Exploring the adaptive mechanism of Camellia limonia in karst high calcium environment via an integrative analysis of metabolomics and metagenomics

Chunni Liu  
Guangxi Normal University

Yang Huang  
Guilin University of Electronic Technology

Feng Wu  
Guangxi Normal University

Wenjing Liu  
Guangxi Normal University

Yiqiu Ning  
Guangxi Normal University

Zhenrong Huang  
Guangxi Normal University

Yu Liang (✉ Liangyu@gxnu.edu.cn)  
Guangxi Normal University

Research Article

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Abstract

**Background:** Yellow *Camellia* is a kind of rare plant with high economic and medicinal value. It is known as the "giant panda" of the botanical world. *Camellia* group of plants (Camellia Sect. Chrysantha Chang) is the only golden flower of *Theaceae*. Compared with *Camellia nitidissima*, *Camellia limonia* grows in karst areas, where the soil has the characteristic of high calcium content. However, there are few studies about the *Camellia limonia* in karst soil environment and the adaptation mechanism is not clear.

**Results:** In this study, we found that under high calcium treatment, the chlorophyll content and leaf areas of *Camellia limonia* increased, while those of *Camellia nitidissima* decreased. The photosynthetic efficiency of *Camellia limonia* was more stable and higher than *Camellia nitidissima*. Compared with *Camellia nitidissima*, the conductance was larger and the degree of leaf shrinkage was smaller in *Camellia limonia*. The metabolomics analysis showed that the kaempferol-3-o-rutinoside, tyrosol, 6-o-methyldeacetylisoipecoside and (r)-mandelic acid are the main differently metabolic compounds. The results of karst high calcium soil metagenomics showed that microbacterium-testaceum, intrasporangium-calvum and rubrivivax-gelatinosus significantly changed. Through metabolomics and metagenomics integrative analysis, flavone and flavonol biosynthesis is suggested to be the main regulation pathway, which is regulated by apigenin, kaempferol, astragalin, isoquercitrin metabolites and TT7, UGT78D1, UGT78D2 genes. This metabolic pathway involves the synthesis of flavonoids. Flavonoids have the functions in drought and salt resistance, which play an important role in the adaptation of *Camellia limonia* in karst high calcium environment.

**Conclusion:** This omics study identified key regulation metabolites and genes for *Camellia* and provided important basis for the adaptive mechanism of plants to adapt to the high-calcium environment and the protection of *Camellia* species.

Introduction

*Camellia* is originated from China and have golden flowers, glossy leaves and beautiful appearance with transparent waxy surface [1]. It is a kind of rare plant that has not only high ornamental and medicinal value but also is very useful genetic resource. *Camellia* is mainly distributed in the southern China and the north of Vietnam [2]. *Camellia* species are heat-loving tropical plants, belonging to the tropical tree species, which have a high requirement for heat and mainly grow under shady and moist evergreen broad-leaf forests or along streamside in the mountains with the altitudes that is less than 700 meters [3].

*Camellia limonia* are an evergreen shrub with 1-2m in height. And their elliptic leathery leaves are 5-10cm in length and 2-3.5cm in width with in margin serrate. Their base is broadly wedge-shaped with dark brown glandular spots below. Flowers solitarily have 8 lemon yellow petals [1]. *Camellia limonia* mainly grow in karst rock mountain area, whereas *Camellia nitidissima* grow in normal soil mountain. Karst landscape is a typical ecosystem with very fragile ecological environment, mainly formed by carbonate dissolution. Karst regions cover about 20% of the dry ice-free land of the earth [4]. It is approximately 22
million square kilometers, accounting for about 12% of the total land area in the world [5]. China is one of the countries with the extensive karst landscapes and is famous for its diverse karst landforms [6]. Chinese karst landscape covers an area of 3.443 million square kilometers, accounting for one third of the country's land area. In southwest China, karst landforms cover an area of about 540,000 square kilometers, which is the largest and most representative karst fragile environment [6]. Located in the humid subtropical monsoon climate zone, the rainfall is abundant, but the precipitation has seasonal characteristics [7]. However, due to the obvious seasonal rainfall and dual hydrogeological structure, water in the karst area is formed only during the rainy season. As a result, karst soil water storage capacity is very limited, and has significant seasonal variation, which result in the shortage of water resources [4]. Because of the development of rock crevasses and falling-water caves, the situation of soil erosion becomes more serious. In these regions, the soil is shallow and discontinuous. Vegetation grows slowly with low biomass [5].

Karst landforms are mainly distributed in monsoon and valley rain forests, which is beneficial for the growth of *Camellia*. The soil types, ashes and elemental components (especially calcium) content of the *Camellia* in the two landform types are significantly different. Studies have shown that the leaf gray matter and calcium contents of *Camellia* in karst landforms are higher than the *Camellia* that grows in normal erosion landforms [8]. Karst is a very special environment whose shape is mainly the typical topography of the surface and underground part is formed by dissolving water on soluble rocks, which is the most typical topography in karst landforms [9]. Moreover, the soil in the karst areas is chiefly formed by weathering of bedrock carbonate rock, which causes the soil in the karst area having higher calcium content. Plants in karst areas have been adapted to grow in a drought and high calcium environment for a long time through their own physiological processes and structural adjustments in the evolution. Soil moisture and calcium carbonate content have the greatest impact on vegetation spectral characteristics in karst areas [10].

In the present research, we focused on the adaptation mechanism and physiological changes of *Camellia limonia* in the karst high calcium environment. In order to systematically obtain the regulation mechanism of *Camellia limonia* in the karst high calcium environment and find key pathway and important metabolites, integrative analysis of metabonomics and metagenomics was conducted and *Camellia nitidissima* was used as comparison. It is shown that flavone and flavonol biosynthesis is the main regulation pathway, which involves the synthesis of flavonoids. Flavonoids further play roles in functions in drought and salt stress, which might contribute to the karst adaptation of *Camellia limonia*. This study can improve the research about adaptive mechanism of plants in karst environments. Also, a multi omics study of *Camellia* can provide a practical foundation and valuable data resources for endangered plant conservation research.

**Methods**

**Plant material and high calcium stress treatment**
*Camellia limonia* and *Camellia nitidissima* used in this study were collected from Guangxi Institute of Botany. All the plants grew in outdoor greenhouse (located in Guangxi Normal University, Guilin, China) for shading cultivation. Ten plants of each variety with the same phenotype were selected as an experimental group. Each treatment plant was irrigated with 300mM calcium chloride solution every two days, while the control group was irrigated with pure water. Leaves were selected for metabolomics. Soil ponds with roots were selected as metagenome. The samples were collected on the 15th day after treatment when the phenotype of plants showed significant differences between *Camellia limonia* and *Camellia nitidissima*.

**Morphological measurement**

The morphological data of plants in high calcium treatment were collected every 5 hours from 7:00 to 18:00 every day. For each plant, at least 3 healthy leaves with the same leaf size and age were randomly selected to measure the photosynthetic parameters, leaf area, temperature, humidity, nitrogen content and chlorophyll content of leaves, respectively. Photosynthetic parameters were measured by Li-6400 photosynthetic apparatus [11]. Plant morphological meter TYS-4N was used to measure nitrogen content, temperature and humidity of leaves. The parameters of leaf length, width and leaf area of plant leaves were measured by a leaf area meter YMJ-B. By repeated freezing and thawing extraction to extract the chlorophyll of leaves were extracted and measured by the methods mentioned in previous relative references [12]. The *Camellia nitidissima* and *Camellia limonia* leaves after were selected for scanning electron microscope (SEM) and paraffin sections conservation based on the previous study [13].

Soil moisture, soil temperature and chemical element content were measured daily, including nitrogen (N), phosphorus (P), and potassium (K). The measurements were taken every five hours from 7:00 to 18:00 every day after high calcium treatment. The contents of N, P and K were measured by extracting soil of plants roots [14]. The PH, temperature and humidity of soil were measured by SOIL SENSOR.

**Metabolomic analysis**

The leaves under calcium treatment for 15 days were used to conduct metabolomic analysis. All the samples were stored at -80°C refrigerator and six biological repeat for *Camellia limonia* and *Camellia nitidissima*, respectively.

Multivariate analysis technologies including Principal Component Analysis (PCA) and Partial Least Squares (PLS) regressions were used at present study to identify several uncorrelated variables (principal components, PCS) from a large set of data [15]. Additionally, methodological advances in untargeted metabolomics and data analysis of tandem mass-spectrometric (MS/MS) data have enabled comprehensive analysis of molecular components in metabolic extracts [16]. These MS/MS spectra can be viewed as barcodes or fingerprints of metabolites and some software tools have been developed to take advantage of this structural information [17].

**Metagenomics analysis**
In order to study the microbial community composition and different express genes in the soil microorganism, we selected plants with obvious different phenotype traits after high calcium treatment to take the soil samples. The soil with the selected plant was taken with a small amount of roots from each plant. Then the samples were stored at -80°C refrigerator until sequencing and two biological repeat for *Camellia limonia* and *Camellia nitidissima*, respectively [18].

Illumina PE sequencing strategy was used to extract total DNA from the soil and then agarose gel electrophoresis was used to detect the quality of the samples and finally the library was obtained. In addition, the Velvet software was used for sequence assembly and finally functional annotation and comparison analysis were performed according to the literature [19, 20].

**Data processing and statistics analysis**

Data analysis was conducted by using the SPSS 20.0 program [4]. Independent-sample was used to detect the indexes of morphology and soil. Leaf morphology indicators include: net photosynthetic rate, stomatal conductance, transpirational rate, leaf area, chlorophyll, nitrogen content, leaf surface moisture and leaf surface temperature; Soil morphology indicators include: pH, temperature and humidity. The methods of mathematical statistics are used to calculate the error value and the significant difference. The results were taken into account as not significant at P > 0.05, significant at 0.01<P<0.05, and extremely significant at P<0.01. PCA and PLS are used to discriminate and analyze the error value and significant difference. A cluster analysis of the identified metabolites was conducted after auto-scaling (mean-centered and divided by the standard deviation) by using MultiExperiment Viewer [21].

**Results**

**Effects of calcium on the karst adaptive characteristics of Camellia**

Abiotic stress signals can cause fluctuations of cytoplasmic calcium ions and changes in phosphorylation forms of some proteins. Calcium ions mediated by calcium channels, change the concentration of calcium ions in cytoplasm and amplify the stress signals with intracellular calcium signal receptors [22]. However, high calcium stress can destroy the cytoskeleton and biofilm structure, resulting in intracellular metabolic imbalance.

In order to study the adaptability of the karst environment with high calcium content of *Camellia limonia*, we first processed *Camellia limonia* with high calcium content and take *Camellia nitidissima* as the control, the results showed that the adaptability of *Camellia limonia* to the environment with high calcium content was stronger.

Under the calcium stress, the phenotypes of *Camellia nitidissima* and *Camellia limonia* became different. The leaves of *Camellia nitidissima* turned yellow and wilting, while the leaves of *Camellia limonia* kept relatively healthy (Figure 1). The SEM and microscopic observation also showed that the leaves and roots of *Camellia limonia* are less wrinkled and dehydrated than *Camellia nitidissima* (Figure 2). The stomatal
conductance and transpiration rate of the two kinds of *Camellia* showed a downward trend. The stomatal conductance of *Camellia nitidissima* decreased more than the one of *Camellia limonia*. While the reduce degree of transpiration rate *Camellia limonia* is less than in *Camellia nitidissima*. The chlorophyll content of *Camellia limonia* increased by 53.12% and *Camellia nitidissima* decreased by 56.91%. The net photosynthetic rate of *Camellia limonia* was more stable than *Camellia nitidissima*. The average net photosynthetic rate of *Camellia limonia* and *Camellia nitidissima* was 3.0853 μmol CO$_2$ m$^{-2}$s$^{-1}$ and 2.4456 μmol CO$_2$ m$^{-2}$s$^{-1}$, respectively. Stomatal conductance affects the oxygen and carbon dioxide, indicating that the water might be the more important factor influencing the net photosynthetic rate when gas were equivalently affected [23]. According to the data, the average leaf humidity of *Camellia limonia* is 21.29% RH, *Camellia nitidissima* leaf humidity averages of 17.88% RH. The average transpiration rate and leaf temperature of *Camellia nitidissima* were 0.64 mmolH$_2$O m$^{-2}$s$^{-1}$ and 31.94 °C. *Camellia nitidissima* were 0.92 mmolH$_2$O m$^{-2}$s$^{-1}$ and 30.95 °C, suggesting the water content of *Camellia limonia* is more than *Camellia nitidissima* (Figure 3). According to the results above, *Camellia nitidissima* has relatively stronger adaptability to the environment with high calcium.

The soil moisture and temperature of the two kinds of *Camellia* did not obviously change. The pH value of the soil of *Camellia limonia* decreased slightly. In addition, the nitrogen and phosphorus content in the soil of *Camellia limonia* increased by 58.60% and 53.99%, respectively, while the nitrogen and phosphorus content in the soil of *Camellia nitidissima* decreased by 13.01% and increased by 49.63%, respectively (Figure S1).

Available nitrogen and phosphorus are the main nutrient elements that restrict plant growth, while plant growth is mainly restricted by nitrogen elements under low ratio of nitrogen and phosphorus [24]. Under high calcium stress, nitrogen content of *Camellia limonia* leaf increased by 14.44% and the available nitrogen in soil of *Camellia limonia* is increased by 58.60%, while nitrogen content of *Camellia nitidissima* leaf decreased by 28.57% and the available nitrogen in soil of *Camellia nitidissima* decreased by 13.01%, suggesting *Camellia limonia* has better nitrogen use efficiency (Figure 3), which may also help *Camellia limonia* survived in high calcium environment.

**Metabonomics of Camellia leaf in karst high calcium environment**

In order to analysis the different metabolites of *Camellia* leaf in karst high calcium environment, the metabonomics were conducted. The PCA score chart showed the degree of aggregation and dispersion of *Camellia limonia* and *Camellia nitidissima* (Figure S2). The distribution points of *Camellia limonia* are relatively close, indicating that the composition and concentration of the variables/molecules contained in *Camellia limonia* are closer.

A total of 9133 metabolites were identified through mathematical statistical analysis and screening, of which 6422 were in the positive ion mode and 2711 were in the negative ion mode (Figure S3). 8966 differential metabolites discemed include metabolites of *Camellia limonia* and *Camellia nitidissima*. The accurate molecular weight of the metabolites (molecular weight error <15ppm) were confirmed and the
fragment information were divided according to the MS/MS mode. We identified 220 different metabolites in HMDB (Human Metabolome Database, HMDB), metlin, massbank, lipiduaps, mzdound databases and obtained the statistical analysis results (Table S1). Under the high calcium stress, *Camellia limonia* has 53 metabolites up-regulated and 167 metabolites down-regulated, of which the main up-regulated metabolite is kaempferol-3-o-rutinoside and tyrosol. *Camellia nitidissima* has 167 up-regulated metabolites and 53 down-regulated metabolites, of which the main metabolites up-regulated are n-acetylhistidine, myristic acid, and pelargonin (Figure 4). The five different metabolites mentioned above have the largest significant difference. The content of these metabolites were changed to affect the metabolic process of *Camellia*, indicating that the significantly different metabolites mainly affect the growth of *Camellia limonia* and *Camellia nitidissima* after the treatment of high calcium stress.

Among them, kaempferol-3-o-rutinoside, tyrosol, 6-o-methyldeacetylisopecoside, n-acetylhistidine, myristic acid and pelargonin are positively correlated. But the three metabolites of *Camellia limonia* were negatively correlated with the three metabolites of *Camellia nitidissima* (Figure 5). It is inferred that the up-regulated metabolites of *Camellia limonia* had the opposite trend to the up-regulated metabolites of *Camellia nitidissima*, indicating that *Camellia limonia* have different metabolite trends in high calcium stress.

MetPA is a part of metaboanalyst (www.metaboanalyst.ca) and is mainly based on the KEGG metabolic pathway. The MetPA related metabolic and KEGG pathway analysis show 42 main metabolic pathways. The most important is biotin metabolism, tyrosine metabolism, flavone and flavonol biosynthesis, inositol phosphate metabolism, alpha-linolenic acid metabolism, ubiquinone and other terpenoid-quinone biosynthesis, phenylpropanoid biosynthesis, lysine biosynthesis, phenylalanine metabolism, glyoxylate and dicarboxylate metabolism, pantothenate and CoA biosynthesis, arginine and proline metabolism and other metabolic (Table S2). The pathways with most significant differences are tyrosine metabolism, flavone and flavonol biosynthesis, phenylpropanoid biosynthesis and biotin metabolism. And more differential metabolites are enriched in these four pathways, indicating that these pathways are more sensitive to high calcium stress for *Camellia limonia*.

**Metagenomics of soil in karst high calcium environment**

Under biotic and abiotic stresses, plants can actively change their own physiological conditions and exudates of their roots. That also has effects on the related microbial communities. Consequently, this directly or indirectly affects the health of plants. The initial central microorganism and exudates of plant root together form a microbial community, including plant central flora, core flora and other microbial communities. Compared with the plants under high calcium treatment, the root microbial populations of the plants in normal condition are different. Therefore, these different populations could affect the growth of plants. Metagenomics analysis of the soil of *Camellia limonia* and *Camellia nitidissima* root were conducted to study the effect of microbial communities under high calcium stress. The result showed that tyrosine metabolism, galactose metabolism, inositol phosphate metabolism, biotin metabolism, phenylpropanoid biosynthesis, arginine and proline metabolism were significantly affected. The
microorganism regulated these metabolic pathways. At the same time, they regulate the expression of responsive proteins through responsive genes. Finally, these microorganisms influence the metabolic pathways of *Camellia*.

The software Velvet was used to evaluate the assembly results, including the statistical table of assembly results, the distribution chart of the cumulative length of contigs and the distribution map of GC content (Table 1).

**Table 1 Assembly result statistics**

| Sample       | *Camellia limonia* | *Camellia nitidissima* |
|--------------|--------------------|------------------------|
| Total length (bp) | 103126589          | 86385177               |
| N50 Length (bp)   | 743                | 687                    |
| N90 Length (bp)   | 531                | 525                    |
| Max Length (bp)   | 55384              | 23635                  |
| Min Length (bp)   | 500                | 500                    |
| Sequence GC (%)   | 61.25%             | 62.85%                 |

We examined nearly 20,000 genes from each sample of rhizosphere microbes. And we found that reduced pathogenicity and loss of toxicity were the most important factors. In order to get a more detailed classification result of species, we match the sequence with the bacterial reference sequence or the virus reference sequence. Moreover, the MetaPhlAn was used to comment the species classification (Table 2). In all samples, rubrivivax is the main member. In root soil of *Camellia limonia*, rhodanobacter, intrasporangium, microbacterium, ilumatobacter are taking disadvantages. Different from *Camellia limonia*, the *Candidatus_Solibacter* was the dominant species of *Camellia nitidissima* (Figure 6). Further, in order to reveal the distribution and evolution relationship of species, phylogenetic analysis was conducted (Figure S4). The three colors represent the most important four species (*dehalogenimonas lykanthroporepellens, micavibrio aeruginosavorus, polymorphum gilvum* and *candidatus accumulibacter phosphatis*) and their evolution relationship. The above results about the metagenomic analysis indicated that, compared with *Camellia nitidissima*, the types of soil microorganisms of *Camellia limonia* under high calcium stress were different. And these microorganisms are related to the salt tolerance and drought resistance of plants.
Camellia limonia adaptive regulation to karst

In order to study the karst adaptation of Camellia limonia, we conducted the integrative analysis of metabolomics and metagenomics. The result showed that flavone and flavonol biosynthesis pathway which is one of the most affected pathways can be regulated by apigenin, kaempferol, astragalin, isoquercitrin metabolites and TT7, UGT78D1, UGT78D2 genes. This metabolic pathway involves the synthesis of flavonoids. Flavonoids have functions in drought and salt resistance [25], so flavonoids may also contribute to the karst environment adaptation for Camellia limonia. Flavonoids include kaempferol, rutin, hesperidin, anthocyanin, etc. Among them, kaempferol has the largest difference in content between Camellia limonia and Camellia nitidissima, and the content of Camellia limonia is greater than Camellia nitidissima. Therefore, Camellia limonia grow better than Camellia nitidissima under the same calcium stress, which indicated that kaempferol may play a critical role in the high calcium tolerance.

The citrate cycle (TCA cycle, Krebs cycle) is an important aerobic pathway for the final steps of the oxidation of carbohydrates and fatty acids. The citrate cycle is also an important pathway for differential metabolites. In the relative pathway of TCA cycle, the sdhA, frdA genes regulate the synthesis of succinate dehydrogenase/fumarate reductase-flavoprotein subunit. Compared with Camellia nitidissima, the sdhA, frdA were differently expression genes in Camellia limonia, which suggested that this two genes might simultaneously regulate the synthesis of fumarate and succinate and further affect the high calcium tolerance of Camellia limonia.

Discussion

Changes in gene regulation and metabolic material network lead to Camellia karst adaptation

Calcium ion is a necessary mineral element for plant growth and development [26]. It is also an important component of plant cell wall and has the function of maintaining intracellular ion balance and cell membrane stability [27]. Calcium ions in the cytoplasm are generally kept at a low concentration, and high concentration of calcium ions will disturb the physiological functions of plant cells [28]. The absorption of calcium by plants is positively correlated with the concentration of calcium ions in soil. If the concentration of calcium ions in soil is too high, the plants will suffer the high calcium stress. High concentration of calcium ions can seriously affect plant photosynthesis and inhibit plant development.
[29]. From the microscopic point of view, calcium ions function as a signal molecule of plant cells to environmental changes, but if the concentration of calcium ions in cells is too high, it will lead to cell dysfunction [30].

The phenotypic characteristics of *Camellia nitidissima* were significantly different from *Camellia nitidissima* after high calcium treatment. The leaves of *Camellia nitidissima* are easier to fall off, which is probably caused by increased abscisic acid in plants after high calcium treatment [31]. Moreover, the color of the *Camellia* leaves has changed obviously. Compared with *Camellia nitidissima*, leaf temperature, soil moisture and potassium contents of *Camellia limonia* are basically maintained at the same level after high calcium treatment. Chlorophyll content, phosphorus content, nitrogen content and leaf moisture of *Camellia limonia* are generally higher than *Camellia nitidissima*. While the pH of soil, transpiration rate of *Camellia limonia* are relatively lower than *Camellia nitidissima*. The Camellia plants lost much water which was mainly caused by higher transpiration under the calcium treatment. For *Camellia limonia*, its transpiration effect was relatively weak, indicating that this may be one of the results of adapting to the karst high calcium environment. *Camellia limonia* can properly undermine transpiration, which is beneficial to live in a karst high calcium environment. Transport of Ca$^{2+}$ to leaves depends on several factors, such as xylem sap Ca$^{2+}$ concentration, water absorption, transpiration and so on. Calcium accumulation may occur in organs with high transpiration. Low transpiration rates can ensure plants well hydrated, which is beneficial to absorb the extra calcium [32]. Therefore, *Camellia limonia* can adapt better to the high calcium karst environment under a certain transpiration intensity. While *Camellia nitidissima* had excessive water loss in the plant body due to relatively strong transpiration, which could affect various life activities and could not survive in karst high calcium environment.

The metabolites of *Camellia* have undergone tremendous changes under calcium stress. Some key genes regulate the synthesis and changes of metabolites in the high calcium environment (Figure 7; Figure 8). Among the changed metabolism pathways, biotin metabolism has the highest impact factor. At the same time, flavone and flavonol biosynthesis involve many metabolic pathways and their impact factors are also high. Flavone and flavonol biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, phenylpropanoid biosynthesis and flavonoid biosynthesis play a regulatory role in metabolite changes and regulation and some of these pathways are interconnected (Figure 7). Special metabolites take part in the defense of plants with both biotic and abiotic environmental factors [33]. Glycosylation is a common modification of the structure of plant specific metabolites. It broadens the structural diversity and improves the substrate molecule solubility, stability and transportability [34]. A variety of glycosylated phenolic metabolites have been accumulated in plant tissues, such as anthocyanins and flavonoids [35]. Our results show that flavonoid biosynthesis and anthocyanin biosynthesis are the metabolic pathways of anthocyanins and flavonoids. This suggests that anthocyanins and flavonoids produced by these two pathways could contribute to resisting adverse environment, such as over-acidity, over-alkalinity, high calcium and drought. It is speculated that the up-regulation of these two metabolites may play the key roles for *Camellia limonia* adaptation to the karst high calcium environment.
Moreover, the changes of different metabolites are also related to the overall metabolomic changes. Under high calcium stress, the chlorophyll content of the leaves of *Camellia nitidissima* decreased significantly and the leaves grew wrinkled (Figure 1; Figure 2). The leaves of *Camellia limonia* lack green luster on the surface, but their chlorophyll content remained relatively high. Plants with strong drought resistance have higher chlorophyll content and less biomass loss [36]. High calcium in the soil could cause the plants run out of water. Our result shows that the chlorophyll content of *Camellia limonia* is higher than *Camellia nitidissima*, which further suggested that enough chlorophyll content could ensure plant stability under high calcium condition. Under the high calcium treatment, some important pathways are very abundant, including energy metabolism associated with TCA Cycle, pathways associated with secondary metabolites, such as flavonol biosynthesis and flavonoids and other important pathways (such as biotin metabolism). All these pathways could also function in the adaptation of *Camellia limonia*.

**Response of Camellia to karst high calcium environment and its regulation model**

In order to further explore the regulation mechanism, we mapped the integrative analysis pathways, including differential metabolites, genes and enzymes (Figure 8). The analysis focused on the relationship of the pathways with higher influencing factors, such as biotin, flavone and flavonol biosynthesis, flavonoid biosynthesis, tyrosine metabolism, inositol phosphate metabolism. The results showed that tyrosine, p-coumaroyl-CoA and fumarate are important metabolites. They are the metabolites connecting key metabolic pathways.

L-tyrosine and fumarate connected tyrosine metabolism, phenylalanine tyrosine and tryptophan, TCA cycle, phenylpropanoid biosynthesis and other metabolic pathways. P-coumaroyl-CoA played a very important role in phenylpropanoid biosynthesis. P-coumaroyl-CoA is also an important hub in the regulation network. It links flavone and flavonol biosynthesis, flavonoid biosynthesis, phenylpropanoid biosynthesis. P-coumaroyl-CoA was involved in the phenylpropanoid pathway. P-coumaroyl-CoA mainly catalyzed the synthesis of 4-coumaroyl-CoA with the substrates of 4-coumaric acid and coenzyme A. The reaction is one of the rate-limiting steps which greatly affect the yields of lignins and flavonoids in phenylpropanoid pathway [37]. In the metabolic regulation processes, enzymes act as biocatalysts in many important bioreactions. In the phenylpropanoid biosynthesis, L-tyrosine is continuously catalyzed by 4-coumarate-CoA ligase, which is regulated by gene *ACOS5*. Shikimate O-hydroxycinnamoyl transferase is regulated by gene *SHT* (AT2G19070). 5-O-(4-coumararyl)-D-quinate 3’-monooxygenase is regulated by gene *CYP98A3*. Caffeate O-methyltransferase is regulated by gene *OMT1* (AT5G54160). All these genes mentioned above were changed in the present study. These enzymes finally lead to the synthesis of differential metabolite ferulic acid. Phenylpropanoid biosynthesis have an overlapping reaction chain. L-tyrosine can be turned to P-coumaric acid. P-coumaric acid can be transformed to P-coumaroyl-CoA catalyzed by 4-coumarate-CoA ligase (4CL). *ACOS5* and *CYP98A3* regulated the synthesis of coumarin. Coumarin is involved in the flavonoid synthesis pathway [38]. Studies about *Arabidopsis thaliana* have shown that flavonoids and flavonoids affect the drought-resistance of plants [39]. *OMT* (AT5G54160) and *SHT* (AT2G19070) regulate the synthesis of lignin [40]. Studies about *Arabidopsis*
*thaliana* have shown that lignin can improve plant tolerance in salt stress [41]. These pathways connect some key differently metabolites in the metabonomics of *Camellia*. Genes regulate the metabolic processes of *Camellia* which were also found similar function in other plants [41], suggesting these gene may play an important role in the karst adaption of *Camellia*.

Plants in karst areas have been in the high calcium environment for a long time. And they have also responded to various environmental stresses through their own physiological processes and structural adjustments in the long-term adaptive evolution. But the regulation mechanism of *Camellia* in karst high calcium environment still remain unclear. The present research explored the regulation mechanism of *Camellia* in high calcium environment by integrative analysis of metabolomics and metagenomics. These omics results and analysis shown that some key metabolites and genes may affect the *Camellia limonia* adaption of karst high calcium environment and lead to the difference of phenotype of *Camellia limonia* and *Camellia nitidissima*. The comprehensive omics level study on *Camellia* can provide practical research foundation and valuable data resources for future plant adaptability research in karst environments and conservation research of rare and endangered plants.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

Y.L., Y.H. and C.L. wrote the main manuscript text. F.W. prepared figures 1-3. W.L. prepared figures 4-5. Y.N. prepared figures 6 and all the tables. Z.H. prepared figure 8 and supplementary materials. All authors reviewed the manuscript.

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**Figures**

**Figure 1**

Phenotype of Camellias in high calcium stress. A: Camellia nitidissima plants, bar=25 cm; B: Camellia limonia plants, bar= 25 cm; Leaves of Camellia nitidissima (lower leaves) and Camellia limonia (upper leaves), bar=15 cm.
Figure 4

Hierarchical cluster analysis of metabolites. The relative content in the figure is shown by the different colors. The columns represent samples and the rows represent metabolites.
Figure 5

Differential metabolite-associated thermogram. Positive correlation tends to 1, and negative correlation tends to -1. At the same time, the cor.test function in R (v3.1.3) is used for statistical test of the metabolite association analysis, and there is a significant correlation with p-value ≤ 0.05.
Figure 6

Genus level and species level abundance (A and B). The horizontal axis represents samples and the vertical axis represents species. The color ranges from red to blue and the abundance ranges from low to high. The cluster diagram can only be made when the sample number is $\geq 3$. 
Figure 7

Pathways flavone and flavonol biosynthesis and flavonoid biosynthesis. Pathways flavone and flavonol biosynthesis and Flavonoid biosynthesis are vital. The figure showed their relationship.

Supplementary Files

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