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The Interrelationship between Latitudinal Differences and Metabolic Differences in the Natural Distribution Area of *Tilia amurensis* Rupr.

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Abstract: *Tilia amurensis* Rupr. is a crucial species widely used in our life, because its wood is easy to process due to its low specific gravity and good elasticity. To understand the effect of the latitudinal gradients on *T. amurensis* metabolites profiles, we collected data from six different latitudes about physiological indicators such as temperature, light, and precipitation, then analyzed the differences in *T. amurensis* metabolite profiles from these different latitudes. The metabolomes of the six latitudes (SFS 49°28′53.26″ N, WY 48°06′51.314″ N, LS 47°11′1.71″ N, BL 45°7′55″ N, BH 43°50′16.8″ N, and TS 40′30.89″ N) were compared using GC–MS/LC–MS, and significant differences in primary and secondary metabolites were found. A total of 29 primary metabolites were screened by orthogonal partial least squares discriminant analysis (OPLS-DA), and 34 flavonoids were determined using the targeted metabolomics methods. A total of 11 flavonoids in secondary metabolites were significantly different in the LS region compared with other areas. The main physiological indicator that differs between the LS region and other regions was the annual sunshine percentage. This indicates that the metabolic differences in *T. amurensis* at different latitudes may be affected by environmental factors such as annual sunshine percentage. As a vital species, *T. amurensis* metabolites change with different environmental factors, indicating that this species has different adaptability to the environment of different latitudes.

Keywords: *Tilia amurensis* Rupr.; latitude difference; metabolic responses; GC–MS/LC–MS

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1. Introduction

Tilia amurensis Rupr. (Tiliaceae) is up to 25 m tall, 1 m d.b.h., bark dark gray, exfoliating, branchlets white or reddish stellate pubescent, glabrescent; terminal bud glabrous, bud scales 3. Petiole slender is 2–3.5 cm, glabrous, leaf blade broadly ovate or ovate-orbicular, 4.5–6 cm × 4–5.5 cm, Petals are 6–7 mm. Stamens 20, 5–6 mm [1]. *T. amurensis* Rupr. has two subspecies with a broadly overlapping range; *T. amurensis* and *T. amurensis* ssp. *taquetii* (or var. *taquetii*) [2]. The difference between the two subspecies is that *T. amurensis* ssp. *taquetii* branchlets and cymes are reddish stellate pilose, leaf blade base usually truncate or slightly cordate and bract stalk 1–1.5 cm [3]. *T. amurensis* in China is named "ziduan" and commonly known as a famous tree species of honey source and is widely distributed in Korea, China, and Japan [4]. *T. amurensis* can reach 20 m in height and 1 m in diameter, with strong resistance to cold. With its biological characteristics of fast growth and good adaptability after transplanting, it has been widely planted as a landscaping and roadside tree [5]. The timber of *T. amurensis* trees is soft and easily worked, and the wood is popular

for carving and making musical instruments. Beekeepers value *Tilia* species as their nectar produces a very pale but richly flavored monofloral honey [6]. *T. amurensis* is a famous nectar tree species. It is also an essential material of high-quality plywood. The wood of *T. amurensis* is easy to process because of low specific gravity and good elasticity.

Differences in a latitudinal location often make plants in different provenances show different adaptations [7]. A wide array of external stimuli can trigger changes in the plant cells, leading to a cascade of reactions, ultimately resulting in the formation and accumulation of secondary metabolites [8], which is also the reason for the different adaptations. Because of the difference in factors such as light quality, UV light, and temperature, changes in the geographical environment affect plant secondary metabolites accumulation. For example, the duration and quality of radiation are different at different latitudes and unstable even in the same area [9]. The received solar radiation varies with the change in the sun angle and duration, so the light in different geographical locations produces different effects on plants. Although UV-B is only a small part of sunlight, it can also have a significant impact on plant metabolism and cause tissue damage [10]. Many studies found that flavonoids increase with UV-B intensity, especially quercetin or kaempferol. [11]. Low temperature has been shown to induce anthocyanin synthesis in various plant species [12]. Different environmental factors will affect plant metabolism, and these effects may be superimposed or offset. Understanding the changes in metabolites can make it clear what environmental factors dominate these changes, so studying the metabolites of *T. amurensis* at different latitudes is meaningful.

Metabolomics focused on the comprehensive study of endogenous low-molecular-weight metabolic entities and is a method applied to characterize changes in metabolites comprehensively [13–15]. Metabolomics has also unraveled the relationship between plant metabolites and the growth environment [16]. The primary analysis platforms in metabolomics studies are gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and nuclear magnetic resonance (NMR) [17,18]. In this study, the metabolic profiling through GC–MS and LC–MS were used to discriminate *T. amurensis* at different latitudes and identify the significant metabolites that produced the differences.

Previous studies have mainly focused on extraction conditions and the determination of polysaccharides in *T. amurensis* flowers [19]. The effects of selective cutting on the distribution pattern and interspecific association of arbor seedlings in *T. amurensis* secondary forest were also reported [20]. The chemical composition of *T. amurensis* and its antitumor and anti-inflammatory activities were also investigated [4]. However, relatively few studies have been conducted concerning the metabolic responses of *T. amurensis* to the environmental factors. This research aims to analyze the changes in metabolic profiles of *T. amurensis* from different latitudes by using GC–MS and LC–MS metabolomics techniques, to understand the adaptation of *T. amurensis* to different latitudes. Understanding the adaptation of *T. amurensis* in different environments is of great significance for future development.

2. Materials and Methods

2.1. Materials and Reagents

In this study, selecting six locations, *T. amurensis* was used as the research object (Figure 1A). Six samples of *T. amurensis* were collected from six different latitude regions of China from July to August 2019, geographical coordinates: SFS 126°46′42.43″ E 49°28′53.26″ N, WY 129°13′48.86″ E 48°06′51.314″ N, LS 128°52′47.59″ E 47°11′1.71″ N, BL 130°59′17″ E 45°7′55″ N, BH 126°36′2.7″ E 43°50′16.8″ N, and TS 117°3′14.54″ E 36°40′30.89″ N. These six sites are all natural distribution areas of *T. amurensis* in northern China. They have different average temperatures, annual sunshine percentages and total rainfall. Natural forests with a concentrated distribution of *T. amurensis* were selected as the research object for each sampling point. Select the *T. amurensis* trees with the growth degree the same and the south slope aspect; they grow well, and there is no human interference as the object and the positions of experimental materials are all tender leaves of

tertiary branches. The diameter at breast height of linden selected in the experiment was between 14 and 20 cm, and the distance between each tree was more significant than 300 m. Afterwards, select leaves that grow well and are free of pests and diseases, and select a total of 50 leaves in four directions for each tree. At least four repetitions were set for each location. The plants were identified as *Tilia amurensis* Rupr. by Professor Li-Qiang Mu and Professor Bao-Jiang Zheng (expert in plant taxonomy of Northeast Forestry University). All collected leaves were carefully washed and frozen (liquid N2), then stored at −80 °C until the GC–MS and LC–MS analysis.

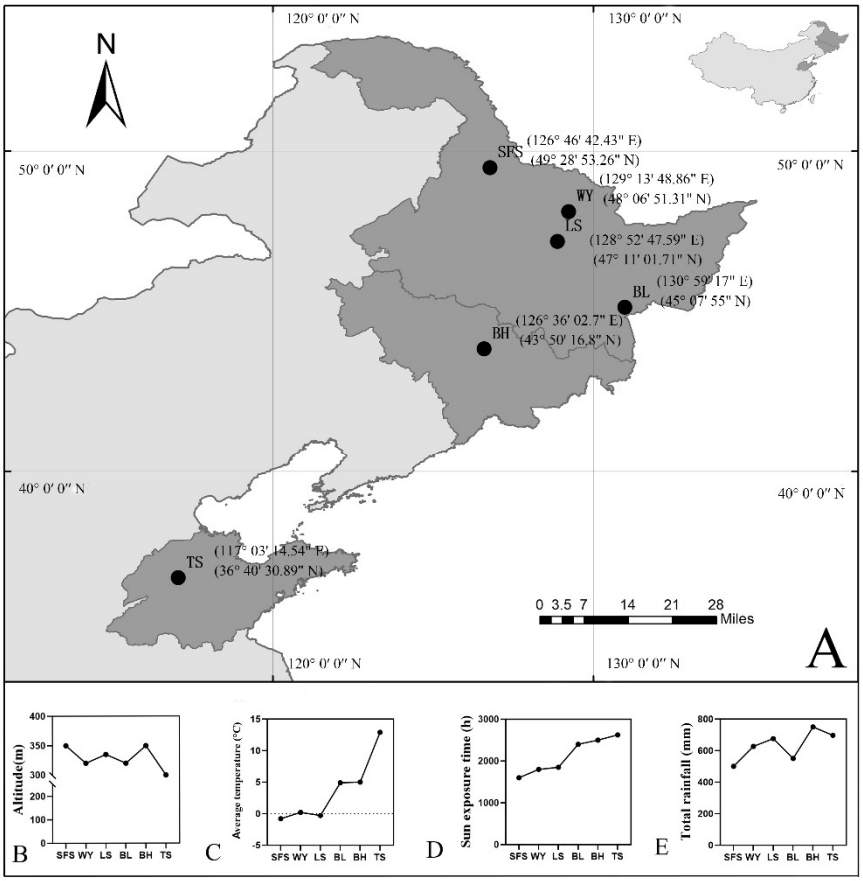

Figure 1. (**A**) distribution area of sampling points; (**B**) altitude (m) of sampling point; (**C**) average temperature (°C); (**D**) annual sunshine percentage (%); and (**E**) total rainfall (mm). Annual sunshine percentage means actual sunshine hours as a percentage of available sunshine hours over a one-year period.

2.2. GC–MS and LC–MS Analysis

Metabolites were extracted from leaf samples for GC–MS and LC–MS analyses following a previous related study [21]. The chromatographic separation process was based on an ultra-high-performance liquid chromatography system (LC, Waters, Milford, CT, USA), which was equipped with an LC20AD pump, temperature controller, column oven, and STL-204 white column injector, which enabled Minami ACQUITY UPLC BEH C18 Column (1.7 μm, 2.1 mm × 50 mm), were equipped with an in-line filter, and the column temperature was maintained at 25 °C. The mobile phase composition of water (A)-acetonitrile (B) was used at a flow rate of 0.25 mL/min.

An electrospray ionization source (ESI) QTRAP 5500 ion trap mass spectrometer (AB SCEX LLC, Framingham, MA, USA) was used to carry out SEM mass spectrometry and multiple-ion reaction monitoring (MRM) scanning mode. The optimized mass spectrometry conditions were as follows: the spray voltage of the electrospray ion source was 5500 V, the

atomization temperature of the ion source was 500 °C, the atomization air pressure was 25 psi, and the air curtain air pressure was 20 psi.

A total of 0.1 g of fresh sample was placed in a 3 mL test tube, and 540 µL of methanol and 60 µL of the internal standard were added, shaken, mixed, and sonicated for 30 min. Then, 300 µL of chloroform and 600 µL of water were added, and the sonication continued for other 30 min, then the supernatant was centrifuged for 10 min at 14,000 rpm at 4 °C. Next, 700 µL of the supernatant were evaporated, and 200 µL of methoxyamine pyridine solution were added to redissolve the solution, which was then incubated at 37 °C for 90 min. Then, 200 µL of BSTFA derivatization reagent and 40 µL of *n*-hexane were added. The sample was vortexed for 2 min, derivatized at 70 °C for 60 min, centrifuged to extract the supernatant, and analyzed by GC–MS.

A total of 0.5 g of fresh sample was weighed and added to a 10 mL test tube, then 3 mL of 70% methanol were added, extracted ultrasonically (100 kHz) for 30 min, and centrifuged at 6000× *g* for 10 min. The process was repeated, and the supernatants were combined and evaporated. They were reconstituted with 1 mL of 70% MS-grade methanol, ultrasonicated for 10 min, and centrifuged at 12,000× *g* for 10 min. The supernatant was aspirated and used for GC–MS analysis.

2.3. Data Preprocessing

GC–MS and LC–MS raw data were transformed into CDF format with data analysis software (Agilent GC–MS 5975, Santa Clara, CA, USA), and the raw data were uploaded to the XCMS platform for preprocessing, including row peak identification, peak filtering, peak alignment, etc. [22]. For further analysis, the XCMS output was treated, exported to Microsoft Excel, and then imported into SIMCA-P version 14.1 software (Umetrics, Malmö, Sweden) for multivariate statistical analysis (comparison of the global). Orthogonal artial least squares discriminant analysis (OPLS-DA) was employed to compare the different latitudes of *T. amurensis* to identify the significant critical compounds to elucidate the degree of difference in metabolites. The permutation test was used to calculate the validity of the OPLS-DA model against over-fitting 999 times of permutation employed in all models. GC–MS compounds with a variable influence on projection (VIP) value greater than 1.0 and a *p*-value below 0.05 were identified as potential biomarkers that could be obtained from the OPLS-DA model [21]. Heatmap Hierarchical Cluster Analysis using the metware cloud platform was completed. GC–MS pathway analysis using MetaboAnalyst 5.0 (accessed on 11 March 2022) was completed. MetaboAnalyst 5.0 (http://www.metaboanalyst.ca, accessed on 11 March 2022) is a free tool that applies the high-quality KEGG metabolic pathway database as its backend knowledge base for pathway analysis and visualization in this study. We searched the information of metabolic pathways through the Kyoto Encyclopedia of Genes and Genomes. In addition, the KEGG (http://www.genome.jp/kegg/, accessed on 15 March 2022) was the resource for the pathways search of the metabolites.

3. Results

3.1. Physiological Indicators of Influence on T. amurensis Rupr. Metabolic Differences

The primary climate type in northeast China is a warm-summer continental climate (Dwb), which gradually changes to a hot-summer continental climate (Dwa) with decreasing latitude. The six groups of *T. amurensis* samples in this study were taken from three different provinces and six regions, including Heilongjiang provinces ShengFeng Mountain (SFS), WuYing (WY), LiangShui (LS), Jilin provinces BoLi (BL), BeiHua (BH), and Shandong provinces TaiShan (TS), and the six groups showed noticeable differences in physiological indexes between different regions (Figure 1A). The growth and development of *T. amurensis* in different areas are influenced by a combination of factors such as average temperature, annual sunshine percentage, total rainfall, and other physiological indicators in that region. At the same time, the differences between habitats are still crucial factors affecting plant metabolism. We collected information on the average temperature, annual sunshine percentage, total rainfall, and altitude of these six regions in the same year through published

data (Figure 1B–E), which represent essential indicators of the growth and development of *T. amurensis*.

Through the analysis, we can find that the annual average temperature of the six collection sites showed a gradually increasing trend with decreasing latitude; the SFS area had the lowest annual average temperature of −0.8 °C, and the highest annual average temperature in the TS area was 12.9 °C, while the annual sunshine percentage showed a trend of decreasing and then increasing. The annual sunshine percentage in the SFS and TS areas is higher at 60%, while the annual sunshine hours in the LS area are the lowest at 43%. The total rainfall in each region also showed an increasing trend in general. Still, the BH area is lower than other regions except for SFS at 550 mm only, and the altitude showed an overall decreasing trend, with the highest altitude in the SFS area at 500 m and the lowest in the TS area at 300 m (Figure 1B–E). More detailed physiological indicators values are added in the Supplementary Materials (Table S1).

3.2. Primary Metabolite Profiling of T. amurensis at Different Latitudes

To study the metabolic differences of *T. amurensis* from different latitudes, we used the OPLS-DA model for data, which is a regression modeling method of multiple dependent variables on multiple independent variables and is characterized by the ability to remove data factors in the independent variable X that are not related to the categorical variable Y and can be interpreted and analyzed separately for orthogonal and non-orthogonal variables, allowing more accurate analysis of metabolite differences and associations in different samples [23]. The OPLS-DA model was fitted to the OPLS-DA model by the permutation test, and the obtained temperament data R2Y = 0.995 met our expectations for the experimental data model, indicating that the OPLS-DA model established in this study can effectively account for metabolic differences in *T. amurensis* at different latitudes. The OPLS-DA score plot (Figure 2) showed a more pronounced separation of the GC–MS data.

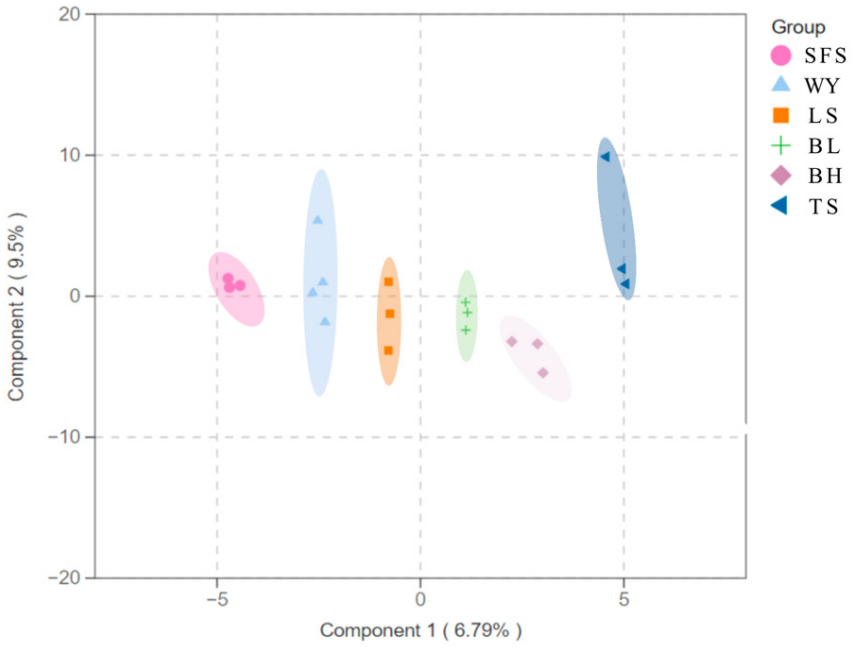

Figure 2. OPLS-DA score plot of primary metabolites at different latitudes of *T. amurensis.* Different colors represent different sampling points.

A total of 154 primary metabolites were detected in this study, mainly including alcohols such as ethenediol and benzenediol, sugars such as d-fructose and d-glucose, and some flavonoids and glycosides. To further screen the differential metabolites, the VIP (variable importance in the projection) value (VIP $\geq$ 1) in the OPLS-DA model was used, along with the combined analysis with the *p*-value ($p \leq 0.05$) of the t-test results to

determine the signature differential metabolites of the GC–MS data. A total of 29 differential metabolites were screened, including sugars (arabinofuranose, d-fructose, d-glucose, d-mannose, d-xylose, and maltose), glycosides (d-glucopyranoside and methyl galactoside) and precursors of synthetic polysaccharides (d-gluconic acid and myo-inositol). The differential metabolites could be classified as upregulated and downregulated by heatmap hierarchical clustering analysis, so the accumulation patterns of metabolites at the five different latitudes of *T. amurensis* samples could be visualized by heatmap hierarchical clustering analysis (Figure 3). The clustering shows that the metabolites screened by the six groups of samples are significantly different. Through the clustering analysis, we could find that the accumulation of d-lyxose and talose in *T. amurensis* from the WY region, myo-inositol, and d-gluconic acid from the LS region, malic acid, citric acid, and methyl p-benzoate from the BH region, D-mannose were significantly increased. Compared with the other five groups, TS had more upregulated metabolites. However, the relative content of l-rhamnose, gallic acid, and quininic acid in *T. amurensis* from the SFS region has a lower value.

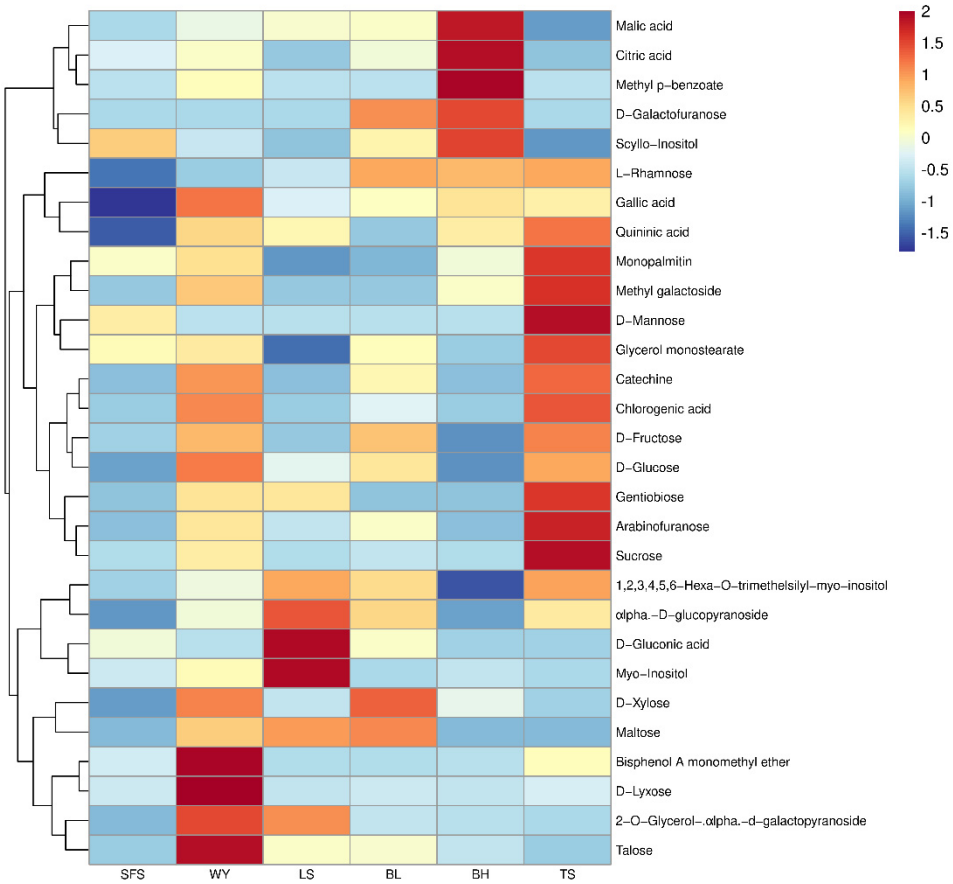

Figure 3. Distribution of primary metabolites at different latitudes of *T. amurensis*. Heat map visualization of the relative difference of potential biomarkers at different latitudes of *T. amurensis*. Data of the content value of each compound were normalized to complete linkage hierarchical clustering. Red indicates high abundance, whereas low relative compounds are blue (color key scale above heat map).

To further analyze the effect of latitude on different metabolic pathways, we imported the differential metabolites from the air GC–MS data into MetaboAnalyst 5.0 (accessed on 11 March 2022) for metabolic pathway analysis, and 17 pathways were identified (Figure 4). Based on -log *p*-values and pathway impact scores, the relevant metabolic pathways were identified as galactose metabolism, Citrate cycle (TCA cycle), starch and sucrose metabolism. By matching the heat map hierarchical clustering analysis, we found that myo-inositol and

d-gluconic acid accumulation increased in the LS region compared with other areas. Some studies have shown that the myo-inositol oxidation pathway provides the nucleotides required for cell wall synthesis. By comparing the data of average temperature, annual sunshine percentage, and total rainfall, we found that the annual sunshine percentage at the LS sampling site was 43%, significantly shorter than the other five areas. At the same time, the lower average temperature could also indicate that the growing conditions of LS are more demanding. Thus, the cell wall formation helps to resist the stress of a low-temperature environment (Figure 1D, Table S1).

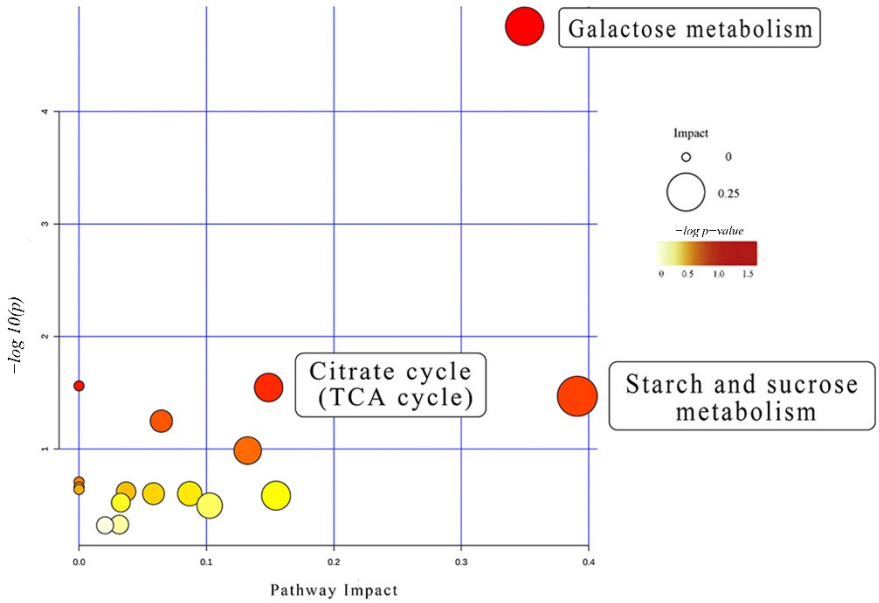

Figure 4. Metabolome map of significant metabolic pathways at different latitudes of *T. amurensis*. Significantly changed pathways are shown based on enrichment and topology analysis. The *x*-axis represents pathway enrichment, and the *y*-axis represents the pathway to impact. The larger size and darker color represent higher pathway enrichment and impact values, respectively.

3.3. Comprehensive Analysis of Flavonoids Secondary Metabolites

Latitude is one of the essential geographical factors that affect flavonoids in plants as a result of the combined effect of multiple environmental factors [11]. We detected 34 flavonoids by targeted metabolomics techniques. To further investigate the secondary metabolites differences among *T. amurensis* from different latitudes, we established an OPLS-DA model for LC–MS data (Figure 5). We found that six regions have apparent separation in LC–MS data. Through the heatmap and hierarchical clustering analysis (Figure 6), we concluded that the accumulation of metabolites such as hesperetin, genistein, apigenin, and other secondary metabolites in *T. amurensis* from the SFS and BL regions increased significantly. The accumulation of metabolites such as kaempferol, luteolin, naringin, and other metabolites decreased significantly. In contrast, the accumulation of metabolites such as kaempferol, luteolin, and naringin significantly increased, and the accumulation of metabolites such as hesperetin, genistein, and apigenin significantly decreased in the secondary metabolites in *T. amurensis* from the LS and BH regions, which is precisely the opposite of the situation in the SFS and BL regions. Interestingly, the three types of metabolites, vanillic acid, syringic acid, and benzoic acid, accumulated significantly in the three areas of SFS, LS, and BH, but decreased substantially in the other three regions. Finally, the TS region significantly increased the accumulation of quercetin, daidzein, chrysin, and naringenin. To further analyze the differences between various secondary metabolites, we performed an analysis of variance (ANOVA) using SPSS; $p < 0.05$ represented significant differences, and a total of 22 secondary flavonoids were significantly different, and their content is represented by a column chart (Figure S1). Figure 7 presents

these 22 secondary metabolites using box line plots. We grouped the significantly variable compounds by different carbon skeletons. We found that apigenin, galangin, genistein, hesperetin, and naringenin have a C6C3C6 skeleton; sinapic acid has a C6C3 skeleton, while syringic acid, benzoic acid, vanillic acid, and gentisic acid have a C6C1 skeleton. We found that the above compound accumulation from the LS region is significantly increased compared to the other five areas. Whereas, compared with the other five regions, the accumulation of glycyrrhizin with a C6C3C6 skeleton and cinnamic acid with a C6C3 skeleton in the BL region significantly increased. Interestingly, Kaempferol and luteolin with a C6C3C6 skeleton in the SFS region and l-phenylalanine with a C6C3 skeleton in the WY region showed a significant decrease in accumulation compared with the other five areas, which indicated that the effect of different latitudes on the secondary metabolites was substantial. The previous study showed that plants grown under low or high temperature conditions exhibited higher phenylalanime ammonia-lyase (PAL) activity, which led to the accumulation of flavonoids [24].

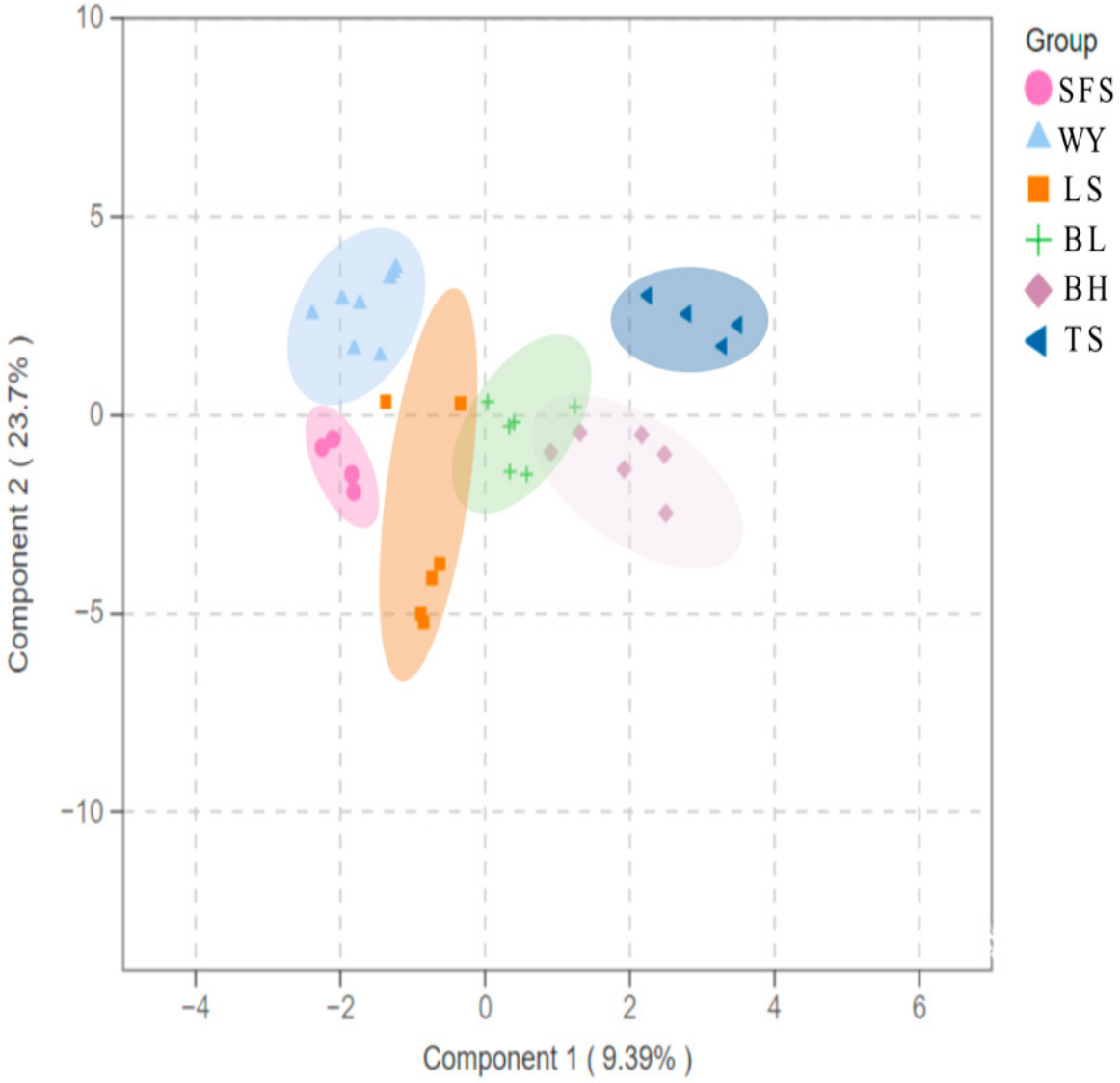

Figure 5. OPLS-DA score plot of secondary metabolites at different latitudes of *T. amurensis*. Different colors represent different sampling points.

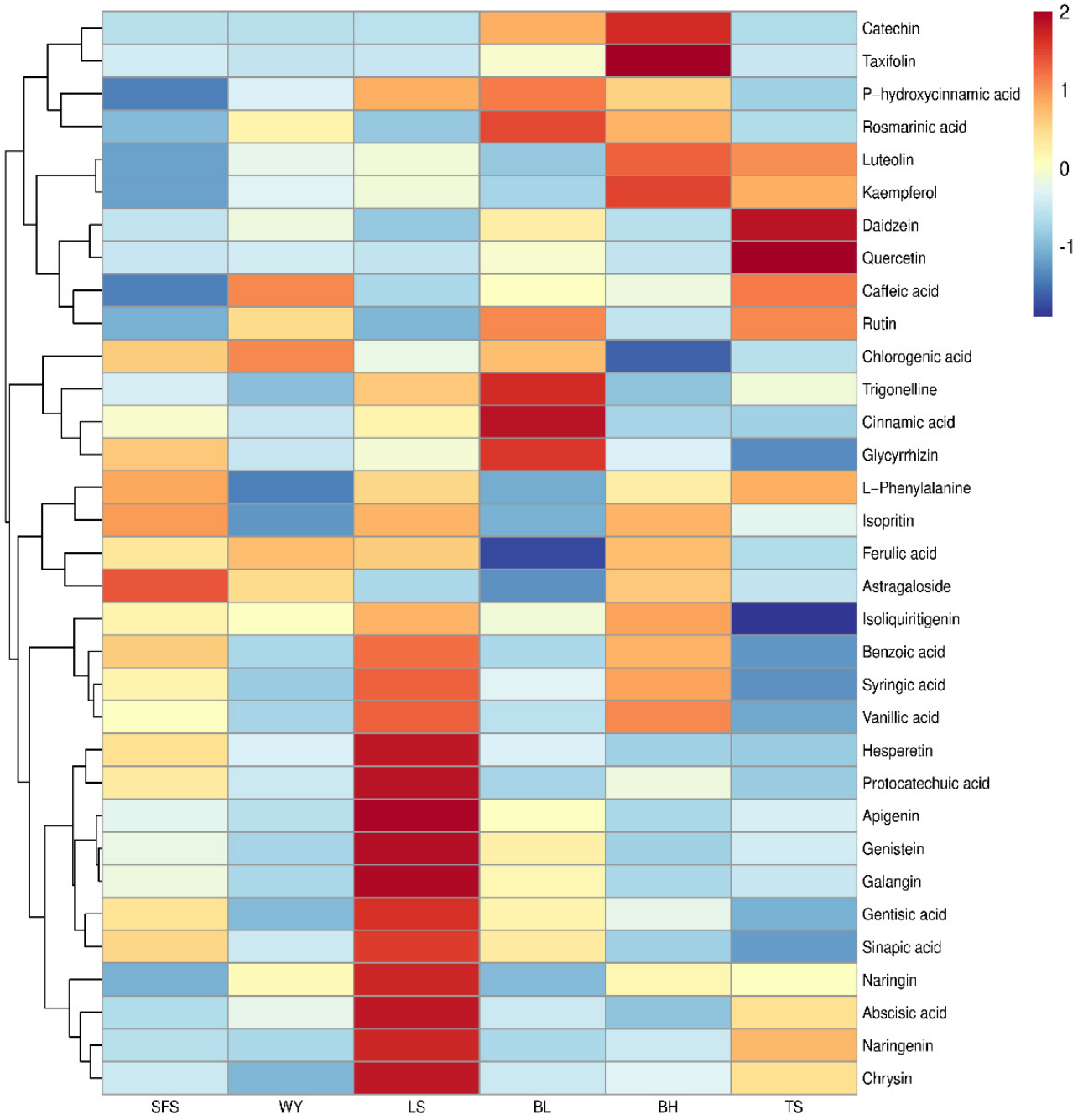

Figure 6. Distribution of secondary metabolites at different latitudes of *T. amurensis*. Heat map visualization of flavonoids at different latitudes of *T. amurensis*. Data of the content value of each compound were normalized to complete linkage hierarchical clustering. Red indicates high abundance, whereas low relative compounds are blue (color key scale above heat map).

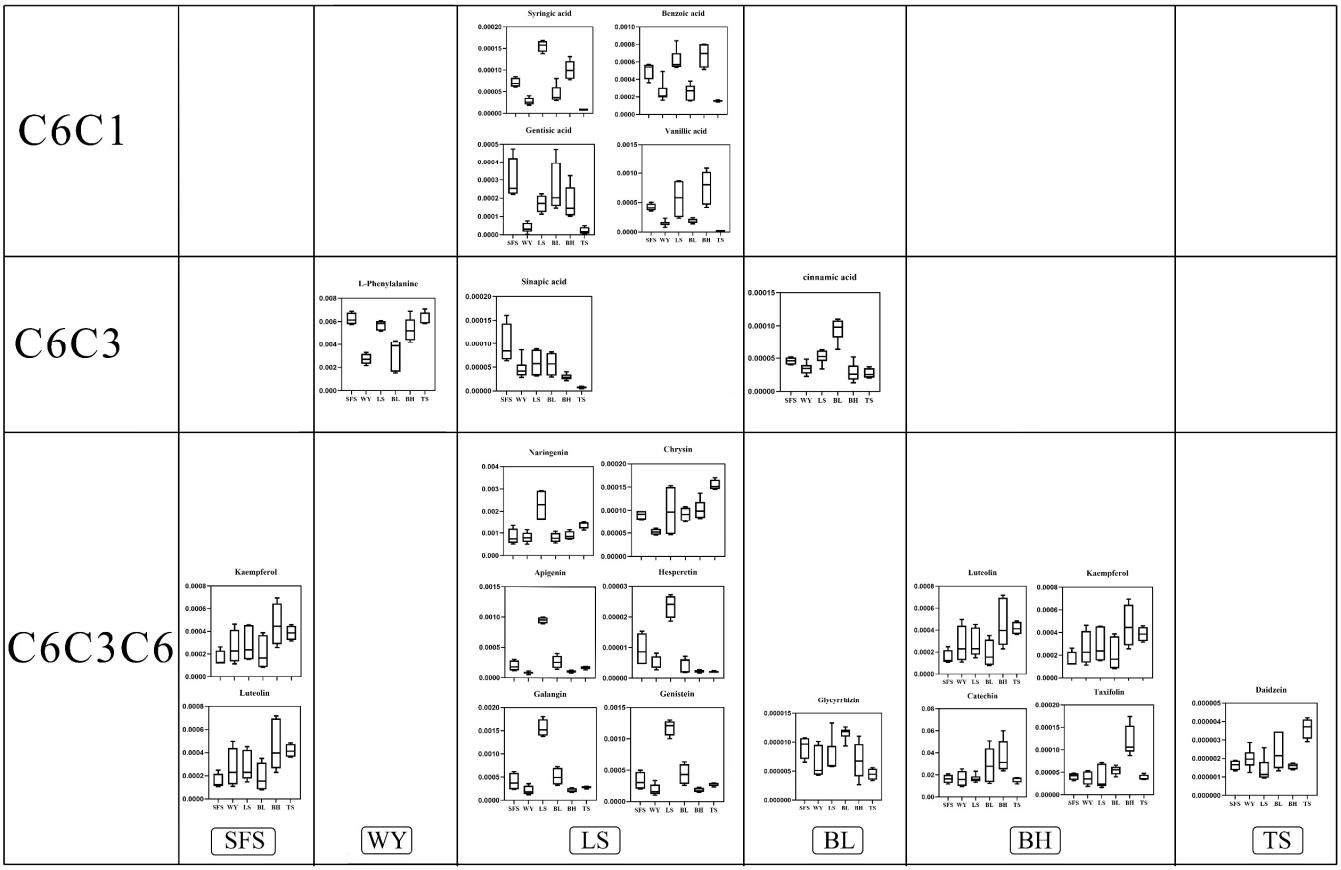

Figure 7. Box and whisker plots of significantly different metabolites. These secondary metabolites were identified through commercial standards. Classification of secondary metabolites that vary significantly at different latitudes by carbon skeleton.

A significant increase in the relative content of various secondary metabolites occurred in *T. amurensis* from the LS region. We can infer that this may be associated with the unique local climatic environment, where precipitation is concentrated in summer (with June–August accounting for more than 60% of the annual precipitation). The climate is variable, with low rainfall in spring and autumn, cold and dry in winter, with an annual sunshine percentage of about 1800 h and an average annual temperature of −0.3 °C. The combined effect of various climatic factors may result in a significant difference in secondary metabolites of *T. amurensis* from the LS region. It is also interesting to note that the degree of variation of secondary metabolites in WY at the same latitude as LS is also different from the LS region. We concluded that this phenomenon may be due to subtle environmental changes at the sampling site. We know from the relevant data that the ecological conditions in WY are longer than LS in terms of annual sunshine percentage. In normal circumstances, more UV-B exposure will lead to flavonoids accumulation, but the experimental results are somewhat different. They indicate that in addition to annual sunshine percentage, other habitat conditions will also affect the secondary metabolism of *T. amurensis*, which is caused by different latitudes. The combined effect of climate differences may be an actual reason for the secondary metabolites of *T. amurensis* at different latitudes. External environmental factors play a critical role in regulating plant secondary metabolism. They can cause significant changes in the content of secondary metabolites, and these external factors include light, temperature, moisture, and radiation. To further investigate the effects of climatic conditions at different latitudes on secondary metabolites of *T. amurensis*, we constructed a visualization of primary and secondary metabolite dynamics in a biochemical pathway map (Figure S2). This pathway map mainly includes flavonoids biosynthesis, flavone

and flavonol biosynthesis, phenylpropanoid biosynthesis, and biosynthesis of secondary metabolites pathway. Protocatechuic acid, phenylalanine, benzoic acid, and gentisic acid accumulation are similar and showed a significant increase in accumulation in the SFS and LS areas compared with other regions. In contrast, *T. amurensis* from the WY area showed a decrease in accumulation of all these substances during the process. Vanillic acid, syringic acid, and ferulic acid accumulation are similar and showed an increase in accumulation of these substances in the SFS, LS, and BH regions. In contrast, they showed a decrease in accumulation of these substances in TS and BL. Taxifolin, catechin, kaempferol, and luteolin belong to flavonoids biosynthesis and showed a significant increase in accumulation of these substances in the BH region compared to the other areas. In contrast, these substances significantly decreased in the SFS, WY, and LS regions. TS showed a significant increase in accumulation of quercetin. Still, interestingly, the accumulation of both substances decreased to varying degrees in all areas except the BL region, where there was a slight change in accumulation of quercetin. Similarly, the accumulation of apigenin, genistein, hesperetin, and naringenin also belongs to flavonoids biosynthesis and increased significantly in this area of LS. In contrast, the accumulation of these four substances decreased to different degrees in the remaining regions.

4. Discussion

Previous reports have described metabolic differences across latitudes in many crops, such as tobacco [25] and Korean domestic soybeans [26]. Until now, metabolic differences in *T. amurensis* from different latitudes have not been reported. Understanding the metabolic differences among latitudes is also significant as a widely used functional tree species. For example, different habitat adaptations may significantly impact introduction efforts; tree species with strong adaptability to the environment may introduce as street tree species in certain habitat conditions in harsher places.

We performed metabolomic measurements and attempted to reveal the primary and secondary metabolic differences from the different latitudes of *T. amurensis*. This study has found that various primary and secondary metabolites vary at different latitudes. We found that the LS region significantly increased the accumulation of d-gluconic acid and myo-inositol in primary metabolites. Inositols are six-carbon cyclohexane hexitols found ubiquitously in the biological kingdom, and their metabolism plays a vital role in growth regulation, membrane biogenesis, osmotolerance, and many other processes. The cyclic polyol inositol and its methylated derivatives play a protective role in plants and increase plant tolerance to abiotic stress [27]. Some studies also suggest that the inositol oxidation pathway provides the nucleotide sugars required for cell wall synthesis. This idea indicates that inositol oxidase utilizes oxygen molecules and catalyzes the oxidative breakage of inositol to produce glucuronic acid. In some views, inositol oxidase uses oxygen molecules and catalyzes the oxidative cleavage of inositol to generate glucuronic acid [28]. The accumulation of myo-inositol and d-gluconic acid in *T. amurensis* from the LS region indicates that low temperature can induce the synthesis of some precursor substances related to cell wall synthesis at specific periods, and the accumulation of upstream substances supplying downstream water-soluble polysaccharide synthesis may enhance the LS region *T. amurensis* cell wall formation. In contrast, water-soluble polysaccharides, an important regional component of the cell wall, play a role in intercellular communication and environmental stress tolerance [29]. In contrast, the accumulation of these substances was reduced in the SFS and BH regions, which may also indicate that, compared to these two regions, the LS regions showed a more positive response at the primary metabolic level in the face of environmental stress. In contrast, talose and d-fructose were increased to varying degrees in the WY area, and saccharides are known membrane protectants. They can stabilize cellular processes [30], and primary metabolism provides critical substrates for secondary metabolic pathways.

The TCA cycle, on the other hand, is the main metabolic pathway for all aerobic processes in the bioregional weave and is a major source of energy and an essential indicator

for growth and development, providing not only the necessary precursors for amino acid biosynthesis and general nitrogen metabolism, but also linking the metabolism of sugars, fats, and proteins [31]. The increased accumulation of citric acid and malic acid in *T. amurensis* from the BH region compared to other regions, and the increased efficiency of the TCA cycle pathway, led us to conclude that the climatic environment in this area is more suitable than other regions for *T. amurensis* growth. The transport of plant carbohydrates relies mainly on sucrose, while storage is dominated by starch, and the interconversion between sucrose and starch is crucial in maintaining intracellular osmotic pressure. Also, changes in environmental conditions usually cause the accumulation of starch and sucrose; in addition to sucrose as a transport, sugar also enters glycolysis and the TCA cycle to produce ATP and NADH. The increased accumulation of sucrose and d-glucose in the TH region may be associated with environmental changes, where plants store sugars such as starch in response to adverse environmental conditions.

In secondary metabolites, flavonoids, including chalcones, flavones, flavonols, anthocyanins, and proanthocyanidins, and their metabolic pathways have been extensively studied using biochemical and molecular biological techniques [32]. Legumes, and to a lesser extent non-legumes, also produce special flavonoids such as isoflavones [33–35]. Plant flavonoids are produced as adaptations to environmental stresses, and plants usually exhibit different flavonoid accumulation characteristics under different geographical and environmental conditions [36]. It has been shown that plants growing under low or high temperature exhibit higher phenylalanime ammonia-lyase (PAL) activity, leading to the accumulation of flavonoids that are highly affected by environmental factors such as average temperature, annual sunshine percentage, total rainfall and are considered to be the main way that plants protect themselves and reduce the damage caused by intense light. When resisting temperature stress, plants can produce flavonoids that play a physiological regulatory role, which is a positive response of plants to temperature stress. All in all, flavonoids intuitively reflect whether plants are under environmental stresses. Flavonoids are a representative group of secondary metabolites that have been recently called "specialized metabolites" because plants synthesize species-specific metabolites [37]. Most flavonoids in plants are produced as adaptations to environmental stresses. Different plants usually exhibit different flavonoid accumulation characteristics under different geographical conditions. Hence, flavonoids are the best class of secondary metabolites that show the effects of environmental changes on plant metabolism [38]. To understand the effect of different latitudes on the secondary metabolism of *T. amurensis*, this study conducted targeted metabolomic measurements of flavonoids in *T. amurensis* at different latitudes. From the results of target analysis, it is obvious that the compound with a C6C3C6, C6C3 and C6C1 skeleton in LS accumulated noticeable differences compared to the other five regions. In plants, many flavonoid biosynthetic genes were induced under stress conditions [39]. For example, the integrated effects of light intensity on growth and flavonoid accumulation in *Cyclocarya paliurus* (Batalin) Iljinsk. were studied, and the results showed that although shade had a negative impact on the contents of flavonoids, kaempferol, quercetin, and isoquercitrin in leaves, high accumulation of total flavonoids in the plant was observed in the intermediate shade [40]. Because of flavonoids' UV-B screening function, such substances already play a critical physiological role in the plant [41,42]. For example, plants accumulate a variety of phytochemicals, including sinapoyl malate, flavonoids, and phenolics, in response to UV-B exposure as a mechanism to ameliorate the deleterious effects of this radiation on cells [43]. Accumulation of these substances will also increase with enhanced UV-B radiation as a response to the intense radiation. The experimental results of our study showed that many secondary metabolites associated with UV-B radiation accumulated significantly in the LS region more than in the other five areas, but the annual sunshine percentage in this region was the lowest among the six groups. Therefore, we infer that the accumulation of corresponding flavonoids in the LS region is not entirely a response to UV-B radiation; this phenomenon is more likely a response to shade conditions. In previous studies on the association of birch (*Betula platyphylla* Sukaczev) leaf flavonoids

content with latitude, it was shown that there was also a negative correlation between latitude and lutein content. Also in the same study, individuals sampled a positive gradient of flavonol content. Correlations are explained by other environmental variables, including factors such as sunlight and temperature. Furthermore, flavonoids compensate each other at different latitudes [44].

Temperature is also the main environmental factor affecting biological metabolism. An increase in temperature can cause energy metabolic disorder [45]. Flavonoids have been implicated in the defense against ROS; ROS-scavenging activity has been invoked mainly for plant tolerance against low temperatures above 0 °C; meanwhile, flavonoids during freezing may be the scavenging of ROS [46,47]. Accumulation of kaempferol and quercetin is significantly increased in *Arabidopsis* under cold stress conditions [46]. However, it is also interesting that the annual mean temperature in the TS region is the highest. Therefore, these differences in flavonoid accumulation patterns due to latitudinal gradients may be due to more complex factors affecting plant secondary metabolites than latitudes alone [48]. These metabolites, especially the secondary metabolites, were closely related to the latitude. There is sufficient evidence that the reduction of annual sunshine percentage changes accumulation of many flavonoids, which is also related to the local climatic environment. The results of metabolomics also verified this conclusion in reverse.

Hence, we can say that we found apparent differences in metabolites accumulation within *T. amurensis* from different natural distribution areas, which may be affected by many environmental factors, mainly the annual sunshine percentage. The experimental results indicate that there are clinical differences in genetics and surely different physical mechanisms of adaptation. It is of practical significance for applying *T. amurensis* in different regions in the future to accurately understand the relationship between the metabolic difference of *T. amurensis* at the physiological level and the environment.

5. Conclusions

Overall, in this study, we use metabolic profiles of *T. amurensis* revealed by a combination of non-targeted GC–MS and targeted LC–MS techniques to investigate the metabolic differences at different latitudes. We found that the primary metabolites of *T. amurensis* were accumulated to varying degrees with different latitudes, including sugars. This may result from adaptation to the unique climatic conditions at this latitude. The data also explained that the secondary metabolites also have the same differences. For example, the accumulation of 10 flavonoids in the LS region is significantly increased compared with other areas; even in a similar region, the secondary metabolites of flavonoids also produce different accumulation patterns. This result shows that even in the natural distribution area, *T. amurensis* may also positively respond to stress, like a low annual sunshine percentage. This can also explain why *T. amurensis* shows different appearances at different latitudes. So, plants usually show different characteristics of metabolites accumulation under different geographical and environmental conditions, which are usually related to latitudinal gradients.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13091507/s1, Figure S1. Column chart of the contents of 22 significantly different flavonoids (Figure S1 A, B, C, D, E, F) and different letter means compounds with similar contents; Figure S2. Visualization of primary and secondary metabolite dynamics in biochemical pathway map; Table S1. Physical indicators at six sampling sites.

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