Unigene58649.p1	34259.1	0.11	down

The primary biochemical metabolic pathways and the distribution of differentially expressed proteins (DEPs) involved in synthetic processes can be identified through KEGG pathway enrichment analysis. The top 20 pathways, ranked by the number of annotated proteins, included photosynthesis (27), metabolic pathways (138), biosynthesis of secondary metabolites (60), and microbial metabolism (35). These pathways constitute the major KEGG pathways associated with the differentially expressed proteins. Notably, energy metabolism and biosynthetic pathways are critical for nitrogen stress tolerance. Significant changes in differentially expressed proteins were observed in energy metabolism (ko00195), fatty acid metabolism (ko01100), and the metabolism of cofactors and vitamins (ko00860) (Figure 4).

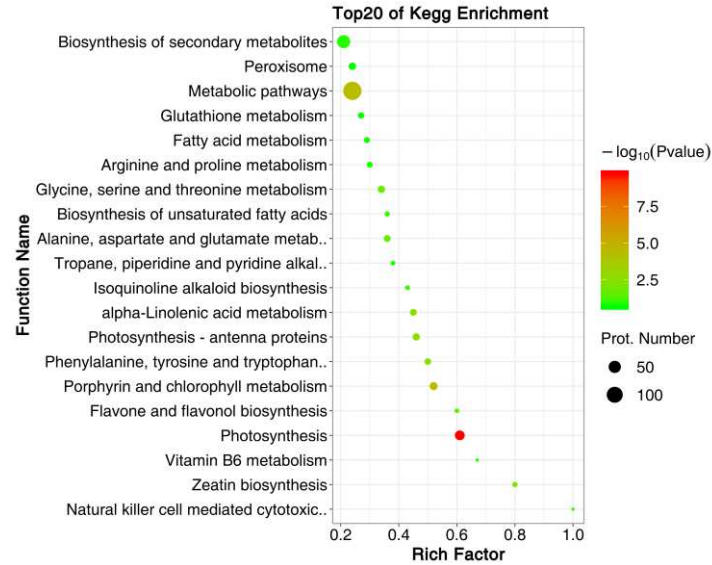


Figure 4 KEGG pathway enrichment of differentially expressed proteins.

2.5 Validation of chloroplast differential protein-coding genes in tall fescue

Real-time fluorescent quantitative PCR (qRT-PCR) was employed to verify the expression levels of genes encoding differentially expressed proteins. To further substantiate the RNA-seq data, five genes encoding proteins that were differentially expressed between low-nitrogen-treated and control (normal) tall fescue plants were randomly selected. These genes included unigene121673.p1 (cynT, can), unigene83458.p1 (PAL, phenylalanine ammonia-lyase [EC:4.3.1.24]), unigene39184.p1 (HemC/HMBS), unigene60878.p1 (glnA, glutamine synthetase, GS), and unigene93353.p1 (protochlorophyllide-dependent translocon component 52, PT52). The expression levels of these genes were analyzed via qRT-PCR, and the results indicated that the gene expression patterns were consistent with the RNA-seq data (Figure 5).

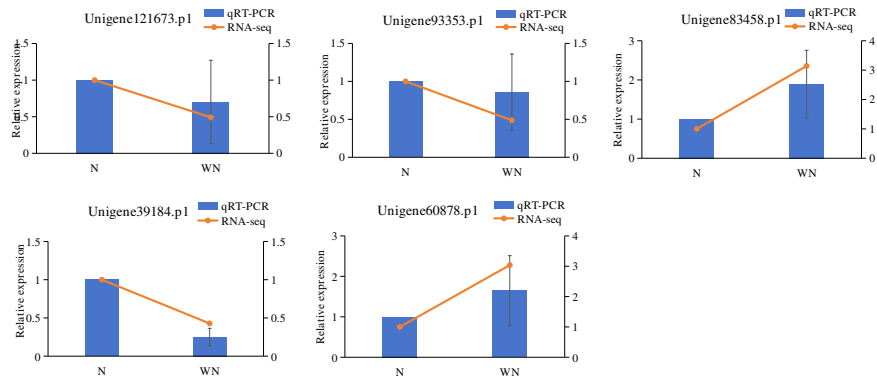


Figure 5 Validation of the DEGs via qRT-PCR analysis.

2.6 Photosynthetic rate of tall fescue under nitrogen stress

The net photosynthetic rate (Pn) of tall fescue under full nitrogen (nonstressed) treatment was $5.60 \pm 0.22 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. In contrast, under nitrogen stress (low-nitrogen treatment), the Pn was $3.30 \pm 1.80 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ greater than that in the full-nitrogen treatment, representing a decrease of 1.7 times. The intercellular CO₂ concentration (Ci), stomatal conductance (Gs), and transpiration rate (Tr) of tall fescue were significantly greater under fully nitrogen-containing conditions than under nitrogen-free conditions. Specifically, the Ci was 1.38 times greater under full-nitrogen (nonstressed) treatment (Table 3). These findings suggest that low nitrogen stress adversely affects the photosynthetic rate of *Festuca arundinacea*.

We measured the chlorophyll content in tall fescue (*Festuca arundinacea*) leaves under low-nitrogen stress. The chlorophyll content significantly decreased in the leaves subjected to low-nitrogen stress. The chlorophyll a, chlorophyll b, and total chlorophyll contents were $2.02 \pm 0.14 \text{ mg/g}$, $0.72 \pm 0.05 \text{ mg/g}$, and $2.74 \pm 0.20 \text{ mg/g}$, respectively, in the fresh leaves of the control group. In contrast, these values decreased to $1.12 \pm 0.15 \text{ mg/g}$, $0.51 \pm 0.04 \text{ mg/g}$, and $1.62 \pm 0.19 \text{ mg/g}$, respectively, in the leaves under low

nitrogen stress (Figure 6). These results suggest that photosynthetic efficiency could be significantly reduced under low-nitrogen stress.

Table 3 Photosynthetic characteristics of nitrogen stress treatment of *Festuca arundinacea*

Different Treatment	Net photosynthetic rate($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Intercellular concentration ($\mu\text{mol}\cdot\text{s}^{-1}$)	CO2	Stomatal conductance ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	Transpiration rate ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)
WN	5.60 $\pm$ 0.22	0.72 $\pm$ 0.21		190.6811 $\pm$ 4.79328	8.182 $\pm$ 0.63571
N	3.30 $\pm$ 1.81*	0.52 $\pm$ 0.22*		182.0942 $\pm$ 5.18559	5.438 $\pm$ 1.72256*

¹ * and ** indicate significant differences at P<0.05 and P<0.01

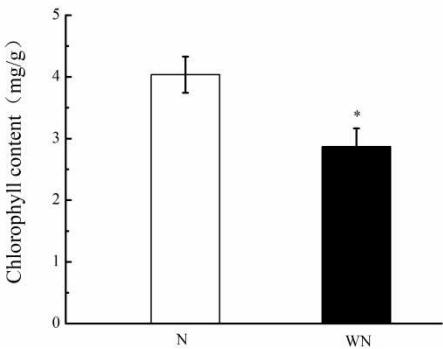


Figure 6 Chlorophyll content of tall fescue under low-nitrogen conditions.

2.7 Characteristics of the light response of tall fescue under nitrogen stress

The light response characteristics of tall fescue under full-nitrogen (nonstressed) and nitrogen-free (low-nitrogen) conditions presented minimal overall differences. The net photosynthetic rate increased gradually with increasing light intensity before stabilizing. At low light intensities ($\text{PAR} \leq 150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), the net photosynthetic rate of tall fescue under full-nitrogen treatment was lower than that under low-nitrogen treatment; however, both rates increased with increasing light intensity. Conversely, at high light intensities ($\text{PAR} \geq 150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), the net photosynthetic rate of tall fescue under full-nitrogen treatment was greater than that under nitrogen-free treatment, with both rates increasing slowly as the light intensity increased. The C_i of tall fescue decreased gradually under both the full-nitrogen and the nitrogen-free treatments, although the C_i under the full-nitrogen treatment remained greater than that under the nitrogen-free treatment. The stomatal conductance (G_s) of tall fescue leaves decreased gradually under full-nitrogen treatment, whereas G_s increased slowly under low-nitrogen treatment. At high light intensities ($\text{PAR} \geq 1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), G_s decreased under nitrogen stress, but the values under full nitrogen treatment were greater than those under low nitrogen treatment (Figure 7). Overall, there was little variation in the transpiration rate (Tr), indicating that the light response curve of tall fescue was influenced by nitrogen stress.

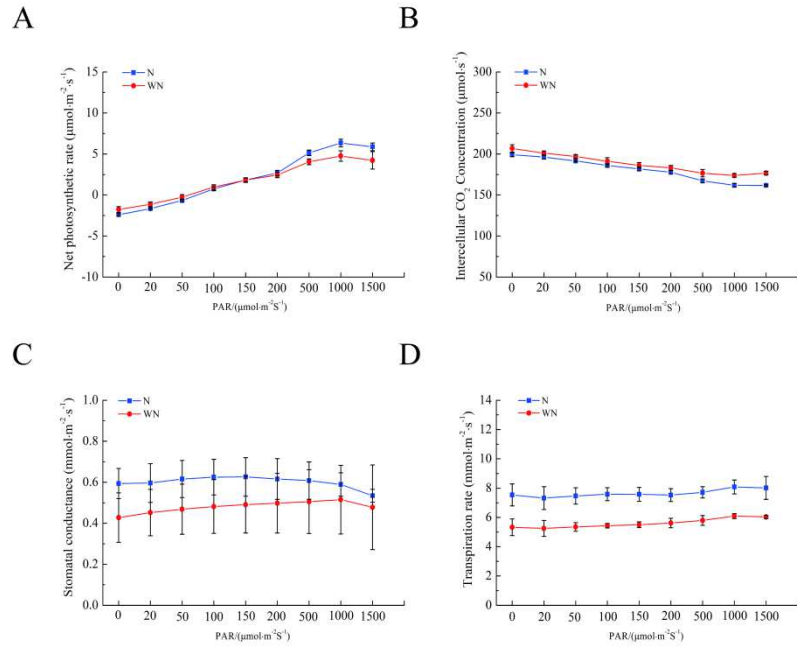


Figure 7 Light response of the photosynthetic rate under low-nitrogen conditions.

(A) Net photosynthetic rate; (B) Intercellular CO₂ concentration; (C) Stomatal conductance; (D) Transpiration rate.

2.8 Analysis of the CO₂ response curve of tall fescue under nitrogen stress

The CO₂ response curve of tall fescue leaves was measured via the LI-6400 photosynthesis system. As the CO₂ concentration increased, the net photosynthetic rate (Pn) generally tended to increase. However, the Pn of tall fescue under full nitrogen treatment was lower than that under nitrogen-free treatment. Notably, when the CO₂ concentration reached 100 $\mu\text{mol}\cdot\text{mol}^{-1}$, the Pn increased sharply. At CO₂ concentrations of $\geq 200 \mu\text{mol}\cdot\text{s}^{-1}$, the increase in Pn was more pronounced in the full nitrogen treatment than in the nitrogen-free treatment. The Pn of *Festuca arundinacea* leaves under fully nitrogen conditions increased more than that in the low-nitrogen treatment group did (Figure 8).

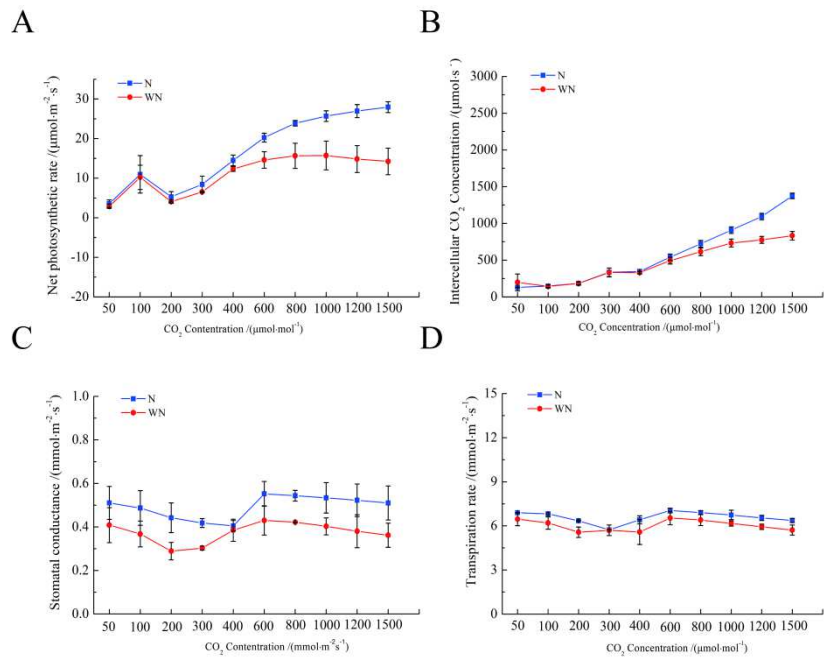


Figure 8 CO₂ response of the photosynthetic rate under low-nitrogen conditions.

3. Discussion

Nitrogen is an essential element for plant growth and plays a direct role in chlorophyll synthesis, protein synthesis, nucleic acid synthesis, photosynthesis, and carbon metabolism. Nitrogen deficiency in plants manifests as leaf chlorosis, reduced tillering, inhibited growth, and a decline in the photosynthetic rate (Nunes-Nesi et al., 2010). Severe nitrogen deficiency can adversely affect both plant yield and quality. Nitrogen deficiency alters the distribution of growth between the roots and above-ground parts of a plant by regulating the levels of plant hormones, such as cytokinins and abscisic acid (Evans & Clarke, 2019). For forage plants such as *Festuca arundinacea*, effective nitrogen management is critical for increasing yield and stress resistance. However, the negative environmental impacts of excessive nitrogen fertilization, such as water pollution and greenhouse gas emissions, have prompted scientists to explore methods for optimizing nitrogen fertilizer use and improving nitrogen use efficiency in plants.

This study investigated the effects of low-nitrogen stress on the ultrastructure and proteome of chloroplasts in *Festuca arundinacea*. The results indicate that chloroplast structures are significantly altered under low nitrogen stress, including the loss of cell protrusions, smoother cell morphology, necrosis or structural abnormalities in mesophyll cells, swelling of chloroplasts, disorganization of thylakoid membranes, formation of giant starch granules, an increase in osmium particles, and even partial disintegration of chloroplasts. This phenomenon aligns with the response mechanisms observed in other plants subjected to low nitrogen stress. Xu et al. (2022) reported that thylakoid membrane species, downregulation of core photosystem II proteins such as PsbA and PsbD, and reduced photosynthetic efficiency in grasses are consequences of low-nitrogen conditions. Additionally, Evans and Clarke (2019) reported that the synthesis of chloroplast membrane lipids may be inhibited, thereby affecting the stability of membrane structures and reducing chloroplast photosynthetic efficiency under nitrogen deficiency conditions. Xu et al. (2022) also demonstrated that the lipid composition of the chloroplast membrane significantly changes, impacting membrane fluidity and stability, which further decreases photosynthetic efficiency under low-nitrogen conditions. These findings suggest that the core organelles involved in photosynthesis are substantially damaged, reflecting a potential stress mechanism in which the plant cell wall is remodeled and that cell death occurs under low-nitrogen stress. This may represent a possible adaptive strategy to low-nitrogen stress, as evidenced by the downregulation of core photosystem II (PSII) proteins, such as HemC and PT52, alongside a decrease in photosynthetic efficiency under low-nitrogen conditions.

In this study, the net photosynthetic rate of *Festuca arundinacea* was reduced by 1.7 times under low nitrogen treatment, which may be attributed to the obstruction of PSII electron transport and a decreased capacity for carbon fixation (Wang et al., 2021). Consequently, the chlorophyll content significantly decreased, further confirming that low nitrogen inhibits chlorophyll synthesis. These results suggest that low nitrogen limits chlorophyll production, thereby diminishing photosynthetic efficiency (Li et al., 2023). Additionally, proteomic analysis revealed that 177 out of 441 chloroplast proteins were upregulated under low-nitrogen conditions, whereas 264 proteins were downregulated. Among these proteins, those associated with photosynthesis and metabolic pathway synthesis, such as PSII core proteins and metabolic pathway synthases, were downregulated. These findings indicate that low nitrogen not only affects core photosynthetic proteins but also may reduce photosynthetic efficiency by altering energy metabolism and fatty acid metabolism pathways. Recent proteomic studies have demonstrated that low nitrogen stress significantly suppresses the expression of core photosynthetic proteins, including photosystem II (PSII) core proteins and metabolic pathway synthases (Wang et al., 2021). The observed downregulation of photosystem II core proteins and metabolic pathway synthases is consistent with previous findings, further suggesting that low nitrogen stress reduces photosynthetic efficiency by disrupting the dynamic balance of the photosynthetic proteome. The proteomic changes induced by low-nitrogen stress in tall fescue chloroplasts involve proteins associated with photosynthesis, carbon metabolism, and stress response processes.

Our KEGG pathway analysis revealed that the differentially expressed proteins were enriched primarily in energy metabolism, fatty acid metabolism, and antioxidant pathways under low-nitrogen stress. The alterations in these pathways underscore how plants adjust their metabolic processes to adapt to low-nitrogen environments. Furthermore, this study identified key proteins related to nitrogen use efficiency in *Festuca arundinacea*, such as metabolic pathway synthases and PSII core proteins, providing new insights into the mechanisms by which plants adapt to low-nitrogen conditions (Zhang et al., 2021). Additionally, this research examined the impact of low-nitrogen stress on the cellular structure and chloroplast proteome of *Festuca arundinacea* and demonstrated that low nitrogen disrupts photosynthetic efficiency by altering chloroplast function and membrane lipid synthesis.

Plants have evolved various strategies to cope with nitrogen deficiency, including increasing their antioxidant capacity and adjusting their energy metabolism. Under nitrogen stress, plants increase the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which help mitigate oxidative damage caused by reactive oxygen species (ROS). These enzymes play crucial roles in protecting plant cells from oxidative stress, maintaining cellular integrity, and supporting

growth. Additionally, plants often adjust their energy metabolism by altering carbon and nitrogen allocation, promoting the synthesis of osmotic regulators and secondary metabolites. These metabolic adjustments not only help plants maintain cellular homeostasis but also increase their overall stress tolerance. Ahanger et al. (2019) demonstrated that nitrogen limitation increases the activity of antioxidants and osmotic metabolites, providing essential protection for wheat under salinity stress. Similarly, Wang and Lü (2020) emphasized how nitrogen metabolism mechanisms contribute to stress resistance by optimizing metabolic pathways. These findings underscore the critical role of metabolic adjustments in enhancing plant resilience to nutrient limitations and environmental stressors. By deeply analyzing the key nitrogen-use efficiency proteins and related metabolic pathways in *Festuca arundinacea*, this study provides a theoretical basis for developing low-nitrogen-tolerant forage varieties.

4. Materials and methods

4.1 Plant materials

Festuca arundinacea Qiancao No. 1 (a cultivar of tall fescue) was developed by the Prataculture Institute of Guizhou in 2005. It is a nationally approved forage species and is cultivated at the Guizhou Institute of Prataculture's Dushan Experimental Base in Dushan, Qiannan, Guizhou, China. It is registered under the number 299 and presents excellent traits such as strong resistance, high yield, and a long green period. Three hundred seeds of *Festuca arundinacea* 'Qian Cao No. 1', which are uniform in size and full in appearance, were selected. The seeds were soaked in clean water and placed in a 50°C constant-temperature drying oven for 24 hours. Then, 50 mL of 75% alcohol was added, and the seeds were gently stirred with tweezers for 30 seconds for disinfection. Finally, the seeds were washed three times with 100 mL of sterile water. Two sterilized filter papers were placed at the bottom of a glass Petri dish, sterile distilled water (5 mL) was added, and the prepared *Festuca arundinacea* seeds were evenly spread on filter paper and placed in a light-controlled incubator for 7 days. Sterile distilled water (3–4 mL) was added daily. Once the seeds had germinated, they were transferred to a hydroponic system containing Hoagland nutrient solution for low nitrogen treatment for 30 days. In the no-nitrogen hydroponic solution, NO_3^- was replaced with Cl^- , while the standard hydroponic solution was a regular Hoagland nutrient solution, and the nutrient solution was replaced every 5 days. Leaves of N-free *Festuca arundinacea* were collected, rapidly frozen with liquid nitrogen, and stored at -80°C for later use. The plant cultivation conditions included 16 hours of light and 8 hours of darkness, with a temperature of $22\pm 2^\circ\text{C}$ and a humidity of $60\pm 5\%$.

4.2 Methods

4.2.1 Cell ultrastructure

Leaf tissue from the same region (middle part of the leaf) of *Festuca arundinacea* under nitrogen stress treatment was cut into 1 cm × 1 cm sections and fixed in a fixation solution (3% glutaraldehyde, pH 7.2). The samples were washed twice with 0.1 mol·L⁻¹ phosphate buffer (pH=7.4), with each wash lasting 5 minutes. The samples were gently attached to conductive tape (with the back side facing up), and critical point drying was performed (Critical Point Dryer Model K850, Quorum Technologies, UK). The samples were then vacuum-coated and observed under a scanning electron microscope (Model Inspect, FEI, USA) at appropriate positions and magnifications. The same part (middle section of leaf tissue) of *Festuca arundinacea* under nitrogen stress treatment was cut into 1 cm × 1 cm pieces and fixed in a fixation solution (3% glutaraldehyde, pH 7.2), followed by further fixation with 1% osmium tetroxide, washing with 0.1 mol·L⁻¹ phosphate buffer (pH 7.4), dehydration stepwise with increasing concentrations of acetone, infiltration, embedding, and sectioning into ultrathin slices (EM UC7, Leica). The sections were stained with uranyl acetate, followed by lead citrate staining. Finally, the samples were observed and photographed under a transmission electron microscope (Model JEM-1400PLUS, JEOL, Japan).

4.2.2 Chlorophyll protein extraction from *Festuca arundinacea* leaves

We weighed 1 g of *Festuca arundinacea* tissue, washed it, dried it, and removed the leaf stalks and coarse veins. The tissue was ground into a powder in a mortar using liquid nitrogen, and the chloroplasts were then isolated according to the instructions provided with the plant chloroplast isolation kit (BB-3622-1, Beijing Beibo). Two hundred microlitres of L3 solution and 1% PMSF were added, and the mixture was subjected to ice bath sonication for 10 minutes at 15% power (sonicating for 2 seconds and pausing for 3 seconds). The sample was subsequently centrifuged at $12,000 \times g$ for 10 minutes, after which the supernatant was collected. 800 µL of cold acetone was added, along with a final concentration of 30 mM DTT, and precipitated for 2 hours. The sample was then centrifuged at $12,000 \times g$ for 20 minutes, and the supernatant was discarded. The remaining pellet was resuspended in 100 µL of 8 M urea, sonicated in an ice bath for 5 minutes at 10% power, and then treated with 1% DTT at 56°C for 30 minutes. Next, 10%

IAM was added, and the mixture was incubated in the dark for 30 minutes. Protein quantification was carried out via the Bradford method, and the samples were aliquoted and stored at -80°C.

4.2.3 Proteolytic hydrolysis and iTRAQ labeling

Protein digestion was performed via the FASP method following Wiśniewski et al. (2009). The digested peptides were labeled via 4-plex/8-plex iTRAQ (Applied Biosystems) following the protocol provided in the kit instructions. The 200 µg protein sample was dissolved in 30 µL of STD buffer (4% SDS, 100 mmol/L DTT, 150 mmol/L Tris-HCl, pH 8.0). The sample was subjected to ultrafiltration dialysis using UA buffer (8 mol/L urea, 150 mmol/L Tris-HCl pH 8.0) to remove DTT and small molecular weight proteins (below 30 kDa). Next, 100 µL of 0.05 mol/L iodoacetamide was added to the UA buffer, and the mixture was incubated in the dark for 20 minutes to block the C-terminus of the peptides. The sample was washed three times with 100 µL of UA buffer, followed by two washes with 100 µL of DS buffer (50 mmol/L triethylammonium bicarbonate, pH 8.5). The sample was dissolved in 40 µL of DS buffer and digested with 2 µg of trypsin (Promega) at 37°C under dark conditions overnight, after which it was collected for further use. The protein concentration was determined by UV absorbance at 280 nm via the extinction coefficient. The iTRAQ reagents were dissolved in 70 µL of ethanol and added to the sample for labeling. After labeling was completed, the sample was vacuum-dried and stored.

4.2.4 Protein separation and chromatographic analysis

The iTRAQ-labeled proteins were purified via a SCX cation exchange column on the AKTA purification system (GE Healthcare). The labeled peptides were dissolved in 2 mL of buffer A (10 mmol/L KH₂PO₄ in 25% ACN, pH 2.7) and loaded onto a 4.6 × 100 mm polysulfonic acid ethyl group separation column (5 µm, 200 Å, PolyLC Inc., Maryland, USA). Elution was performed using 0%-10% buffer B (500 mmol/L KCl, 10 mmol/L KH₂PO₄ in 25% ACN, pH 2.7) at a flow rate of 1 mL/min for 2 minutes, followed by 10%-20% B buffer for 25 minutes, 20%-45% B buffer for 5 minutes, and 50%-100% B buffer for 5 minutes. The samples were collected every minute, and the absorbance was monitored at 214 nm. Similar samples were pooled, and desalting was performed. Each pooled sample was concentrated, redissolved in 40 µL of 0.1% (v/v) trifluoroacetic acid and stored at -80°C for future use.

4.2.5 Liquid mass spectrometry

The prepared samples were analyzed via a nanoLC-MS-MS system. Specifically, 10 µL (approximately 5 µg) of the sample mixture was injected into a C18 reversed-phase chromatography column (15 cm in length, 75 µm internal diameter), which was packed with liquid A (0.1% formic acid) of 5 µm RP-C18 resin. A linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) was used for elution. The separation was carried out at a flow rate of 250 nL/min for more than 140 minutes. Mass spectrometry data were obtained by scanning within the range of 300–1800 m/z, with a focus on the highest-abundance HCD fragments.

4.2.6 Database comparison analysis of the mass spectrum data

The mass spectrometry data were analyzed via MASCOT and Proteome Discoverer 1.3 software. The sequences of the differentially expressed proteins were obtained from the UniProtKB database, and a BLAST search was performed against the NCBI BLAST and SwissProt databases for functional annotation. Sequences with a threshold of $<10^{-3}$ were further analyzed via Blast2GO and Gene Ontology (GO) with a threshold of 10^{-6} .

4.2.7 Total RNA extraction, reverse transcription of cDNA and fluorogenic quantitative PCR

Total RNA was extracted from tall fescue via the DP432 kit following the instructions provided in the kit manual. RNA reverse transcription was performed via the FastKing RT Kit (KR116). DNase was used to treat 1 µL of RNA, and 5 µg of RNA from each sample was reverse transcribed into cDNA according to the manufacturer's instructions. The cDNA was diluted 10 times and stored for future use. Fluorescent quantitative PCR (qRT-PCR) was performed to verify the expression of selected candidate genes. The primers for qRT-PCR were designed via Primer Premier 6.0 software. The primer sequences are provided in Table 4. The PCR mixture consisted of 20 µL of total sample, including 10 µL of Power qPCR PreMix (Genecopoeia), 0.5 µL of each Primer F and Primer R mixture, 1 µL of cDNA, and 8 µL of ddH₂O. The reaction mixture was prepared on ice and placed in an ABI 7500 qPCR machine (Applied Biosystems Inc., USA) with the following program: 95°C for 10 minutes, 95°C for 10 seconds, 60°C for 10 seconds, 72°C for 30 seconds, and 40 cycles followed by a final 72°C for 10 minutes. Gene expression was analyzed via the $2^{-\Delta\Delta Ct}$ method, with three biological replicates and three technical replicates for each sample.

Table 4 Primers for the qRT–PCR analysis of differentially expressed genes

Primer name	Primer sequences (5'-3')
U121673-F	CCAACATGGTCCCTTCCTAC
U121673-R	GAGTGACAGGAGTGCCTTGA
U39184-F	GGAGGCAAGGGTTTATTCA
U39184-R	CTTCTCGTGGGAGGTTACAG
U60878-F	ACGAGACAGCTGACATCCAC
U60878-R	AAGTAGCCCTTGCCGTTCT
U83458-F	TCCTACCCGCTCTACCAGTT
U83458-R	GTTCCACTCCTTGAGGCACT
U93353-F	TTCTATGGCTCACCGCTTC
U93353-R	GATCACCTGCTCTTCCCT
GAPDH-F	GCCATTCCTGTCAACTTTCCGT
GAPDH-R	AAGGGCTGCTAGCTTCAACATCAT

4.2.8 Chlorophyll determination

To measure the chlorophyll content, 10 g of tall fescue leaves were cut into small pieces and soaked in 5 mL of 80% acetone solution for 48 hours. The absorbance was measured at 663 nm and 646 nm. Chlorophyll a (Chl a) and chlorophyll b (Chl b) were calculated via the following formulas: $\text{Chla} = 12.21 \times \text{OD}_{663} - 2.81 \times \text{OD}_{646}$, $\text{Chlb} = 20.13 \times \text{OD}_{646} - 5.03 \times \text{OD}_{663}$, total chlorophyll (TChl) = Chla + Chlb.

4.2.9 Photosynthetic gas exchange parameter determination

Uniform and healthy tall fescue plants that had undergone nitrogen stress treatment were selected and acclimated to outdoor light conditions for 1–2 days. Using a Li-6400 portable photosynthesis system (Li-COR, USA), the following photosynthetic parameters were measured: net photosynthetic rate (Pn), transpiration rate (Tr), intercellular CO₂ concentration (Ci), and stomatal conductance (Gs). Artificial light with photosynthetically active radiation (PAR) of 1000 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ was used, with a leaf temperature of 25±2°C, a relative humidity of 60±5%, a CO₂ concentration of 400 $\mu\text{mol} \cdot \text{mol}^{-1}$, and an air flow rate of 500 $\mu\text{mol} \cdot \text{s}^{-1}$. Three plants were measured per treatment, and each plant was measured three times.

4.2.10 Photoresponse curve

After nitrogen stress treatment, uniform and healthy plants were selected for measuring the light response curve via the LI-6400 portable photosynthesis system (LI-COR, USA). The leaf chamber CO₂ concentration was set at 380±6.0 $\mu\text{mol} \cdot \text{mol}^{-1}$, the atmospheric temperature was 28±2°C, and the air relative humidity was 60±4.0%. The light intensity was controlled via an LED light source with 8 PAR intensity levels, including 1500, 1000, 500, 200, 150, 50, 20, and 0 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, with minimum and maximum wait times set to 120 and 300 s, respectively. Each sample was measured three times, and each treatment included three biological replicates. The photosynthesis measurement system automatically recorded the net photosynthetic rate (Pn, $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), transpiration rate (Tr, $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), stomatal conductance (Gs, $\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), intercellular CO₂ concentration (Ci, $\mu\text{mol} \cdot \text{mol}^{-1}$), and other parameters.

4.2.11 CO₂ response curve

After nitrogen stress treatment, uniform and healthy tall fescue plants were selected for measuring the CO₂ response curve of the leaves via the LI-6400 portable photosynthesis system (LI-COR, USA). The effective radiation intensity (PAR) was controlled at approximately 1500 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, with an atmospheric temperature of 28±2°C and a relative humidity of 60±4.0%. The CO₂ concentration in the leaf chamber was controlled by installing a high-pressure CO₂ cylinder (O-ring), with the CO₂ concentration gradient set at 1500, 1200, 1000, 800, 600, 400, 400, 300, 200, 100, and 50 $\mu\text{mol} \cdot \text{mol}^{-1}$ (11 levels). Each leaf was measured three times. The minimum wait time was set to 120 seconds, and the maximum wait time was set to 300 seconds. The photosynthesis measurement system automatically recorded the net photosynthetic rate (Pn), intercellular CO₂ concentration (Ci), stomatal conductance (Gs), and other related photosynthetic physiological parameters.

5. Conclusions

Under low-nitrogen conditions, the surface of *Festuca arundinacea* leaf cells became smoother, protrusions disappeared, mesophyll cells exhibited necrosis or structural abnormalities, and chloroplasts presented abnormal morphology along with the formation of giant starch granules. These results indicate that the cell structure and metabolic balance of tall fescue were significantly disrupted under low-nitrogen stress. A total of 441 proteins were differentially expressed in *Festuca arundinacea* chloroplasts under low nitrogen stress, with 177 proteins upregulated and 264 proteins downregulated. Notably, the expression of photosystem II core proteins and metabolic pathway synthases was downregulated, directly inhibiting photosynthetic efficiency, whereas the expression of antioxidant-related proteins was upregulated, indicating that the plant responds to stress by increasing redox balance. The upregulation of antioxidant-related proteins (such as SOD and POD) suggested that tall fescue alleviates oxidative damage induced by enhancing its antioxidant capacity under low nitrogen. Oxidative stress is a typical response of plants to low nitrogen stress, and the activation of redox balance mechanisms plays a crucial role in maintaining cellular homeostasis. The differentially expressed proteins identified in this study, particularly those related to photosynthesis, antioxidant activity, and nitrogen metabolism, provide a theoretical foundation for understanding the molecular mechanisms underlying plant nitrogen use efficiency. These findings are highly important for improving the stress resistance of tall fescue and breeding varieties with high nitrogen use efficiency. Low nitrogen stress significantly affects the physiological and metabolic functions of tall fescue plants by disrupting their structure, inhibiting their photosynthetic efficiency, and inducing oxidative stress. The results of this study provide important scientific insights into understanding plant adaptation mechanisms under low nitrogen, optimizing nitrogen fertilization strategies, and breeding turfgrass species.

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Abbreviations

The following abbreviations are used in this manuscript:

TEM	Transmission electron microscopy
SEM	Scanning Electron Microscopy
iTRAQ	Isobaric tags for relative and absolute quantitation
Pn	Net photosynthetic rate
Ci	Intercellular CO2 concentration
Gs	Stomatal conductance
Tr	Transpiration rate

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