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GlPS1 overexpression accumulates coumarin secondary metabolites in transgenic Arabidopsis

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Research Article

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Abstract

The dried root of *Glehnia littoralis* is a traditional Chinese herbal medicine mainly used to treat lung diseases and plays an important role in fighting coronavirus disease 2019 pneumonia in China. This study focused on the key enzyme gene *GlPS1* for furanocoumarin synthesis in *G. littoralis*. In the 35S:*GlPS1* transgenic *Arabidopsis* study, the *Arabidopsis thaliana*-overexpressing *GlPS1* gene was more salt-tolerant than *Arabidopsis* in the blank group. Metabolomics analysis showed 30 differential metabolites in *Arabidopsis*, which overexpressed the *GlPS1* gene. Twelve coumarin compounds were significantly upregulated, and six of these coumarin compounds were not detected in the blank group. Among these differential coumarin metabolites, isopimpinellin and aesculetin have been annotated by the Kyoto Encyclopedia of Genes and Genomes and isopimpinellin was not detected in the blank group. Through structural comparison, imperatorin was formed by dehydration and condensation of zanthotoxol and a molecule of isoprenol, and the difference between them was only one isoprene. Results showed that the *GlPS1* gene positively regulated the synthesis of coumarin metabolites in *A. thaliana* and at the same time improved the salt tolerance of *A. thaliana*.

Key Message

Results showed that overexpression of the *GlPS1* gene in *Arabidopsis* can accumulate more coumarin and increase salt tolerance.

Introduction

In China, Chinese herbal medicines have been used for thousands of years. As early as ancient times, there was a legend that Shennong tasted all kinds of herbs. His classic book *Shennong Bencaojing* has been handed down today (Zhao 2020). Until now, Chinese herbal medicine culture has become an inseparable part of Chinese culture.

Nowadays, due to more adverse reactions caused by the use of western medicine, more and more people use Chinese herbal medicines to treat diseases (Liu et al. 2015). The bioactive components of Chinese herbal medicines play an important role in treating diseases. The development of new drugs cannot be separated from the theoretical guidance of traditional and ethnic medicines. Medicinal plants should be selected from the theoretical knowledge of traditional medicine. It is important to develop new drugs by extracting and isolating new bioactive components with *in vitro* or *in vivo* activities (Yuan et al. 2016). Bioactive ingredients mainly come from metabolites produced by the secondary metabolic pathway of plants. The secondary metabolites of plants mainly include five categories: alkaloids, flavonoids, phenolic acids, lignans, and terpenes, most of which are phenolic substances. Some compounds have biological activity and are widely used in medicine (Panche et al. 2016; Neag et al. 2018; Wink and Michael 2012; Ali et al. 2016; Barker 2019).

Plants produce and accumulate secondary metabolites through different metabolic pathways (Aharoni and Galili 2011). The phenylpropane biological metabolism pathway is important for phenolic substance metabolism, in which aromatic amino acids are finally converted into phenolic acids, flavonoids, stilbenes, coumarins, and other phenolic substances through a series of biochemical reactions (Shahidi and Yeo 2016). *Glehnia littoralis* is a perennial salt-tolerant plant belonging to the genus *Glehnia* in Umbelliferae, an endangered plant. The dried root of this plant can be used as medicine, commonly known as *G. littoralis* root. It is a tonic in traditional Chinese medicine. The whole plant of fresh *G. littoralis* can be eaten as a vegetable, and its roots can also be dried and processed into medicinal materials. In traditional medicine, *G. littoralis* root is mostly used to treat respiratory and pulmonary diseases, for example, cough, hemoptysis, dry throat, thirst, and other symptoms. Since the coronavirus disease 2019 (COVID-19) virus has spread worldwide, traditional Chinese medicine has played an important role in treating COVID-19 pneumonia. The therapeutic effect is very remarkable (Zhao et al. 2020). Some traditional Chinese medicine compounds contain *G. littoralis* root, so some effective ingredients in *G. littoralis* have played a positive role in fighting COVID-19 pneumonia (Can et al. 2020). There are many bioactive components in *G. littoralis*. Phytochemical analysis shows that the main active components of *G. littoralis* are phenylpropanoids, coumarins, lignans, flavonoids, organic acids and their derivatives, terpenoids, polyacetylenes, steroids, nitrogen-containing compounds, etc. Coumarins are the main bioactive components in *G. littoralis* (Song J et al. 2020).

Sixty-seven coumarins have been isolated from *G. littoralis*, including seventeen simple coumarins and six pyran coumarins. The largest number of furocoumarins is 44 (Yang et al. 2019). Coumarins are widely used in the medical field. They are widely existing natural and synthetic compounds with anti-inflammatory, antioxidant, hepatoprotective, antithrombotic, antiviral, antibacterial, antituberculosis, anticancer, antidepression, antihyperlipidemic, and anticholinesterase activities (Emami and Dadashpour 2015). To sum up, coumarins have very important medical and commercial value in medicinal plants. The amount of high-value secondary metabolites determines the market price of medicinal materials (Chizzola and Lohwasser 2020). Therefore, improving the quality of medicinal plants by technical means is a very meaningful scientific research task. Plants accumulate secondary metabolites under stress conditions. Shu et al. found that salt stress treatment of *G. littoralis* can increase the content of three furanocoumarins, psoralen, imperatorin, and isoimperatorin, in *G. littoralis* root. Therefore, appropriate stress is of great economic value in improving the quality of *G. littoralis* (Shu et al. 2019). With the development of science and technology, molecular breeding technology is widely used in medicinal plant breeding. Transcriptome analysis of medicinal plants is used to screen and obtain key enzyme genes of plant secondary metabolism to construct expression vectors. *Agrobacterium*-mediated technology is used to transfer the expression vector into recipient cells, and a new medicinal plant is screened and cultivated (Hao and Xiao 2015; Bandurska et al. 2016; Niazian 2019). The ultimate goal is to improve the quality of medicinal plants and increase their metabolite levels (Yang et al. 2021). Some medicinal plants, such as *Salvia miltiorrhiza*, are mature in terms of molecular breeding (Song et al. 2013).

To improve the quality of *G. littoralis*, this study preliminarily screened the key enzyme genes of cytochrome P450 epoxygenase furocoumarin in the *G. littoralis* transcriptome, including *PAL*, *C4H*, *4CL*,

C2H, *UDT*, *PS*, *MS*, and other secondary metabolic key enzyme genes (Song et al. 2020). The key enzyme genes *GlUDT* (GenBank accession no. MH507300.1) and *GlPS1* (GenBank accession no. MF632070.1) of furocoumarin were cloned and analyzed by bioinformatics. The construction of the overexpression vector has been completed (Song et al. 2018). This study further explored the *GlPS1* gene of *G. littoralis* to determine its protein function.

Materials And Methods

Plant cultivation

Arabidopsis thaliana (ecotype Columbia, Col-0) seeds were surface-sterilized, stratified by incubation in the dark at 4°C for 3 days, and transferred to a chamber with 8 h light/16 h darkness at 75% relative humidity (RH) and 22°C.

Fresh *G. littoralis* seeds were selected and harvested in July and August, dried in the sun, and packaged. The seeds were soaked in running water, put into a container of tap water overnight, discarding the excess water on the next day, and mixed. The seeds were packed into a black plastic bag, sealed, and treated at 4°C low-temperature dark treatment for 3 to 4 months. After ~ 3 months, a bulge was observed on the concave surface of *G. littoralis* seeds. For the swelling point, the shell on the seed was carefully scraped using a scalpel, and the residue was set aside. On a sterile table, the seeds were immersed in a 0.1% $HgCl_2$ solution for 20 min. The $HgCl_2$ solution was recovered, and the seeds were rinsed thrice with sterile water and placed on moist sterile filter paper for later use. A wound was gently cut on the seed convex shaft using an anatomy knife; the wound depth was not too deep. Using two scalpels in the seed pocket, light pressure was applied on both sides of the medial axis of the face to cause the *G. littoralis* seed to wound from the medial axis. The seed was split and divided into two and a half. At this time, elongated embryo nodules were visible to the naked eye. Structure: The embryo was taken out from the seed using an anatomical needle and placed in a tissue culture bottle or cone on MS medium in a bottle. The bottle was sealed, and the embryo was cultured in an illuminated incubator with 8 h light/16 h darkness at 75% RH and 22°C. *G. littoralis* embryos were put into an MS culture medium for ~ 15 days to obtain seedlings for the experiment.

Methyl jasmonate (MeJA) and NaCl-treated G. littoralis **seedlings**

The MeJA solution (200 μmol L^{-1}) was prepared with 50% ethanol solution as the solvent and applied evenly to the upper and lower surfaces of the true leaves of robust sterile seedlings of *G. littoralis* as the experimental group material. In the CK group, the real leaves were only coated with 50% ethanol solution, and the treatment method was the same as the experimental group. Dark treatment was carried out at room temperature for 16 h, the true leaves of the aseptic seedlings of the *G. littoralis* were cut, and the leaves were quickly frozen in liquid nitrogen and stored at − 80℃.

The filter paper was spread flat in the Petri dish, and the NaCl solution (200 mmol L^{-1}) was prepared with distilled water. After the filter paper in the Petri dish was moistened and the Petri dish was tilted, it was then appropriate to observe a very small amount of effusion on the outer side of the Petri dish. Tweezers were used to gently spread the roots of *G. littoralis* aseptic seedlings on the filter paper moistened with NaCl solution, and the roots were in full contact with the surface of the filter paper. The samples were incubated at 25℃ for 24 h for the sampling of the whole plant, quickly frozen in liquid nitrogen, and stored at − 80℃.

Rna Isolation And Quantitative Real-time Polymerase Chain Reaction (Qrt-pcr)

Total RNA was extracted from the leaves (MeJA-treated samples) and whole plant (NaCl-treated samples) using RNAprep Pure Plant Kit (Tiangen, China). RNA reverse transcription was performed using HiScript® III RT SuperMix for qPCR (Vazyme, China) and qRT-PCR using primer references (Table 1). qRT-PCR was performed using ABI QuantStudio 5 (Thermo Fisher, USA). qRT-PCR analysis was performed in a total volume of 25 μL containing 12.5 μL SYBR Premix ExTaq™ (TaKaRa, China), 0.5 μL of each primer, 1 μL cDNA, and 10.5 μL nuclease-free water. Gene expression was calculated using the Power = $2^{-\Delta\Delta Ct}$ method. qRT-PCR analysis was built on at least three biological replicates of each sample with three technical replicates.

Table 1
Nucleotide sequences of primers used in this study

Primer name	Sequence (5′→3′)
GlActin F[a]	ACTGAAGCGCCTCTCAATCC
GlActin R[a]	GGCTCACACCATCACCAGAA
GlPS1 F[a]	GGTTTTCGCAGACCGACCTT
GlPS1 R[a]	CACATGTGCTCTTTACTTGTCTCC
GlPS1 KpnI F[c]	CGGGGTACCGGTTTTCGCAGACCGACCTT
GlPS1 SalI R[c]	ACGCGTCGACCACATGTGCTCTTTACTTGTCTCC
At Actin semi F[b]	GATGGTTGGGATGGGGCAAAA
At Actin semi R[b]	CATCTCCAGAGTCGAGCACA
GlPS1 semi F[b]	ACTGAAGCGCCTCTCAATCC
GlPS1 semi R[b]	GGCTCACACCATCACCAGAA

NOTE: [a]Designed primers for qPCR amplification of the *GlPS1* and *GlActin* genes. [b]Designed primers for semi-qRT-PCR amplification of *Arabidopsis* genes. [c]Designed *Kpn*I and *Sal*I restriction sites primers for *GlPS1* ORF(Open Reading Fragement) PCR amplification.

Transformation and regeneration of Arabidopsis

The *GlPS1* gene was cloned by PCR from *G. littoralis* cDNA using *Kpn*I and *Sal*I restriction sites. The amplified fragment was inserted into the pCambia1300-221-3HA vector between the *Kpn*I and *Sal*I sites upstream of the 35S gene to form the vector pCambia1300-221-3HA:*GlPS1*. The pCambia1300-221-3HA:*GlPS1* vector was transformed into *Arabidopsis* ecotype Columbia using the *Agrobacterium tumefaciens*-mediated floral dip method (Xie et al. 2021). Transformed *Arabidopsis* seeds were selected on 1/2 Murashige and Skoog (MS) medium containing 30 $\mu g.mL^{-1}$ hygromycin. Three independent lines of the T3 generation were randomly chosen for further analysis. Refer to the DNA extraction method of transgenic *Arabidopsis* by Doyle (1991). The primers used are shown in Table 1.

Semi-qRT-PCR determination 35S:GlPS1 **transgenic** Arabidopsis

Total RNA was extracted from the whole transgenic Arabidopsis plant using RNAprep Pure Plant Kit. RNA reverse transcription was performed using HiScript® III RT SuperMix for semi-qRT-PCR and qRT-PCR using primer references (Table 1). Semi-qRT-PCR was performed using ABI VeritiPro PCR (Thermo Fisher). Semi-qRT-PCR analysis was performed in a total volume of 25 µL containing 12.5 µL Green™ Premix Ex Taq™ II (TaKaRa), 0.5 µL of each primer, 1 µL cDNA, and 10.5 µL nuclease-free water. The GlPS1 and GlActin gene-specific primers used for semi-qRT-PCR are shown in Table 1. Statistical data were evaluated by Student's t-test.

Phenotypic analysis of transgenic Arabidopsis **salt stress**

The seeds of three strains randomly selected from CK strains were fully mixed. Similarly, the seeds of three strains of GlPS1 were fully mixed and washed thrice with 75% ethanol for 60 s each time in a sterile environment. The seeds were placed on sterile filter paper for drying. The seeds were sprinkled onto a 1/2 MS solid plate with gradient NaCl concentrations of 0, 100, 150, and 200 mmol L^{-1}. The Petri dish was sealed with a sealing film and placed vertically in an illumination incubator for culture for 2 weeks. The culture conditions of transgenic *Arabidopsis* were 8 h light/16 h darkness at 75% RH and 22°C. At the end of the culture, *Arabidopsis* seedlings were taken out, and the root length (from the hypocotyl to the root tip) was measured and photographed. Statistical data were evaluated by Student's *t*-test.

Metabolomics analysis of transgenic Arabidopsis

Sample preparation and extraction: The freeze-dried leaf was crushed using a mixer mill (MM 400; Retsch) with a zirconia bead for 1.5 min at 30 Hz. A 100 mg powder was weighted and extracted overnight at 4°C with 1.0 mL of 70% aqueous methanol. After centrifugation at 10,000 *g* for 10 min, the extracts were absorbed (CNWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 mL; ANPEL, Shanghai, China;

www.anpel.com.cn) and filtrated (SCAA-104, 0.22 μm pore size; ANPEL) before liquid chromatography (LC)-mass spectrometry (MS) analysis.

High-performance LC (HPLC) conditions: The sample extracts were analyzed using an LC-electrospray ionization (ESI)-tandem MS (MS/MS) system (HPLC; Shim-pack UFLC Shimadzu CBM30A System; MS, Applied Biosystems 6500 QTRAP).

Analytical conditions: HPLC: column, Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 · 100 mm); solvent system, water (0.04% acetic acid)/acetonitrile (0.04% acetic acid); gradient program, 100:0 (v/v) at 0 min, 5:95 (v/v) at 11.0 min, 5:95 (v/v) at 12 min, 95:5 (v/v) at 12.1 min, 95:5 (v/v) at 15.0 min; flow rate, 0.40 mL min^{-1}; temperature, 40°C; injection volume: 2 μL. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

ESI-QTRAP-MS/MS: LIT and triple quadrupole (QQQ) scans were acquired on a triple QTRAP-MS, API 6500 QTRAP LC/MS/MS system, equipped with an ESI turbo ion spray interface, operating in a positive-ion mode and controlled by Analyst 1.6.3 software (AB Sciex).

ESI source operation parameters: ion source, turbo spray; source temperature 500°C; ion spray voltage, 5500 V; ion source gas I, gas II, and curtain gas were set at 55, 60, and 25 psi, respectively; the collision gas was high. Instrument tuning and mass calibration were performed with 10 and 100 μmol L^{-1} polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to 5 psi. DP and CE for individual MRM transitions were done with further DP and CE optimization. A specific set of MRM transitions was monitored for each period according to the metabolites eluted within this period.

Results

Effects of MeJA and NaCl stress on GlPS1 **gene expression of** G. littoralis

Proper salt treatment can induce salt stress and secondary stress in plants. Proper salt stress is helpful in improving the quality of medicinal plants. MeJA plant hormone can induce plants to produce defensive metabolites that can alleviate biotic and abiotic stresses faced by plants. MeJA also positively improves the quality of medicinal materials (Yu et al. 2019; Yang and Guo 2018; Zhou et al. 2021; Gengmao et al. 2014). To verify whether the *GlPS1* gene of *G. littoralis* is involved in secondary metabolism and salt stress, *G. littoralis* were treated with MeJA and NaCl salt stress. qRT-PCR was used to analyze the *GlPS1* gene of *G. littoralis* (Fig. 1a and b). After 24 h of treatment, *GlPS1* gene expression in *G. littoralis* treated with MeJA and NaCl was significantly higher than in the untreated control. The relative expression was nearly four times that of the control, indicating that the *GlPS1* gene successfully participated in the reaction process of secondary metabolism and NaCl stress in *G. littoralis*.

GlPS1 **overexpression enhanced the NaCl tolerance of transgenic** Arabidopsis

It is a good technical means to study gene function through model plants, especially in the study of abiotic stress of plants. *Arabidopsis* plants that successfully expressed exogenous genes were treated with abiotic stress and compared to the control group in morphology and physiological and biochemical indices. The gene function was deduced (Liu et al. 2020; Li et al. 2012). To speculate whether the *GlPS1* gene could improve the salt tolerance of *A. thaliana*, and the salt tolerance of plants was closely related to the accumulation of plant metabolites (Aharoni and Galili 2011), *A. thaliana* plants were prepared with 35S overexpression of the *GlPS1* gene. DNA detection results showed that the positions of the bands of *GlPS1* transgenic *A. thaliana* strains and the positive control were correct, whereas the negative control had no electrophoresis bands (Figure S1). The semiquantitative experimental results showed that the semiquantitative electrophoresis band of the *GlPS1* gene was not detected in the PCR results of the CK group. The semiquantitative electrophoresis band of the *GlPS1* gene was detected in all *GlPS1* transgenic *Arabidopsis* strains, indicating that the *GlPS1* gene was successfully expressed in *GlPS1* transgenic *Arabidopsis* strains (Fig. 1c).

To test the salt tolerance of transgenic *A. thaliana* with 35S:*GlPS1*, transgenic *Arabidopsis* seeds were planted with the CK and *GlPS1* groups on 1/2 MS plates with different NaCl concentrations, and the root length of the seedlings of the two groups was compared after 2 weeks for comparative analysis. *A. thaliana* seeds germinated into seedlings successfully in all 1/2 MS media. *A. thaliana* grew well in the medium supplemented with 0 and 100 mmol L^{-1} NaCl. The aerial part had two healthy intact cotyledons and true leaves. The root was long. *A. thaliana* grown on the medium without NaCl was stronger. On the medium supplemented with 150 mmol L^{-1} NaCl, the hypocotyl length of *A. thaliana* was obviously shortened. The true leaves and cotyledons showed slight wilting. On the medium supplemented with 200 mmol L^{-1} NaCl, the length of the *A. thaliana* plant was < 1 cm, indicating that the plant shrank seriously. The hypocotyl shortening was very serious. The leaves were pale green, and no true leaf structure was found (Fig. 2).

The hypocotyl length of two groups of *A. thaliana* was measured and analyzed. Results were presented in the form of histograms. When NaCl was not added, the average root length of *A. thaliana* in the CK and *GlPS1* groups was 4.3 and 3.3 cm, respectively. There was a significant difference between the groups. At a NaCl concentration of 100 mmol L^{-1}, the average root length of *A. thaliana* in the CK and *GlPS1* groups was 4.8 and 4.6 cm, respectively. There was no significant difference between the groups. At a NaCl concentration of 150 mmol L^{-1}, the average root length of *A. thaliana* in the CK and *GlPS1* groups was 2.0 and 2.5 cm, respectively, whirly half of the length compared to the previous value. However, the root length of *A. thaliana* in the *GlPS1* group was longer than that in the CK group. Data were reversed with significant differences. At a NaCl concentration of 200 mmol L^{-1}, the root length of *A. thaliana* in the CK and *GlPS1* groups was < 1 cm. There were significant differences between the groups (Fig. 1d).

Results showed that the salt tolerance of *A. thaliana* root transformed with the *GlPS1* gene was better under salt stress conditions. At the same time, it was speculated that the *GlPS1* group of *A. thaliana* was

more salt-tolerant because *GlPS1* overexpression accumulated metabolites in *A. thaliana*. Thus, it was necessary to systematically analyze the metabolites in the two groups of *A. thaliana* plants.

35S:GlPS1 **transgenic** Arabidopsis **metabolomics analysis**

To determine the secondary metabolites in the two groups of *A. thaliana*, plant metabolomics analysis was conducted on the CK and GlPS1 groups, and the 226 detected secondary metabolites were divided into 19 classes. The classification results are shown in Fig. 3a, including 32 coumarins, 29 phenolic acids, 27 flavonols, 27 glucosinolates, 24 alkaloids, 9 flavonoids, 5 plumeranes, 5 phenolamines, 5 lignans, 4 flavonoid carbonosides, 3 dihydroflavones, 3 isoflavones, 2 piperidine alkaloids, 2 sesquiterpenoids, 2 flavanols, 2 anthocyanins, 1 isoquinoline alkaloid, 1 amphetamine alkaloid, and 43 others.

To compare the accumulation of secondary metabolites in the two groups of *A. thaliana*, the 226 detected secondary metabolites were analyzed by heat mapping. The secondary metabolites were classified into five clusters marked as Roman numerals I to V, respectively. Compared to the secondary metabolites in the *GlPS1* group in clusters I and II, the secondary metabolites in the CK group were upregulated. In cluster III, most secondary metabolites were upregulated, and a few were downregulated. In clusters IV and V, most secondary metabolites were upregulated (Fig. 3c). Principal component analysis (PCA) showed that the two groups of experimental samples were separated obviously on the first principal component but not on the second principal component, indicating differences in the accumulation of secondary metabolites between the two groups. There was little difference in the types of secondary metabolites (Fig. 3b). PCA and cluster heat map analysis showed that there were differences in the accumulation of secondary metabolites between the two groups. The content of the total components in the *GlPS1* group was higher than in the CK group.

To further analyze the content and types of differential metabolites in the two groups, the logFC (fold change) value was used as the abscissa and the variable importance in projection VIP) value as the ordinate to construct a volcano map. The VIP value of 1 was set as the dividing line to distinguish the differential metabolites. Thirty different metabolites were obtained, of which 26 were upregulated and 4 were downregulated (Fig. 3d). Among the 30 differential metabolites, there were 12 coumarins, 2 glucosinolates, 5 alkaloids, 2 flavonols, 2 phenolic acids, 1 isoflavone, 1 anthocyanin metabolite, 1 phenolamine metabolite, 1 lignan metabolite, 1 flavonoid carbonoside, and 2 others. Eleven compounds, including 8-methoxypsoralen, isopimpinellin, imperatorin, isooxypeucedanine, wedelolactone, esculin, glucosinalbin, caffeoylcholine 5-glucoside, feruloylcholine glucoside, caffeic acid, and isorhamnetin-O-hexoside-O-rhamnoside-O-rhamnoside, had no effective relative peak areas in the CK group, but effective peak areas were detected in the *GlPS1* group (Table 2). Therefore, these 11 compounds were unique secondary metabolites of *GlPS1* transgenic *Arabidopsis*, and they were regulated by the *GlPS1* exogenous gene.

Table 2
Statistics of differentially accumulating secondary metabolite in CK and 35S:*PS1* transgenic *Arabidopsis*

	Compounds	Peak area				VIP ≥ 1	
Class		CK	GlPS1	P	FC		Type
Coumarins	Aesculetin	50838 ± 23536	138087 ± 7375	0.02	2.72	1.13	up
Coumarins	Daphnetin	44132 ± 21347	139460 ± 3102	0.01	3.16	1.22	up
Coumarins	Isoscopoletin	12829667 ± 2622924	81176333 ± 2510380	0.00	6.33	1.57	up
Coumarins	4-Methylesculetin	265270 ± 73755	1009583 ± 77513	0.00	3.81	1.33	up
Coumarins	6-Hydroxy-7-methoxy-coumarin	3096100 ± 1584212	16908333 ± 510308	0.00	5.46	1.50	up
Coumarins	8-Methoxypsoralen	9 ± 0	80331 ± 12445	0.00	8.93	3.49	up
Coumarins	Isopimpinellin	9 ± 0	25894 ± 2008	0.00	2.88	3.27	up
Coumarins	Imperatorin	9 ± 0	134537 ± 6147	0.00	1.49	3.59	up
Coumarins	Isooxypeucedanine	9 ± 0	129987 ± 5478	0.00	1.44	3.59	up
Coumarins	Phellopterin	12178 ± 4061	443910 ± 24754	0.00	3.65	2.20	up
Coumarins	Wedelolactone	9 ± 0	19161 ± 5170	0.02	2.13	3.20	up
Coumarins	Esculin	9 ± 0	6645067 ± 151901	0.00	7.38	4.26	up
Glucosinolates	Gluconapin	253103 ± 26409	650300 ± 70250	0.00	2.57	1.12	up
Glucosinolates	Glucosinalbin	9 ± 0	1512167 ± 68381	0.00	1.68	4.02	up
Alkaloids	Caffeoylcholine 5-glucoside	9 ± 0	74471 ± 11383	0.00	8.27	3.48	up

NOTE: Diffffferentially accumulating compounds were identifified using thresholds of VIP (variable importance in projection) ≥ 1 and fold change ≥ -1 (up-regulated) or ≤ 1 (down-regulated) in CK (transgenic *Arabidopsis* with empty vectors) compared to GlPS1(transgenic *Arabidopsis* with 35S:*GlPS1*). The mean and SD values for the peak area of each metabolite are listed. A two-tailed Student's *t* test of the means was performed and p values which were over 0.05 (indicating non-signifificance) have been indicated in bold.

	Compounds	Peak area				VIP ≥ 1	
Class		CK	GlPS1	P	FC		Type
Alkaloids	Feruloylcholine glucoside	9 ± 0	20634 ± 1846	0.00	2.29	3.22	up
Alkaloids	N-isobutyl-4,5-epoxy-2E-decaenamide	126642 ± 112367	3310333 ± 155769	0.00	2.61	2.13	up
Alkaloids	Camalexin	370595 ± 298636	23468333 ± 962923	0.00	6.33	2.41	up
Alkaloids	Cocamidopropyl βine	2299567 ± 1453144	50595333 ± 4161829	0.00	2.20	2.05	up
Flavonols	Isorhamnetin-O-Hexoside-O-rhamnoside-O-rhamnoside	9 ± 0	51902 ± 12447	0.02	5.77	3.40	up
Flavonols	Kaempferol-3-O-rhamnosyl-sophorotrioside-7-O-glucoside	191457 ± 63010	43307 ± 7138	**0.05**	2.30	1.36	down
Phenolic acids	Caffeic acid	9 ± 0	129453 ± 6377	0.00	1.44	3.59	up
Phenolic acids	Glucosyloxybenzoic acid	4567353 ± 3606822	19471000 ± 483242	0.02	4.26	1.39	up
Isoflavones	2'-Hydoxy,5-methoxy Genistein-4',7-O-diglucoside	119761 ± 114995	336410 ± 31108	0.07	2.81	1.18	up
Anthocyanins	Cyanidin	112889 ± 52934	312433 ± 11394	0.02	2.77	1.14	up
Phenolamine	p-Coumaroylputrescine	521777 ± 394617	1562433 ± 28175	0.04	2.99	1.20	up
Lignans	Pinoresinol-acetylglucose	371567 ± 218773	94396 ± 16341	**0.16**	2.50	1.12	down
Flavonoid carbonoside	8-C-Hexosyl-chrysoeriol O-feruloylhexoside	99158 ± 24712	39812 ± 4298	0.04	4.00	1.07	down

NOTE: Diffffferentially accumulating compounds were identifified using thresholds of VIP (variable importance in projection) ≥ 1 and fold change ≥ -1 (up-regulated) or ≤ 1 (down-regulated) in CK (transgenic *Arabidopsis* with empty vectors) compared to GlPS1(transgenic *Arabidopsis* with 35S:*GlPS1*). The mean and SD values for the peak area of each metabolite are listed. A two-tailed Student's *t* test of the means was performed and p values which were over 0.05 (indicating non-signifificance) have been indicated in bold.

	Compounds	Peak area				VIP ≥ 1	
Class		CK	GlPS1	P	FC		Type
Others	3,5,7,4'–Tetrahydroxy-Coumaronochromone	1925333 ± 1012556	5431333 ± 79192	0.03	2.82	1.15	up
Others	Androsin	424733 ± 270074	91641 ± 29987	**0.16**	2.20	1.20	down

NOTE: Diffffferentially accumulating compounds were identifified using thresholds of VIP (variable importance in projection) ≥ 1 and fold change ≥ -1 (up-regulated) or ≤ 1 (down-regulated) in CK (transgenic *Arabidopsis* with empty vectors) compared to GlPS1(transgenic *Arabidopsis* with 35S:*GlPS1*). The mean and SD values for the peak area of each metabolite are listed. A two-tailed Student's *t* test of the means was performed and p values which were over 0.05 (indicating non-signifificance) have been indicated in bold.

To analyze the metabolic pathways involved in 30 differential metabolites in the experimental metabolome and the related annotation information in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, differential metabolites were searched in the KEGG pathway, and 11 compounds were annotated. Three were involved in the biosynthesis of phenology, two in metabolic pathways, two in the biosynthesis of secondary metabolites, one in phenology biosynthesis, one in the degradation of aromatic compounds, one in arginine and proline metabolism, and one in anthocyanin biosynthesis (Fig. 4a). Phenylpropanoid biosynthesis was the metabolic pathway that involved the *GlPS1* gene. In the literature, this pathway was divided into 11 submetabolic pathways, among which coumarin biosynthesis was the metabolic process that this study focused on.

To analyze the significance and enrichment degree of differential metabolites, an enrichment map of the Rich factor and P values of differential metabolites was drawn (Fig. 4b). The focus was on the three compounds in phenanthroponoid biosynthesis. Compared to the other six metabolic pathways, the enrichment of phenanthroponoid biosynthesis was the most significant. Therefore, the *GlPS1* gene was significantly involved in *A. thaliana* biosynthesis.

To show the results of this metabolomics experiment more clearly in the published metabolic pathway, the results of the thermogram analysis of different metabolites were put into the published phenylpropanoid biosynthesis (Fig. 5). Phenylpropanoid and coumarin biosyntheses are two submetabolic pathways of phenylpropanoid biosynthesis, also the focus of this experiment. Phenylpropanoid biosynthesis was located upstream of coumarin biosynthesis, and the p-coumarate-CoA provided by it was an important reaction substrate for downstream coumarin biosynthesis. Imperatorin was similar to xanthotoxol in chemical structure, and both had a psoralen structure. It was speculated that accelerin is formed by the dehydration reaction between xanthotoxol and a molecule of isopentenol, but there might be a series of biochemical reactions between them. Therefore, it was speculated that imperatorin is the downstream product of coumarin biosynthesis. Results indicated that the functional protein induced by the *GlPS1* gene of *G. littoralis* played an important role in phenylpropanoid and coumarin biosyntheses.

Discussion

Phenylpropanoid biosynthesis is an important pathway of plant secondary metabolism, which uses several intermediates in the shikimic acid pathway as the core raw materials to synthesize metabolites. The shikimate pathway is located upstream of phenylpropanoid biosynthesis. In this process, hydroxycinnamic acid and some esters undergo a series of cascade reactions, such as reductase, oxygenase, and transferase. Combinatorial decomposition is carried out in different parts of plants, resulting in plant tissue differences (Vogt 2010). Phenylalanine produced by the shikimate pathway provides raw materials for downstream of phenylpropanoid biosynthesis. The catalytic products of L-phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumaric acid CoA ligase (4CL) provide a material basis for all subsequent branching metabolic processes. 4CL introduces the generated p-coumaroyl CoA into different biosynthetic pathways. It is the direct precursor of flavonoid, coumarin, or lignin biosynthesis (D'Auria 2006). When plants are stressed, the change in the concentration of the shikimic acid pathway intermediates may lead to the formation of new phytoalexins, volatiles, flavonoids, and anthocyanins in the whole phenological biosynthesis and protein resynthesis (Schoch et al. 2006). Most metabolites produced by plant secondary metabolism are stored in vacuoles in the form of glycosylation to participate in the subsequent biosynthesis reaction. Phenolic compounds, such as coumarins, are related to the survival strategy of plants. After extracellular information induction, the intermediate in the vacuoles is released into the cytosol for the next metabolism. Vacuoles are important organelles for the accumulation of secondary metabolic intermediates and also important for the biosynthesis and transport of intermediates (Shitan and Yazaki 2013; Xu et al. 2015; Shitan 2016; Park et al. 2017; Martinoia 2018; Brito and Martinoia 2018; Zhang et al. 2018; Taguchi et al. 2000; Shitan and Yazaki 2019).

MeJA is a plant endogenous hormone molecule usually used as an inducer and elicitor of plants (Zhao et al. 2005; Pauwels et al. 2009; Shi et al. 2019). MeJA has been used for a long time to regulate the secondary metabolism of plants to trigger stress reactions and produce plant secondary metabolites (Rahnamaie et al. 2019). External application of MeJA to plants can upregulate or downregulate genes related to plant secondary metabolism in a short time. At the same time, the amount of secondary metabolites in plant cells is increased. Rahnamaie et al. analyzed the transcriptome of leaves of *Polygonum minus* plants with MeJA and obtained 2374 differentially expressed genes, of which 1419 were upregulated and 955 were downregulated, including secondary metabolic synthetase gene and transcription factor (Rahnamaie et al. 2017). In agricultural production, the quality of agricultural products can be improved by applying MeJA externally. Using MeJA externally in apples can induce a blush phenomenon in a short time. Many phenolic compounds accumulate in the peel, which improves the apple's quality (Shafiq et al. 2013). Exogenous MeJA can increase volatile components in tea, especially geraniol, linalool, and their oxides. Furthermore, MeJA can activate the secondary metabolic pathway, especially terpene volatile components (Shi et al. 2017). Spraying healthy *Ocimum basilicum* L. with 0.5 mM MeJA can accumulate total phenol content in plants and increase significantly (Kim et al. 2006). MeJA-induced treatment can promote the release of volatile organic compounds from leaves of *P. minus*, and its components are mainly terpenoids. Terpenoids mediated by MeJA are related to plant

resistance to biotic and abiotic stresses (Rahnamaie et al. 2019). On the molecular mechanism of MeJA regulation, exogenous MeJA can regulate JA biosynthesis and JA signaling pathway and regulate downstream gene expression, including genes related to the key enzymes of the phenylpropanoid pathway, such as PAL, C4H, 4CL, chalcone synthase, chalcone flavanone isomerase, flavanone 3-hydroxylase, and dihydroflavonol reductase. Many genes encoding transcription factors were induced by MeJA (Rahnamaie et al. 2017).

Salt stress affects the secondary metabolic process of medicinal plants (Yang et al. 2018; Gupta and Huang 2014; Sytar et al. 2018). The increase of salinity in the soil makes plant roots suffer from salt stress. In the initial stage, due to osmotic stress mediated by high salt concentrations (Xu et al. 2016; Munns 2002; Adak et al. 2019), plant roots lose water and the water absorption capacity of roots drops sharply. The roots are dehydrated, vacuoles and cytoplasm lose water, and the cytoplasm becomes thicker (Mahajan and Tuteja 2005). To resist the water loss caused by salt stress, plants accumulate specific metabolites, such as phenolic compounds, to resist biotic and abiotic stresses, resulting in reduced water potential of plants and water absorption in the soil (Yi et al. 2010; Fu et al. 2011; Abideen et al. 2015; Parida et al. 2004; Ksouri et al. 2007; Hanen et al. 2008; Lim et al. 2012; Petridis et al. 2012; Borgognone et al. 2014; Falcinelli et al. 2017; López et al. 2009; Valifard et al. 2017; Imen et al. 2012; Daneshmand et al. 2010; Petrusa and Winicov 1997; Aziz et al. 1998; Pedrazani et al. 2003). The accumulation of secondary metabolites in plants is related to the ability of plants to cope with salt stress. In this experiment, the successful heterologous overexpression of the *GlPS1* gene in *A. thaliana* and the subsequent metabolomics analysis confirmed that the *GlPS1* gene might accumulate secondary metabolites in *A. thaliana* because transgenic *Arabidopsis* has more obvious salt tolerance. Additionally, the *GlPS1* protein may play a direct role.

The psoralen synthase (PS) protein sequence has been cloned and sequenced in many species, but its protein function is rarely published. In 2006, the first *PS* gene in *Ammi majus* plant by molecular biology was cloned and verified (Larbat et al. 2007). In 2020, the protein activity of the PS gene *CYP71AJ49* of *Peucedanum praeruptorum* was confirmed by yeast experiments *in vivo* and *in vitro*. HPLC-ESI-MS verified that the *CYP71AJ49* gene could transform marmesin into psoralen. It was subsequently speculated that *CYP71AJ49* might have other activities (Ramos et al. 2018; Larbat et al. 2007; Kim et al. 2017, 2021; Xie et al. 2019; Silva-Junior et al. 2018; Pecrix et al. 2018; Raymond et al. 2018; Bredeson et al. 2022; Hufnagel et al. 2022; Jian et al. 2020). The *GlPS1* gene was heterologously expressed in model plant *A. thaliana*, and metabolomics analysis was carried out. The heterologous expression of the exogenous gene in the model plant *A. thaliana* through the study of the gene function of plant metabolomics is rarely reported in the literature. This study made a breakthrough in previous metabolomics research methods of *A. thaliana* and proved the function of the *GlPS1* protein of *G. littoralis* by analyzing relevant metabolomics data (Saito 2013; Meng et al. 2019; Lan et al. 2021; Jaini et al. 2017; Zhu et al. 2019).

Coumarin biosynthesis of *G. littoralis* is similar to the published metabolic pathway. Based on the analysis of different metabolites between the CK and *GlPS1* groups, 11 metabolites were not detected in

the CK group. Three metabolites, isopimpinellin, esculin, and caffeic acid, were noted in the metabolic map (Fig. 4). The imperatorin site was a putative pathway. It was speculated that these four coumarin metabolites might not exist in wild *A. thaliana*. In contrast, the content of the *GIPS1* group was significantly higher than the CK group. The unique variable *GIPS1* protein played an important biological function. The metabolites of psoralen could not be detected due to the limitation of standard quantity samples; however, the structural formulas of isopimpinellin and imperatorin both contained psoralen units. Thus, it was speculated that the psoralen content in the *GIPS1* group was higher. Imperatorin was formed by the dehydration reaction with isopentenol based on psoralen. It was also speculated that this process is completed by isopentenyl transferase (IPT). The *IPT* gene has been reported in many species. It mainly functions as isoprene tRNA and participates in the *de novo* synthesis of a cytokine through ADP/ATP-IPT. However, whether IPT plays a role in coumarin biosynthesis is unknown (Chen et al. 2021; Jameson et al. 2015). Therefore, relevant experimental data are needed to confirm it. In addition, the *GIPS1* gene is also involved in other metabolic pathways of *A. thaliana*, upregulating two metabolites (p-coumaroylputrescine and cyanidin 3–O–3",6"-O-dimalonylglucoside). P-coumaroylputrescine is a secondary metabolite of phenolic amides induced by biotic and abiotic stresses in plants, especially by trauma. Therefore, these metabolites can be used as antimicrobial, herbivorous compounds to reduce ultraviolet stress (Andama et al. 2022; Alamgir et al. 2016; Shinya et al. 2021).

Conclusions And Perspectives

There are few related studies on *G. littoralis* because it is difficult to study the species. When *G. littoralis* seeds and plants are stored and grown in the laboratory, the requirements on temperature, air humidity, soil type, water content, light intensity, etc., are strict. The experiment would fail if it was careless. Therefore, it was a good choice to study the genes related to the secondary metabolism of *G. littoralis* through the model plant *A. thaliana* and metabolomics; however, genetic engineering and breeding of *G. littoralis* are also very important scientific research tasks. Based on this study, the experimental method of infecting *A. thaliana* can be considered to prepare *G. littoralis* transgenic plants.

Abbreviations

Methyl jasmonate (MeJA), L-phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumaric acid CoA ligase (4CL), p-coumaroyl CoA 2'-hydroxylase (C2H), umbelliferone dimethylallyl transferase (UDT), psoralen synthase (PS), marmesin synthase (MS).

Declarations

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Figures

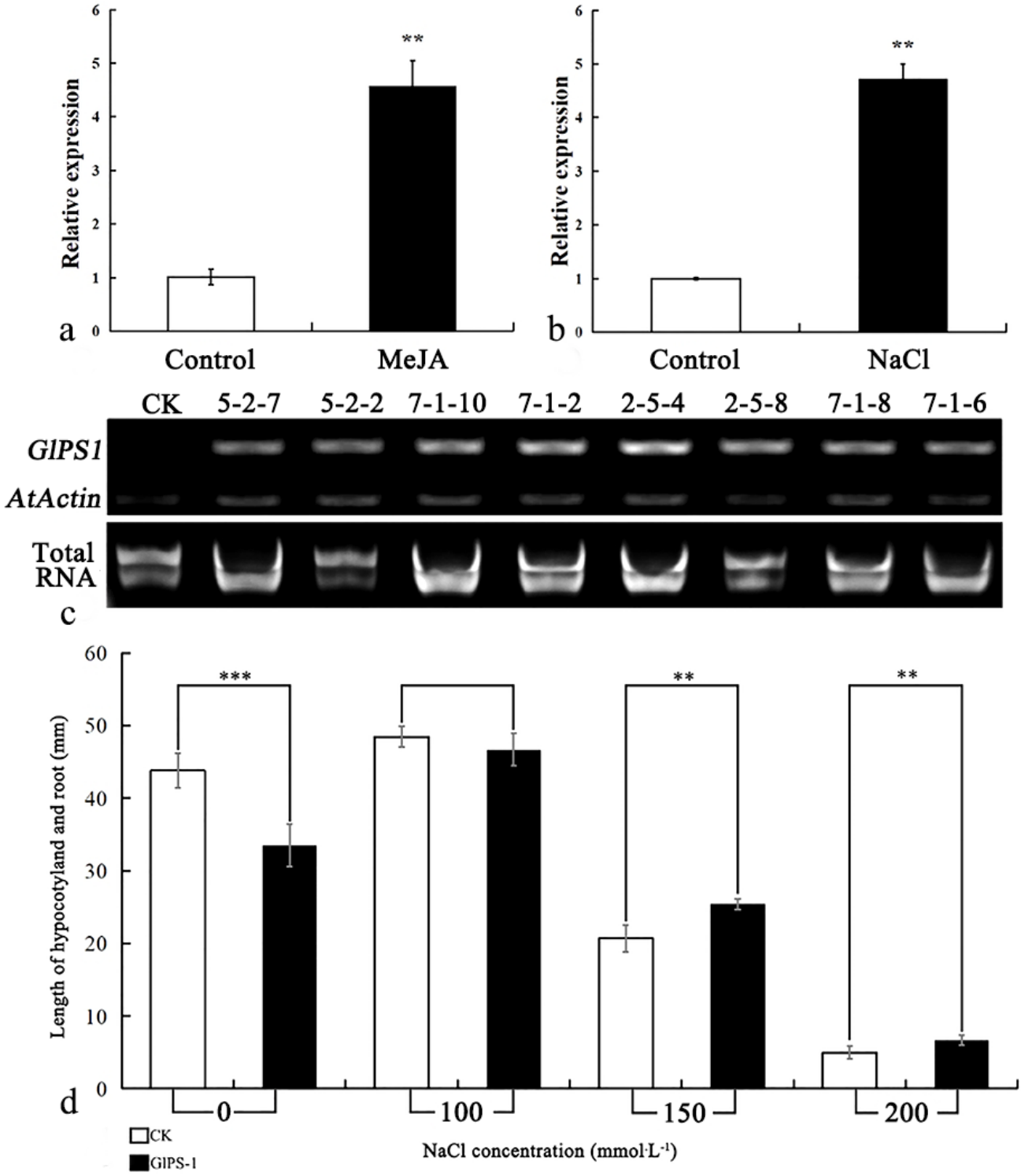


Figure 1

a, *GlPS1* gene expression of *G. littoralis* treated by MeJA. b, *GlPS1* gene expression of *G. littoralis* treated by NaCl stress. c, Semi-quantitative detection of CK (transgenic *Arabidopsis* with empty vectors) and GlPS1 (transgenic *Arabidopsis* with 35S:*GlPS1*). d, Root length measurement and analysis under salt treatment in CK and GlPS1.This experiment was repeated three times. Data represent the mean ± SD, significant difference from the wild type, as indicated by Student's *t*-test: **, 0.01<P < 0.05.

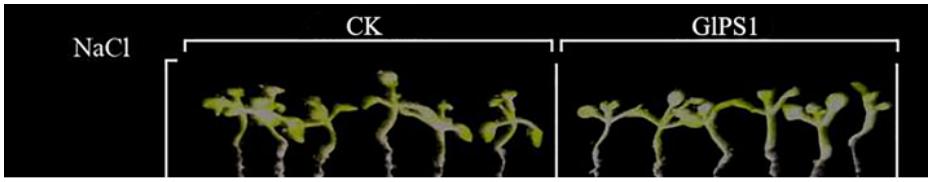


Figure 2

Comparison of CK and 35S:*GlPS-1* transgenic *Arabidopsis* under salt stress. Note：0, 100, 150, 200 $mmol^{-1}$ was the concentration of NaCl treatment, The hypocotyls of *Arabidopsis* with the same NaCl concentration were arranged in a straight line. Scale bar 1 cm.

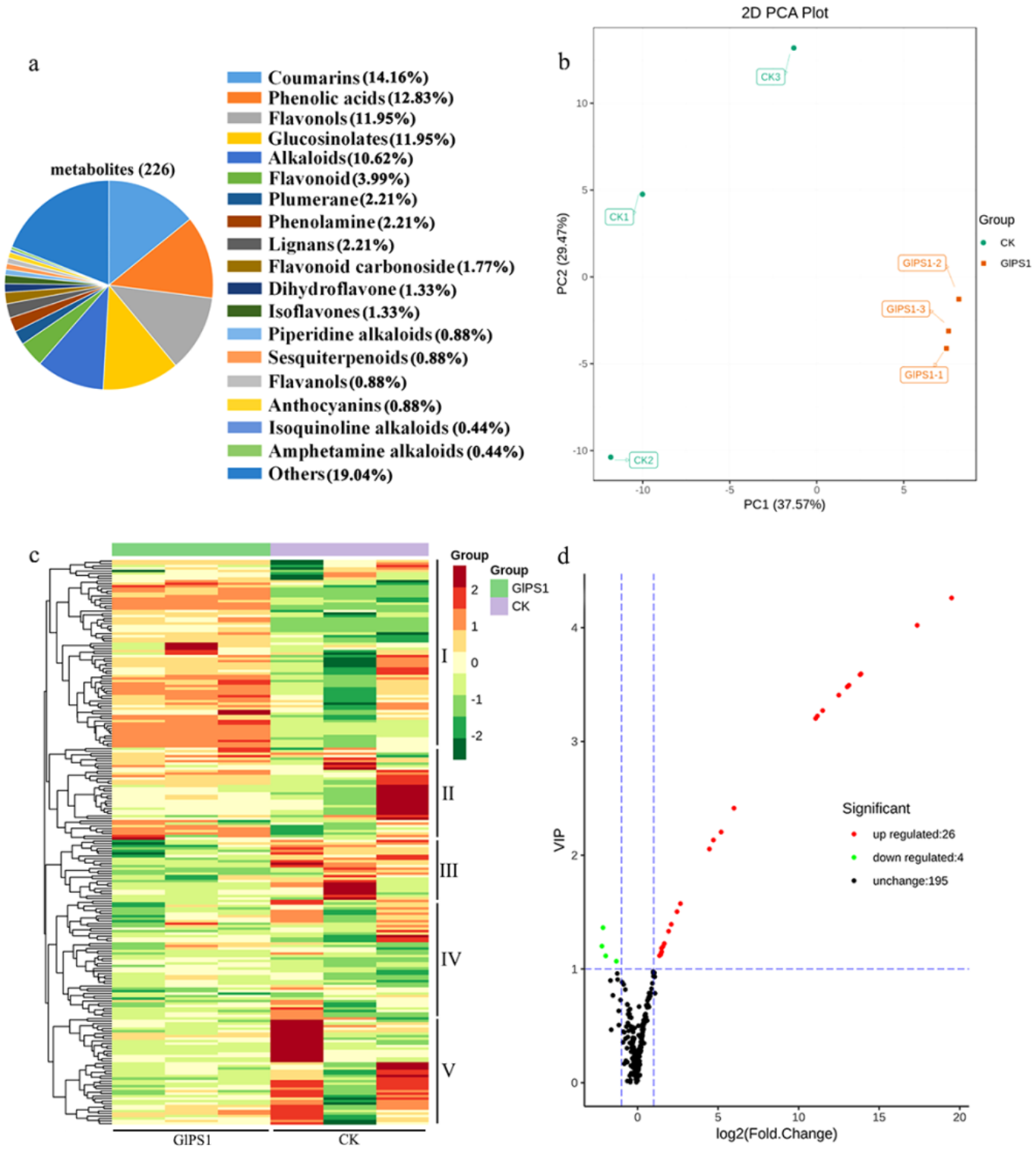


Figure 3

a, Classification of the 226 metabolites of CK and GIPS1. b, PCA analysis of metabolites identified from CK and GIPS1. Equal volumes of CK and GIPS1 fruit samples were mixed for use as a quality control (QC). c, cluster heat map analysis of metabolites from samples of CK and GIPS1. The colour indicates the level of accumulation of each metabolite, from low (green) to high (red). The Z-score represents the deviation from the mean by standard deviation units. d, Volcouldo plot of the 226 metabolites identified.

Differential metabolites were defined as metabolites with fold change≥-1 or≤1 in CK compared to GlPS1. A threshold of VIP≥1 was used to separate differential metabolites from unchanged metabolites.

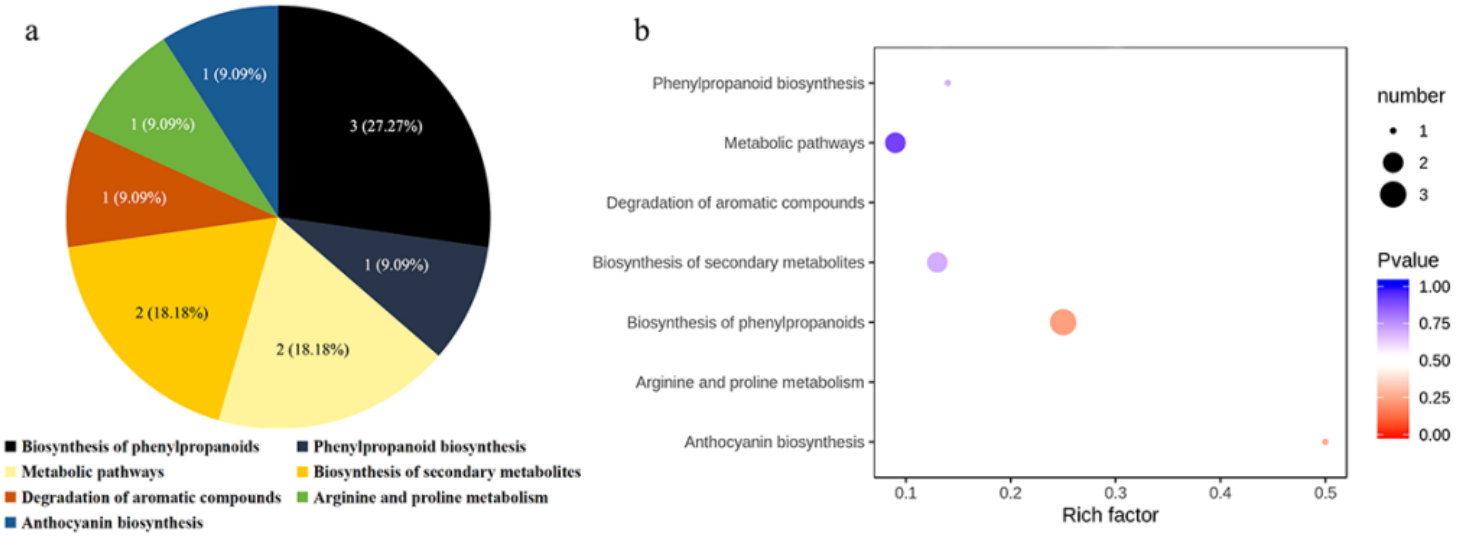


Figure 4

a, Pie chart analysis of differential metabolites in KEGG annotation of differentially accumulating secondary metabolite in CK and GlPS1 group. b,KEGG annotations and enrichment of differentially accumulating secondary metabolite of CK and GlPS1 group.

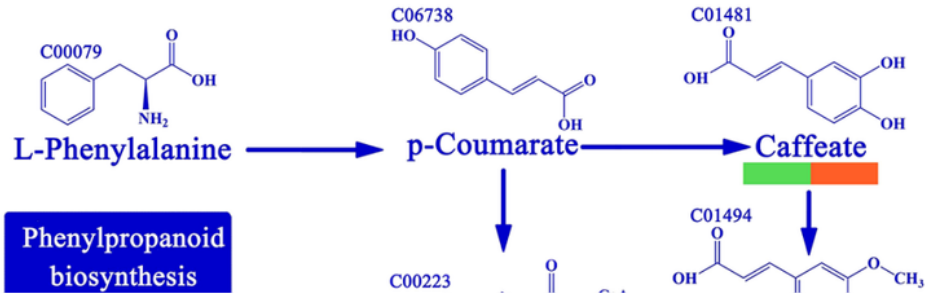


Figure 5

Phenylpropanoid biosynthesis and Coumarinbio synthesis Pathway in *G. littoralis.* **NOTE:** The red color small rectangle indicates that metabolite content is significantly upregulated, the green small rectangle indicates that metabolite content is significantly down regulated. The blue character and formula represent Phenylpropanoid biosynthesis. The green character and formula represent Coumarin biosynthesis.The dotted line part is a speculated coumarin metabolic pathway