GABA and Its Shunt's Contributions to Flavonoid Biosynthesis and Metabolism in the Tea Plat (Camellia Sinensis)

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Research article

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Abstract

Background: γ-Aminobutyric acid (GABA), a signal molecule, is regarded as the intersection of carbon and nitrogen metabolism, but its contributions to flavonoid metabolism in tea plants during the whole growth cycle remain unclear, and the correlation between the GABA shunt and flavonoid metabolism in tea plants is worth exploring. Secondary metabolites and their correlations with the taste qualities of tea plants (*Camellia sinensis*) during different seasons have been investigated.

Results: Related secondary metabolites and transcript profiles of genes encoding enzymes in the GABA shunt, flavonoid pathway and polyamine biosynthesis were measured throughout the whole tea plant growth season and after exogenous GABA applications. In addition, levels of differentially expressed proteins were measured after treatment with or without exogenous GABA. The tea leaves showed the highest metabolite concentrations in spring. *CsGAD*, *CsGABAT*, *CsSPMS*, *CsODC*, *CsF3H* and *CsCHS* were found to be important genes in the GABA and anthocyanin network.

Conclusion: GABA and anthocyanin concentrations showed a positive correlation, to some extent, and *CsF3H* and *CsCHS* played important roles in the GABA and anthocyanin network. Further studies should focus on exploring GABA and flavonoid metabolism through the transgenic engineering of tea plants.

Background

Tea is a popular beverage worldwide. The tea plant (Camellia sinensis) is an important commercial crop in China because of its importance in Chinese traditional culture and the special sensory qualities of tea produced from specific regions. However, tea quality is easily affected by the environment, especially the climate in which the tea plants are grown, This is part of tea's appeal—special taste characteristics that can only be discerned by connoisseurs.[1] In different seasons, the metabolites and taste qualities of tea have been studied preliminarily.[2, 3] Flavonoid compounds, like flavonols and flavan-3-ols, contribute greatly to tea quality and tea plant growth. Anthocyanins are tea flavonoid compounds that play important roles in plant coloring and in preventing damage caused by abiotic and biotic stresses, like cold, drought, UV irradiation and fungal infection.[4] Anthocyanins in plants have been studied in depth because they are not only bioactive components but their consumption is also beneficial for human health, including reducing the risk of developing cancer and diabetes. Additionally, amino acid levels play significant roles in changes in the qualities of tea harvested from plants during different seasons. γ-Aminobutyric acid (GABA), a non-protein amino acid, has been a hot research subject for years owing to its roles in a variety of biological functions, such as improving the medicinal value of plants and their resistance to environmental stress.[5–7] In addition, exogenous GABA influences the contents of free amino acid components in tea plants under both suitable environmental conditions and cold stress.[6] In plant cells, the GABA shunt, a part of the tricarboxylic acid cycle, is responsible for GABA synthesis through an irreversible reaction involving α-decarboxylation and glutamate, as well as polyamine (PA) degradation by glutamate decarboxylase (GAD), GABA transaminase (GABA-T) and succinic semi-aldehyde (SSA).[8] Ornithine/arginine (Arg) and PA biosynthetic pathways are associated with seed

growth, while GABA has been predicted to play important roles in seed development, but its detailed modes of action remain poorly understood.[9] In this study, tea leaves were collected during regular stages over a whole year to investigate the contribution of the GABA shunt to seasonal differences in tea growth and quality. Although numerous studies regarding anthocyanins and GABA biosynthesis have been reported[4], the correlations between GABA and anthocyanins have not been investigated.

In this work, isobaric tags for relative and absolute quantification (iTRAQ)-based proteomic analyses were performed to select the differentially expressed proteins (DEPs) involved in flavonoid metabolism, the GABA shunt and amino acid transport. The tea plants were treated with exogenous GABA and compared to normal untreated controls. DEPs involved in the GABA shunt and flavonoid metabolism were identified. Furthermore, we discuss the morphological, chemical and transcript profiles related to the GABA shunt and flavonoid metabolism at different developmental stages over the whole year. This study will increase our understanding of the effects of the GABA shunt on the synthesis of anthocyanins and possible correlations between the GABA shunt and flavonoid metabolism in tea plants during different seasons.

Methods

Plant materials and treatments

Tea plants (*C. sinensis* cv. *Longjingchangye*) formal identified by Feng Li, were collected from the tea garden of the Zhongshan Palace in Nanjing, China. A voucher specimen of this material has been deposited in Department of Tea Science, Nanjing Agricultural University. In this study, single buds having two leaves were obtained seasonally, including 7th Jan. 2018 (A), 30th Sep.2018 (B), 30th Dec. 2018 (C), 15th Apr.2019 (D), 18th Apr. 2018 (E), 21st Apr. 2019 (F), 24th Apr. 2019 (G), 27th Apr. 2019 (H), 30th Apr. 2019 (I), 3rd Mar. 2019 (J), 5th Mar. 2019 (K). For each stage, three samples per mother tree were collected. The leaves were mixed and divided into three subsamples. Some samples were stored at −80°C.

Treated tea leaves were collected from one-year-old tea plants planted in a growth room at Nanjing Agricultural University. Planting conditions were as follows: a photoperiod regimen of 14-h light (25°C, 200 μmol m^{-2} s^{-1}, 75% humidity) and 10-h dark (25°C, 75% humidity). A treatment of 5 mM GABA was chosen for this experiment on the basis of a previous experiment. The control (T1) plants were not treated with GABA. For the treatment (T2), the nutrient solution was supplemented with GABA and applied at 25°C. One bud and three leaves were collected on days 0, 4 and 7 and stored at −80°C. Physiological parameters were determined on days 0, 4 and 7, and iTRAQ was performed on day 7.

Chemicals

Standard PAs, catechins and amino acids were purchased from Shanghai Yuanye Bio-technology Co. Ltd. (Shanghai, China).

Extraction and measurement of PAs and free amino acid components

Tea leaves were subjected to extraction and analyzed using previously reported methods.[6, 8]

Determination of catechin and anthocyanin concentrations

Methods of measuring the catechin and anthocyanin contents were described previously by Wang.[10] A Waters e2695 system (Waters, USA) was employed for catechin determination: flow rate, 1 ml/min; temperature, 35°C; column, Phenomenex 00G-4337-E0, 250 × 4.6 mm; injection volume, 10 μl; mobile phases, A = 1% formic acid (v/v) and B = 100% acetonitrile (v/v), 0–42 min; detection, λ = 280.

Determination of chlorophyll concentration

The determination of the chlorophyll concentration was performed in accordance with a previous report.[11]

Gene expression level analysis

A plant RNA extraction kit (Aidlab, China) was used to isolate total RNA from 100 mg fresh leaves. A cDNA Synthesis SuperMix (TransGen, China) was used for cDNA synthesis. Quantitative real-time PCR (qRT-PCR) was performed using a Bio-Rad iQ5 fluorescence quantitative PCR platform. The primer pairs are listed in Table S1. *CsGAPDH* was used as an internal control gene in this study. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Protein extraction, digestion, iTRAQ labeling, mass spectrometry analysis and data analysis

Protein extraction, digestion, iTRAQ labeling, mass spectrometry analysis and data analysis were followed as previously reported.[6] Briefly, 1 ml phenol extraction buffer was mixed with 0.1 g frozen tea leaves and then shaken for 40 min at 4°C with 1 ml saturated phenol (Tris–HCl, pH 8.0). Then, 0.1 M ammonium acetate: methanol and lysate solutions were used to extract proteins. An iTRAQ Reagent 4-plex Multiplex Kit (AB Sciex) was used to label samples after being reduced, alkylated and trypsin-digested.

A Triple TOF 5600 mass spectrometer (SCIEX, USA), a capillary C18 trap column (3 cm × 100 μm) and a C18 column (15 cm × 75 μm) were used to perform the analyses, load samples and separate samples, respectively. Additionally, a 2.4-kV ion spray voltage, 35-psi curtain gas, 5-psi nebulizer gas and an interface heater were used to acquire data.

DEP annotations and a protein–chemical interaction analysis

All the DEPs in the GABA shunt, flavonoid metabolism pathway and amino acid transport were identified using iTRAQ-based quantitative proteomics[6]. In addition, the predicted interaction networks of proteins and chemicals in the GABA shunt, amino acid transport and flavonoid metabolic pathway were constructed using STITCH (http://stitch.embl.de/).

Statistical analysis

The average value of replicates was calculated. SPSS17.0 (SPSS Inc., Chicago, IL, USA) was used to determine expression level differences. A heatmap was constructed using Tbtool vo.66583 (China).

Results

Morphological changes in tea leaves during seasons

The coloring of the tea leaves occurred gradually during different seasons under different temperatures and light conditions (Fig. 1A–K). In summer, the bud and first leaf were purplish red; they were a bit yellow and red in autumn, more yellow in winter and green in spring. In spring (D–K), there were less sprouting buds in progressive stages, such as H, I and J.

Changes in the chemical contents and gene expression levels in the GABA shunt

A heatmap was constructed to identify correlations between transcripts and metabolite profiles in the GABA shunt at different times over the whole year. The spermine, spermidine, GABA, Arg and proline (Pro) contents showed obvious increasing trends when spring began. The PA content was highest in stage “G”, while that of GABA was highest in stage “J”. Additionally, the stages having the lowest GABA contents occurred in summer and winter, while those of spermine and spermidine occurred in summer and winter, respectively. Additionally, the GABA and glutamate contents showed opposite trends over the whole study period. Except for stages “J” and “K”, the GABA and spermidine contents showed similar changes. Moreover, the spermine and spermidine contents changes in a consistent manner (Fig. 2B).

The relative expression levels of 20 genes (*CsGAD1*, *CsGAD2*, *CsGAD3*, *CsGDH1*, *CsGDH2*, *CsGABA-T1*, *CsGABA-T2*, *CsCuAO*, *CsADC1*, *CsADC2*, *CsSPDS1*, *CsSPDS2*, *CsSPMS1*, *CsSPMS2*, *CsODC*, *CsP5CS*, *CsSMADC1*, *CsSMADC2*, *CsSSADH1* and *CsSSADH2*) were characterized using qRT-PCR. We noted that even though the *GAD* gene was very important in GABA synthesis, the mRNA levels of *CsGAD1* and *CsGAD2* were very low over the whole year compared with other genes, expect *CsODC*, while that of *CsCuAO* was high. The expression level of *CsGAD1* was highest in stages “A” and “C”, while at stage “C”, those of *CsGABAT1*, *CsGABAT2*, *CsADC1* and *CsSPDS1* were highest (Fig. 2A).

Changes in amino acids, chlorophyll and metabolite contents, as well as gene expression profiles in the flavonoid pathway

In winter, the anthocyanin content was highest, while it was lowest in stage “F”. The chlorophyll *a* and *b* concentrations peaked at stage “D”, while they were lowest at stages “C” and “J” (Fig. 3A). The epigallocatechin gallate and epicatechin gallate contents experienced steady downward trends from stages “A” to “K”. The epicatechin content was highest in stage “D” and lowest in stage “B”. The catechin content was lowest at stage “D”. (Fig. 3B). The expression level of *CsANR* was highest in stage “D”, while that of *CsUFGF* was highest in stage “C”. The expression levels of *CsCHS* and *CsFLS* were highest in

stage “K”, while that of *CsFLS* was lowest in stages “C” and “D”. Additionally, the expression level of *CsCHS* was at stage “D”, and that of *CsCHI* was at stage “C” (Fig. 3C).

The Val, Ile, Leu and Try contents showed similar trends in the study, experiencing fluctuations throughout the whole period. The contents of other amino acid components showed an increasing trend from the beginning of spring (Fig. 3D).

The GABA concentration was significantly negatively correlated with the gallocatechin gallate (GCG), epigallocatechin (EGC) and chlorophyll contents, while the glutamate content was significantly negatively correlated with the GCG and chlorophyll contents and extremely significantly negatively correlated with the EGC content. However, over the season, the contents of chemicals related to the GABA shunt were negatively correlated with the anthocyanin content (Table S2). In flavonoid metabolism, the expression levels of *CsPAL*, *Cs4CL*, *CsF3H*, *CsCHS*, *CsFLS*, *CsLAR*, *CsANS* and *CsANR* were strongly correlated with the catechin content, while the anthocyanin content was only significantly positively correlated with the *CsANR* expression level. Additionally, the GABA content was also positively correlated with the *CsF3H* expression level (Table S3).

Interaction networks of proteins and chemicals

STITCH was used to analyze the interaction networks of chemicals and proteins in the GABA shunt and flavonoid metabolic pathway. In the flavonoid metabolic pathway, p-coumaric acid, catechin, EGC and epicatechin had no interactions with chemicals related to the GABA shunt. In addition, ketoglutarate and phenylalanine were the most important bridges to chemicals related to the GABA shunt and flavonoid metabolism. Moreover, the proteins P5CS, CHS1, CHI and F3H played important roles in the correlations of these two pathways.

DEPs and chemicals related to GABA and anthocyanin metabolism in tea leaves after exogenous GABA applications compared with the control

The expression levels of proteins CHS, FLSI, F3H, ANR and ALDH12A1 were down-regulated when an exogenous GABA treatment was applied compared with the control, while those of proteins SDH-2, LPD1, GLN1-1, MS1 and AGT1 were up-regulated. (Fig. 5A) The PA contents decreased when compared with the control treatment. The putrescine and spermine concentrations underwent significant downward trends compared with the control on day 4. Additionally, the GABA and anthocyanin concentrations in the GABA-treated samples increased dramatically compared with those of the control during the whole study period (Fig. 5B). The contents of all the amino acid components, except cysteine, methionine and tyrosine, increased after both treatments. In the GABA shunt, the CsGAD and CsSPMS2 expression levels were significantly positively correlated with the anthocyanin content, while the *CsGAD1*, *CsGABAT1*, *CsSPMS2* and *CsODC* levels were heavily correlated with chemicals related to the GABA shunt (Table S4).

Discussion

Effects of climate on tea leaf morphology and chemical compound contents

During the tea growth cycle, tea quality and morphology are affected by water levels, temperature and sunlight radiation intensity and duration. The anthocyanin contents were higher in summer than in winter.[12] However, in our results, the anthocyanin contents and the *UFGF* expression were highest in winter (stage “C”), while the purple color of tea leaves was the deepest in summer. The sharp enhancement in the anthocyanin concentration in winter may result from the increasing level of water present in the environment (snow was present in stage “C”). In stage “C”, yellow-colored tea leaves had higher anthocyanin concentrations and lower chlorophyll *a* and *b* concentrations. During stage “A”, purple-red-colored tea leaves showed the second highest anthocyanin and both chlorophyll *a* and *b* concentrations. Unfortunately, we did not measure the correlations among tea leaf colors and pigment levels. In the flavonoid synthetic pathway, *UFGT* and *CHI* were key anthocyanin biosynthetic genes, because their expression levels were highly positively correlated with the anthocyanin content.

In spring, because of warming, bud are hastening to mature and open.[13] In plucking seasons, chemical compounds in tea leaves are influenced by temperature, sun exposure time and water levels. The amino acid content is highest in spring, and amino acid and catechin levels are affected by temperature, water levels and sun exposure time. [3] In our results, fluctuations in the catechins contents were moderate in spring, and epigallocatechin gallate and epicatechin gallate contents were highest in summer. Green tea leaves had higher amino acid contents compared with leaves harvested in later seasons.[14] In our results, the amino acid contents, especially those of Ala, Pro and Arg, in tea leaves were higher in summer and spring than in other seasons.

Chemicalaccumulations and transcript profiles of genes encoding enzymes involved in the GABA shunt and PA biosynthesis during the whole tea growth cycle

The metabolites present and taste qualities of tea leaves harvested in different seasons have been investigated.[15, 16] GABA may be regarded as a signal molecule, and it accumulates to adjust to stress reactions, especially those related to temperature.[6, 17, 18] However, GABA concentrations in tea leaves were higher in spring than those in winter and summer. Additionally, the expression *CsGAD1* level increased significantly in summer and winter, while the*CsGABAT1* and *CsGABAT2* levels decreased. The *CsCUAO* expression level was fairly low in winter. Perhaps, *CsCUAO* could be used to explore the roles of the GABA shunt in the plant growth cycle. However, the contents of all the chemical compounds in the GABA shunt and PA biosynthesis were lower in summer and winter compared with in spring. In previous studies, the concentrations of most chemical compounds in plants were found to be highest in spring.[12, 14] At the same time, PAs, Met and Pro concentrations increase to improve plant resistance to temperature stress.[19–23] In winter and summer, there are many other factors that influence plant growth, like the sunlight and water levels. It is worthwhile exploring how the genes *CsGAD*, *CsGABAT*, *CsSPMS* and *CsODC* function during the tea growth cycle, because their expression levels were strongly correlated with the chemical compounds in the GABA shunt and PA biosynthetic pathway.

The possible contributions of flavonoid metabolism to tea plants during the tea growth cycle

Flavonoid accumulations in grapes change from summer to winter in response to many factors, including temperature, sunshine and water levels.[12] Unfortunately, the flavonoid regulatory mechanisms that act in response to changes in climatic conditions remain elusive. Because of global warming, climatic influences on tea quality must be taken into account. Flavonoids are produced by plant secondary metabolism.[24] Correlations between GABA and environmental conditions have been investigated.[6, 17, 25] We explored this point by investigating correlations between GABA and flavonoid metabolism. The GABA shunt and flavonoid metabolism may affect each other because they both contribute to the regulation of physiological processes of plants in response to biotic and abiotic stresses.[16] Here, the contents of compounds in the GABA shunt were correlated with catechin levels (Table S2). In particular, Glu and GABA concentrations were strongly negatively correlated with GCG and EGC levels. *CsPAL1*, *CsC4H*, *CsCHS*, *C'F"5'H*, *CsDFR*, *CsDFR*, *CsLAR* and *CsANR* play important roles in catechin synthesis.[17] Here, we were unable to define clear correlations between the GABA contents and transcript profiles of genes involved in flavonoid metabolism. In the future, the overexpression or gene-silencing of *CsODC*, *CsSPMS2* or *CsSPDS1* will be performed (Fig. S1).

Effects of exogenous GABA on flavonoid metabolism

The correlations of GABA and anthocyanins with flavonoid metabolism have been studied, and anthocyanin biosynthesis in plants is influenced by many factors, like hormones, temperature and light.[26–28] However, there is no research regarding correlations between GABA and anthocyanins, which are important compounds in flavonoid metabolism. We found that the addition of exogenous GABA increased the anthocyanin concentrations in tea leaves (Fig. 5B). In the anthocyanin biosynthetic pathway, there are many functioning transcription factors .[29, 30] The CHS, FLS1, F3H and ANR expression levels were down-regulated after the application of exogenous GABA compared with under normal conditions, and the *CsF3H* and *CsCHS* expression levels also decreased (Fig. 5). Tea cultivars with purple leaves are used to produce unique teas with specific flavors. The use of GABA treatments might represent a practical method to regulate flavonoid metabolism and produce tea with optimal desired qualities.

Conclusion

In this study, the secondary metabolite contents and transcripts profiles of genes encoding enzymes in the GABA shunt, PA biosynthesis and flavonoid pathway were measured during the whole tea plant growth period. The results suggested that *CsGAD*, *CsGABAT*, *CsSPMS* and *CsODC* may be effective and helpful in exploring the complicated metabolism of the GABA shunt and PA biosynthesis in tea plants during the whole year. To determine more about the correlations between GABA and flavonoid metabolism, we detected the expression levels of proteins, some chemical compounds and transcripts profiles related to tea plants treated with and without exogenous GABA. GABA and anthocyanin concentrations showed a positive correlation, to some extent, and *CsF3H* and *CsCHS* played important

roles in the GABA and anthocyanin network. Further studies should focus on exploring GABA and flavonoid metabolism through the transgenic engineering of tea plants.

Abbreviations

GABA: gamma-aminobutyric acid; iTRAQ: Isobaric tags for relative and absolute quantification; PAO: Polyamine oxidase; PAs: polyamines; Spd: spermidine; Spm: Spermine; TCA cycle: tricarboxylic acid cycle; GADPH, glyceraldehyde-3-phosphate dehydrogenase; ADC, arginine decarboxylase; GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; ODC, ornithine decarboxylase; P5CS, pyrroline-5-carboxylate synthase; SPDS, spermidine synthase; SPMS, spermine synthase; SSADH, succinic semialdehyde dehydrogenase; CuAO (DAO), Diamine oxidase; SAMDC, S-Adenosylmethionine decarboxylase; PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate coenzyme A ligase; CHS, Chalcone synthase; CHI, chalcone isomerase gene; FLS, flavonol synthase; DFR, dihydroflavonol reductase; LAR, Leucoanthocyanidin reductase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; F3H, flavanone 3-hydroxylase; F3‘H, flavanone 3’-hydroxylase; F3‘5’H, flavanone 3’5’-hydroxylase; UFGT, UDP glucose: flavonoid 3-o-glycosyltransferase; Thr, Threonine; Asp, Asparticacid; Ser, Serine; Glu, Glutamicacid; Gly, Glycine; Ala, Alanine; Cys, Cysteine; Val, Valine; Met, Methionine; Ile, Isoleucine; Leu, Leucine; Tyr, Tyrosine; Phe, Phenylalanine; Lys, Lysine; His, Histidine; Arg, Arginine

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data sets are included within the article and its Additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XZ WF designed the experiments, JL YC TR conducted the experiments, QS YW YM analyzed the data, JL YC drafted the manuscript, XZ supervised the project. All authors have read and approved the final version of this manuscript.

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Figures

Figure 1

Images of leaves from different developmental stages of tea plants (C. sinensis cv. Longjingchangye) growing in a tea garden in Nanjing: A, 7th Jan. 2018; B, 30th Sep.2018; C, 30th Dec. 2018; D, 15th Apr.2019; E, 18th Apr. 2018; F, 21st Apr. 2019; G, 24th Apr. 2019; H, 27th Apr. 2019; I, 30th Apr. 2019; J, 3rd Mar. 2019; K, 5th Mar. 2019.

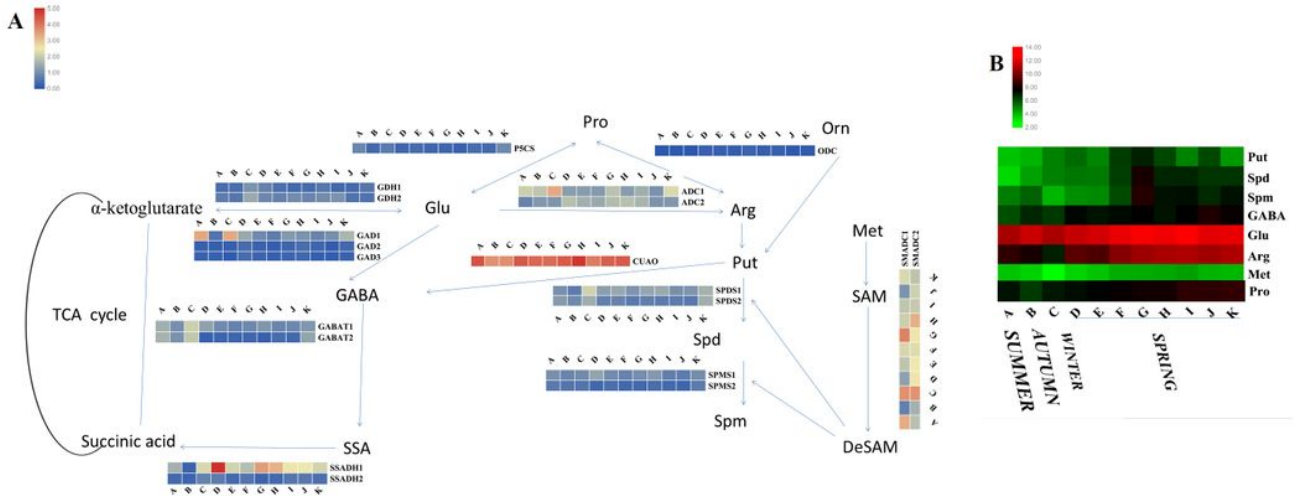

Figure 2

Heatmap analysis of transcripts and metabolic profiles related to the GABA shunt in different developmental stages of tea plants (C. sinensis cv. Longjingchangye). A, expression levels of genes related to the GABA shunt; B, changes in chemicals related to the GABA shunt

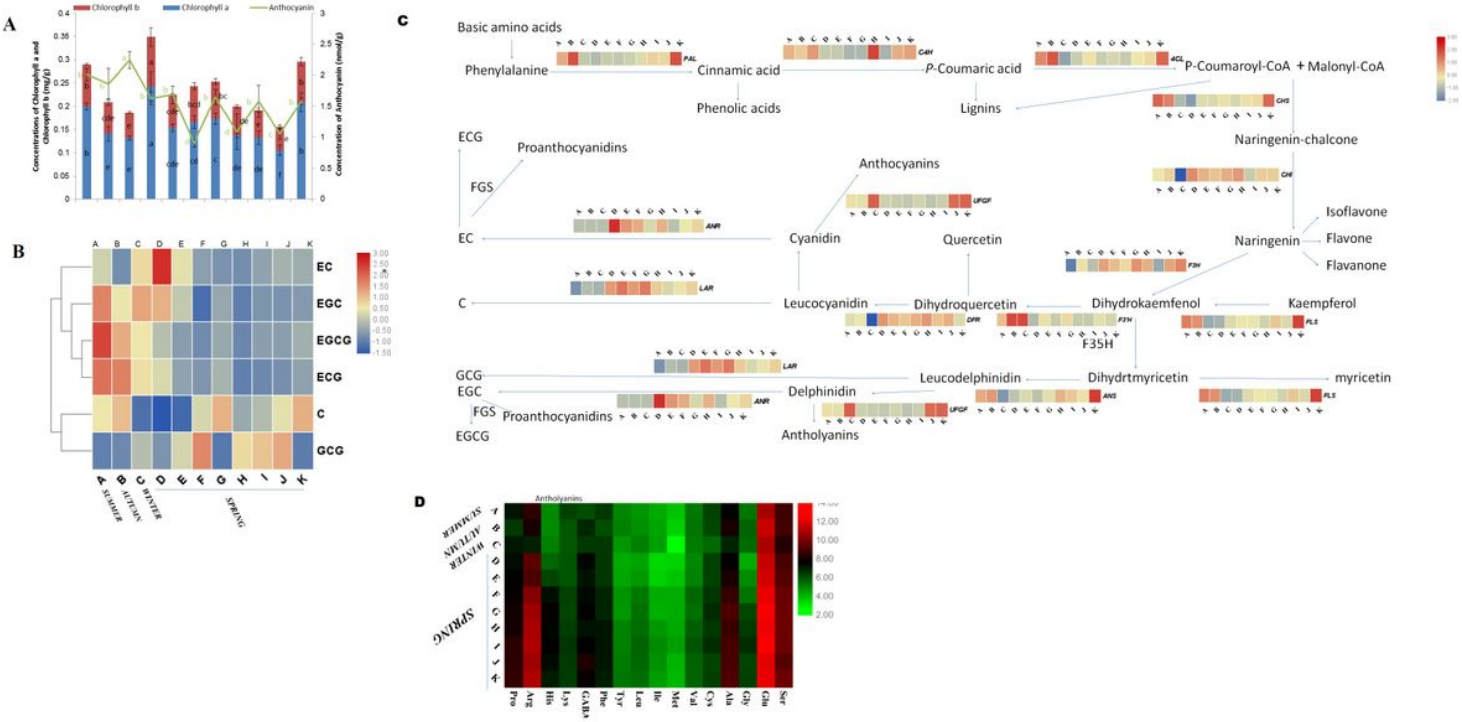

Figure 3

DEPs and chemicals related to GABA and anthocyanin metabolism-related proteins in tea leaves after exogenous GABA application compared with the control. A, fold changes of DEPs in the tricarboxylic acid cycle, flavonoid metabolism and amino acid transport after exogenous GABA application compared with

the control; B, changes in the contents of PAs, GABA and anthocyanin in treated leaves; C, changes in the contents of amino acid components in treated leaves

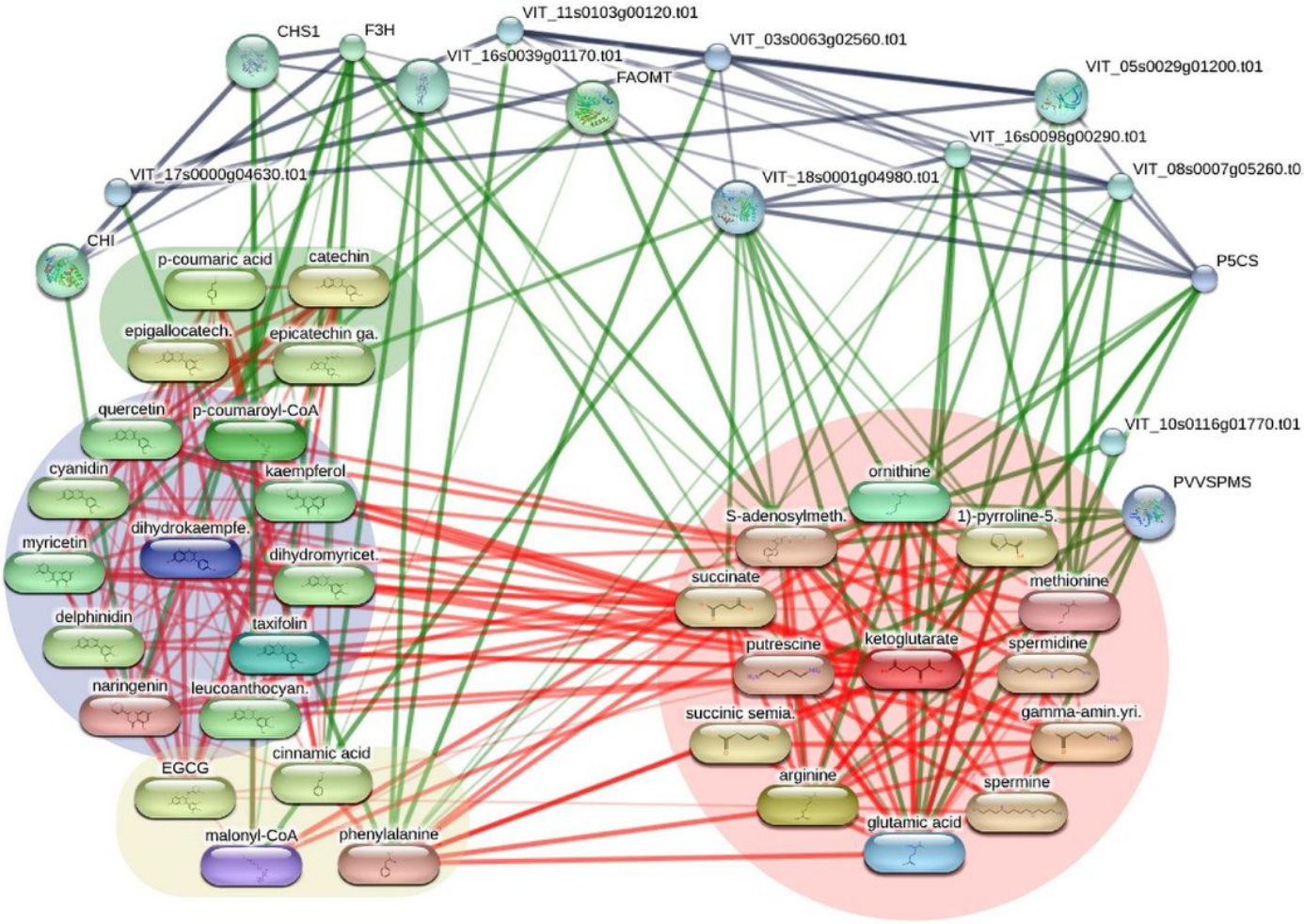

Figure 4

Interaction networks of chemicals, proteins, and GABA in tea. The image presents a comprehensive view. Thick lines represent strong associations. Protein–protein interactions are shown in gray. Chemical–protein interactions are shown in green. Chemical–chemical interactions are shown in red

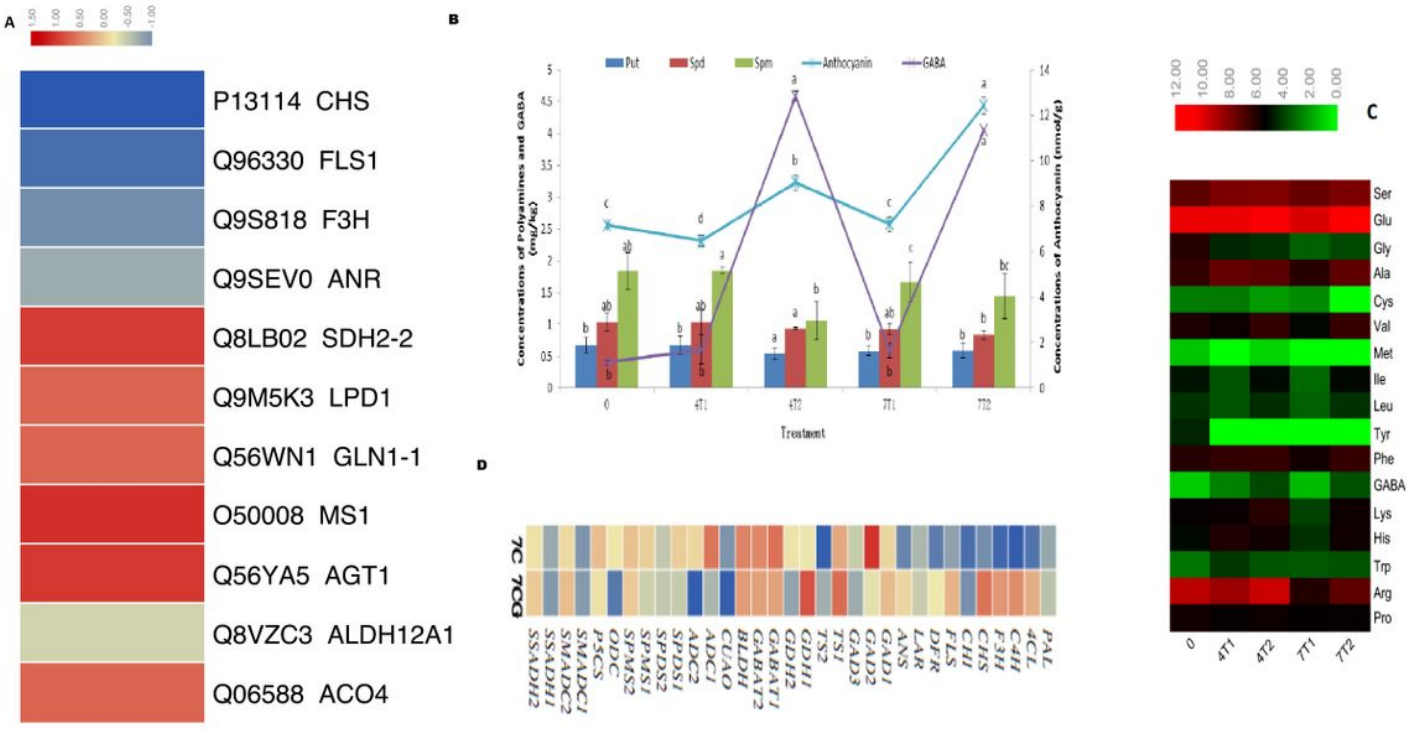

Figure 5

DEPs and chemicals related to GABA and anthocyanin metabolism-related proteins in tea leaves after exogenous GABA application compared with the control. A, DEPs related to GABA and flavonoid metabolism-related proteins in tea leaves after exogenous GABA application compared with the control; B, changes in the contents of PAs, GABA and anthocyanin in the treated leaves; heatmap of the contents of amino acid components during the treatments

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplyfig.1.pdf
- supplytable1.docx
- supplytable2.docx
- supplytable3.docx
- supplytable4.docx
- supplytable5.doc