Postprint: Analysis of Differentially Expressed Metabolites in Drynaria roosii Rhizome in Response to Calcium Stress

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Abstract

Drynaria fortunei is a typical epiphytic fern species in karst regions that primarily attaches to rock surfaces or within crevices via its rhizome. Concurrently, the dried rhizome of D. fortunei is utilized medicinally and holds significant pharmacological value. To investigate alterations in metabolites within D. fortunei rhizomes under Ca2+ stress, non-targeted metabolomic analysis was conducted using ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) on D. fortunei rhizomes subjected to different concentrations (0, 600, 1200 mmol · L^{-1}) of Ca2+ stress. The results demonstrated: (1) a total of 64 differentially expressed metabolites were identified. (2) There were 48 differentially expressed metabolites in the 0 vs. 600 mmol · L^{-1} comparison group, 45 differentially expressed metabolites in the 0 vs. 1200 mmol · L^{-1} comparison group, and 44 differentially expressed metabolites in the 600 vs. 1200 mmol · L^{-1} comparison group. (3) The identified differentially expressed metabolites were categorized into five classes based on their chemical classification information. These findings indicate that Ca2+ stress affects amino acid metabolism, flavonoid biosynthesis, lignin biosynthesis, fatty acid metabolism, and other pathways in D. fortunei rhizomes. Through non-targeted metabolomic analysis, key metabolites involved in the response of D. fortunei rhizomes to Ca2+ stress were preliminarily elucidated, laying the foundation for further investigation into the regulatory mechanisms of D. fortunei adaptation to Ca2+ stress, and offering novel insights for the quality improvement of D. fortunei rhizome medicinal material.

Full Text

Analysis of Differentially Expressed Metabolites in Drynaria roosii Rhizome in Response to Calcium Stress

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Abstract

Drynaria roosii is a typical epiphytic pteridophyte in karst regions that primarily grows on rock surfaces or in crevices through its rhizome. The dried rhizome of *D. roosii* is used medicinally and holds significant pharmaceutical value. To investigate metabolic changes in *D. roosii* rhizome under Ca^{2+} stress, we performed non-targeted metabolomic analysis using ultra-performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) on rhizomes exposed to different Ca^{2+} concentrations (0, 600, and 1,200 $\text{mmol} \cdot \text{L}^{-1}$). The results revealed: (1) a total of 64 differentially expressed metabolites were identified; (2) 48 differentially expressed metabolites were found between the 0 and 600 $\text{mmol} \cdot \text{L}^{-1}$ comparison group, 45 between the 0 and 1,200 $\text{mmol} \cdot \text{L}^{-1}$ group, and 44 between the 600 and 1,200 $\text{mmol} \cdot \text{L}^{-1}$ group; (3) these metabolites were classified into five categories based on their chemical classification information. Our findings demonstrate that Ca^{2+} stress affects amino acid metabolism, flavonoid biosynthesis, lignin biosynthesis, fatty acid metabolism, and other pathways in *D. roosii* rhizome. Through non-targeted metabolomic analysis, we have preliminarily identified key metabolites involved in the response to Ca^{2+} stress, laying a foundation for further investigation into the regulatory mechanisms of *D. roosii* adaptation to Ca^{2+} stress and providing new insights for quality improvement of *D. roosii* rhizome medicinal materials.

Keywords: non-targeted metabolomics, high concentration Ca^{2+}, metabolic pathway, adaptive mechanism, Miao medicine

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Karst regions account for 12% of global land area, with China's karst area representing 15.6% of the world's total. Karst habitats are fragile ecosystems characterized by high Ca^{2+} concentrations in shallow soils. Analysis of typical karst soils in Puding, Huajiang, Libo, and Luodian in Guizhou revealed average exchangeable calcium levels of 3.61 $\text{g} \cdot \text{kg}^{-1}$, several times higher than in non-karst soils elsewhere in China. While Ca^{2+} plays crucial roles in plant growth and development—including signal transduction, membrane permeability, and cell wall remodeling—excessively high concentrations adversely affect soil properties and mineral nutrient uptake. When cellular Ca^{2+} absorption exceeds physiological limits, chloroplasts suffer direct damage, photosynthesis is impaired, and leaf senescence accelerates. Consequently, plants must maintain low intracellular Ca^{2+} concentrations to ensure normal physiological activities. Current research indicates that plants adapt to high-Ca^{2+} karst environments primarily through Ca^{2+} accumulation and excretion, synthesis of osmotic adjustment substances, production of antioxidant enzymes, and regulation of sterol methyltransferase activity. These findings are significant for understanding plant adaptation strategies in karst regions and provide a foundation for rocky desertification control and vegetation restoration.

Drynaria roosii, belonging to the Polypodiaceae family, is a typical epiphytic plant in karst regions and a dominant species widely distributed throughout Guizhou. Its dried rhizome, known as "Gusuibu" in Chinese medicine or by various Miao names, was first documented in *Lei Gong's Treatise on Preparation* and is traditionally used to strengthen bones and tendons, promote blood circulation, and relieve pain, treating conditions ranging from physical injuries to colds and fractures. Currently, large-scale artificial cultivation of *D. roosii* has not been established, and research on its adaptation to abiotic stress remains limited. Previous studies on leaf responses to calcium stress revealed that high Ca^{2+} concentrations primarily inhibit leaf growth through osmotic stress, with proteomic analysis showing that differentially expressed proteins are mainly involved in protein metabolism, amino acid metabolism, sugar and energy metabolism, photosynthesis, and antioxidant defense pathways. Physiological studies on rhizome responses demonstrated that high Ca^{2+} causes decreased tissue water content, inducing osmotic stress that generates excessive reactive oxygen species (ROS), which cells counteract by synthesizing various antioxidant enzymes to prevent oxidative damage.

Metabolomics reveals differences in metabolic profiles among species, tissues, and under various stress conditions. Previous metabolomic analyses showed that differential metabolites in *D. roosii* leaves under calcium stress primarily involve amino acid metabolism, lignin biosynthesis, and flavonoid biosynthesis. Similarly, studies on *Sophora tonkinensis* seeds identified flavonoids and fatty acid metabolites as key differential metabolites during germination under Ca^{2+} stress, indicating that different metabolites play distinct roles in organ-specific adaptation. Metabolomics has become an essential systems biology tool for understanding plant adaptive metabolic responses to abiotic stress. In this study, conducted at the National Miao Medicine Engineering Technology Research Center of Guizhou University of Traditional Chinese Medicine, we performed non-targeted metabolomic analysis using UPLC-QTOF-MS on two-year-old *D. roosii* rhizomes treated with Hoagland nutrient solutions containing 0, 600, and 1,200 $\text{mmol} \cdot \text{L}^{-1}$ Ca^{2+}. Through analysis of differentially expressed metabolites, we addressed: (1) which metabolic pathways are primarily affected, and (2) whether high Ca^{2+} concentrations influence medicinal material quality.

1.1 Materials

Drynaria roosii Nakaike plants were cultivated for two years in a greenhouse after sporophyte formation. The plants were then transferred to pots containing vermiculite as substrate and grown in a phytotron at 24°C with 75% humidity and a 12 h light/12 h dark photoperiod at 2,000 mol $\cdot$ m^{-2} $\cdot$ s^{-1} light intensity. After seven days of Hoagland solution application, plants were treated for 14 days with modified Hoagland solutions adjusted to Ca^{2+} concentrations of 0, 600, and 1,200 mmol $\cdot$ L^{-1} (concentration selection based on Wu et al., 2023). Solutions were replaced daily to maintain stable Ca^{2+} concentrations. Fresh rhizomes were harvested for experiments or rapidly frozen in liquid nitrogen and stored at -80°C. Each treatment included six biological replicates.

Methods

Metabolite analysis followed the method of Li et al. (2022). Differentially expressed metabolites were screened using fold change \$ \$1.5 or \$ \$0.67 with $P < 0.05$ as criteria.

2.1 Quality Control Analysis

Non-targeted metabolomics was employed to determine changes in differentially expressed metabolites in *D. roosii* rhizome under Ca^{2+} stress. Principal component analysis (PCA) plots showed that samples from each group clustered tightly in both positive and negative ion modes, with clear separation between quality control (QC) samples and different treatment samples, indicating good experimental reproducibility [Figure 1: see original paper].

2.2 Differentially Expressed Metabolite Analysis

Up-regulated differential metabolites are shown in Table 1. Compared with the control, amino acid metabolism pathways involved six metabolites, with N-methyl-L-phenylalanine showing the greatest upregulation under 600 mmol $\cdot$ L^{-1} Ca^{2+} treatment, while trans-2-hydroxycinnamic acid, N-methyl-L-phenylalanine, D-aspartic acid, and L-histidine were most upregulated under 1,200 mmol $\cdot$ L^{-1} Ca^{2+} treatment. Flavonoid biosynthesis pathways involved 25 metabolites, with vitexin 4-O-glucoside, rhoifolin, isorhoifolin, luteolin 6-C-glucoside, quercetin 3-rutinoside, quercetin 3-galactoside, myricetin 3-galactopyranoside, herbacetin-3,8-diglucopyranoside, robinin, kaempferol-3-glucoside-3' '-rhamnoside, kaempferol 3-glucoside 7-rhamnoside, and cyanidin-3-O-glucoside showing the greatest upregulation at 600 mmol $\cdot$ L^{-1}, while vitexin 4-O-glucoside, luteolin 6-C-glucoside, quercetin 3-glucoside, quercetin 3-galactoside, herbacetin-3,8-diglucopyranoside, robinin, kaempferol 3-glucoside 7-rhamnoside, phlorizin, and formononetin were most upregulated at 1,200 mmol $\cdot$ L^{-1}. Lignin biosynthesis pathways involved four metabolites, with chlorogenic acid and neochlorogenic acid showing the greatest upregulation at 600 mmol $\cdot$ L^{-1}, and chlorogenic acid at 1,200 mmol $\cdot$ L^{-1}. Fatty acid metabolism pathways involved seven metabolites, with 12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid most upregulated at 600 mmol $\cdot$ L^{-1}, and 1-oleoyl-sn-glycero-3-phosphoethanolamine, (10E,15Z)-9,12,13-trihydroxyoctadeca-10,15-dienoic acid, 12S-hydroxy-5Z,8Z,10E,14Zeicosatetraenoic acid, and 9-hydroxy-10E,12Z-octadecadienoic acid at 1,200 $\mathrm{mmol \cdot L^{-1}}$. Additionally, eight other metabolites were identified .

Down-regulated differential metabolites are shown in Table 2. Compared with the control, one amino acid metabolite was down-regulated. Six flavonoid compounds were down-regulated, with kaempferol 3-O-arabinoside, dihydrokaempferol, and naringenin showing the greatest downregulation at 600 $\mathrm{mmol \cdot L^{-1}}$ Ca^{2+}. Lignin biosynthesis pathways involved two metabolites, and five other metabolites were also identified .

3.1 Effects of Ca^{2+} Stress on Lignin Synthesis

Lignin is essential not only for mechanical support and long-distance transport of water and nutrients but also for plant responses to abiotic and biotic stresses. Exogenous application of p-coumaric acid significantly increases proline content in chia (*Salvia hispanica*), while salt stress elevates p-coumaric acid levels in amaranth (*Amaranthus tricolor*). Our results similarly show increased p-coumaric acid content in *D. roosii* rhizome under 600 $\mathrm{mmol \cdot L^{-1}}$ Ca^{2+} treatment, suggesting that plants may promote p-coumaric acid synthesis to induce proline accumulation and protect biomembranes from damage under stress conditions. p-Coumaric acid and p-coumaroylquinic acid are upstream intermediates in chlorogenic acid synthesis. Transcriptomic analysis of tea plants under salt and drought stress revealed significant upregulation of HCT genes encoding hydroxycinnamoyl transferase, which catalyzes p-coumaroylquinic acid formation. Our results also show increased p-coumaroylquinic acid content in *D. roosii* rhizome under 1,200 $\mathrm{mmol \cdot L^{-1}}$ Ca^{2+} treatment. Exogenous chlorogenic acid effectively reduces membrane damage and lipid oxidation in apple leaves under oxidative stress while stimulating antioxidant and polyphenol oxidase activities. Endogenous chlorogenic acid significantly enhances antioxidant capacity in chrysanthemum, and its content increases markedly in honeysuckle under salt stress and in globe artichoke under drought stress. Our findings of significantly increased chlorogenic acid content under calcium stress suggest it may function as an antioxidant to protect plants from oxidative damage. Additionally, neochlorogenic acid content increased under 600 $\mathrm{mmol \cdot L^{-1}}$ Ca^{2+} treatment, consistent with observations in honeysuckle under salt stress, indicating its role in stress adaptation. These analyses demonstrate that lignin synthesis pathways play important roles in plant adaptation to adverse conditions, with lignin deposition promoting cell wall thickening to combat drought, salt, and cold stresses. Therefore, *D. roosii* rhizome may adapt to calcium stress through similar mechanisms.

3.2 Effects of Ca^{2+} Stress on Flavonoid Compound Synthesis

Abiotic stresses such as salinity, drought, and extreme temperatures cause ROS accumulation and oxidative damage, which flavonoids can mitigate through their antioxidant properties. Flavonoid treatment alleviates oxidative damage from salt and drought stress in rice, legumes, and tobacco. In ground asparagus, robinin, rutin, and apigenin contents increase significantly under salt stress, while anthocyanins, luteolin, apigenin, and quercetin increase in chrysanthemum under drought stress. Peach catechins, quercetin-3-rutinoside, quercetin-3-glucoside, and kaempferol-3-rutinoside also increase under cold stress. Our results show 21 flavonoid compounds increased in *D. roosii* rhizome at 600 $\text{mmol} \cdot \text{L}^{-1}$ Ca^{2+} and 12 increased at 1,200 $\text{mmol} \cdot \text{L}^{-1}$ Ca^{2+} compared with controls. These findings indicate that Ca^{2+} stress promotes flavonoid biosynthesis in *D. roosii* rhizome, enabling ROS scavenging to reduce oxidative damage and enhance calcium stress resistance.

3.3 Effects of Ca^{2+} Stress on Amino Acid Metabolism

Amino acid metabolism plays crucial roles in plant growth, development, and abiotic stress responses. Our results show increased trans-2-hydroxycinnamic acid content at 1,200 $\text{mmol} \cdot \text{L}^{-1}$ Ca^{2+}. This compound, an intermediate in phenylalanine metabolism, possesses antioxidant activity and may participate in ROS scavenging. N-methyl-L-phenylalanine content increased at both 600 and 1,200 $\text{mmol} \cdot \text{L}^{-1}$ Ca^{2+}, suggesting phenylalanine metabolism's important role in calcium stress responses. L-histidine and L-tryptophan increased at 1,200 $\text{mmol} \cdot \text{L}^{-1}$ Ca^{2+}, while DL-arginine and D-aspartic acid increased at both concentrations. Studies on *Dendrobium sinense* identified L-tryptophan as a key amino acid responding to drought stress, with multiple amino acids potentially regulating osmotic balance. Additionally, metabolomic analysis of blueberry leaves under salt stress revealed five affected amino acid metabolic pathways. Therefore, increased amino acid content in *D. roosii* rhizome under Ca^{2+} stress may play important roles in osmotic adjustment to enhance stress tolerance.

3.4 Effects of Ca^{2+} Stress on Fatty Acid Metabolism

Beyond energy storage, fatty acids and their derivatives play vital roles in abiotic stress resistance. As essential membrane components, polyunsaturated fatty acids are crucial for maintaining normal cellular functions. ω-6 and ω-3 desaturases are key enzymes in linoleic acid (18:2 and 18:3) biosynthesis, which constitute important plant membrane components. Fatty acid desaturases 2 (FAD2) and 6 (FAD6) are ω-6 desaturases that synthesize dienoic fatty acids in the endoplasmic reticulum and plastids. Under salt stress, FAD2 and FAD6 expression upregulates in *Arabidopsis* seedlings, and linoleic acid content increases significantly in rapeseed roots. Transgenic tobacco overexpressing FAD3 or FAD8 shows enhanced tolerance to drought and osmotic stress. Our results demonstrate increased unsaturated fatty acid content (three types at 600 $\text{mmol} \cdot \text{L}^{-1}$ and four at 1,200 $\text{mmol} \cdot \text{L}^{-1}$), along with increased phosphocholine and phosphoethanolamine at both concentrations. Choline is essential for phosphatidylcholine synthesis, while phosphoethanolamine can convert to phosphatidylethanolamine or be methylated to phosphocholine, which can further convert to choline. Choline oxidation produces glycine betaine, a potent osmoprotectant. These results suggest that increased unsaturated fatty acids help stabilize membranes, while elevated phosphoethanolamine and phosphocholine may play important roles in osmotic regulation under Ca^{2+} stress.

3.5 Effects of Ca^{2+} Stress on Active Component Content in *D. roosii* Rhizome

The Chinese Pharmacopoeia (2020) evaluates *D. roosii* medicinal quality based on naringin content. Previous studies comparing tree-grown and rock-grown *D. roosii* also used naringin as a key quality indicator. Multi-component quantitative analysis of 80 batches from different habitats evaluated protocatechuic acid, epicatechin, neoeriocitrin, luteolin, and naringin as quality markers aligned with traditional efficacy. Since flavonoids serve as primary active components, their content provides a viable quality assessment metric. Research shows that naringenin and luteolin increase significantly in *Prosopis farcta* under lead stress, epicatechin increases in grape under drought stress, and naringenin increases in lentil under heat stress. Our study found 25 flavonoid compounds increased to varying degrees under calcium stress, suggesting that medicinal quality may improve with moderate soil Ca^{2+} content. However, further in-depth research is needed to fully understand these quality effects.

In conclusion, *D. roosii* rhizome adapts to high-calcium karst environments through multiple mechanisms: lignin synthesis for cell wall thickening, flavonoid synthesis for ROS scavenging, amino acid synthesis for osmotic balance, and unsaturated fatty acid synthesis for membrane stability. High Ca^{2+} concentrations promote synthesis of various flavonoid metabolites, which serve as key quality indicators for *D. roosii* medicinal material. Therefore, appropriately increasing soil Ca^{2+} content during artificial cultivation may help improve medicinal quality.

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Note: Figure translations are in progress. See original paper for figures.