# Coupled Metabolome With Physiology Unveiled Mechanisms for Cadmium Affecting Active Ingredients Synthesis in Salvia Miltiorrhiza, A Non-cd-hyperaccumulator

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**Research Article**

**Keywords:** Salvia miltiorrhiza, Cd pollution, Physiological characteristics, Metabolic profiles

**Posted Date:** September 20th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-886213/v1

# Abstract

## Background

Cadmium (Cd) poses threats to human health by affecting the safety (Cd accumulation) and quantity (contents of active ingredients) of *Salvia miltiorrhiza* due to human activities and Cd characteristics. It remains largely unknown how Cd stress affects the synthesis of active ingredients in *S. miltiorrhiza*.

## Results

Here we investigated physiologies (contents of Cd, malondialdehyde (MDA) and proline, and activities of superoxide dismutase, peroxidase (POD) and catalase (CAT)), transfer factor (TF), bioconcentration factor (BCF) and metabolites of *S. miltiorrhiza* at different levels of Cd contamination with a pot experiment. The results revealed that Cd concentration, as it rose in soil, increased significantly in roots and leaves with TFs and BCFs below 1 in the Cd addition groups; POD and CAT activities and proline content increased and then declined significantly. Besides, amino acids and organic acids (especially D-glutamine (D-Gln), L-aspartic acid (L-Asp), L-phenylalanine (L-Phe), L-tyrosine (L-Try), geranylgeranyl-PP (GGPP), and rosmarinic acid (RA)) contributed more than other metabolites in discriminating roots under different levels of Cd contamination. With Cd concentration rising, the relative content of GGPP declined and then increased significantly; RA content rose significantly; content of L-Phe and L-Try increased and then declined significantly, while the content of D-Gln and L-Try decreased and then increased significantly.

## Conclusions

These results suggested that *S. miltiorrhiza* belonged to a non-Cd-hyperaccumulator with most Cd accumulated in roots; Cd enhanced the RA synthesis via regulating amino acid metabolism but inhibited the tanshinone synthesis mainly by declining the GGPP content, with proline, POD and CAT playing vital roles in resisting Cd stress.

# Background

With the booming economy and society, serious Cd pollution in soils emerged, caused by "three wastes" produced in the long-term mining and smelting and hazardous substances produced in traditional agriculture growth [1, 2]. Cd features strong bioaccumulation, high bioavailability, and strong biotoxicity [3, 4]. Cd migrates easily from soil to plants leading to declining the yield and quality of plants [5]. Eventually, Cd enters the human body through food chains, seriously threatening people's health [6]. Hence, much attention have been put on soil Cd contamination [7, 8].

Phytomedicines (medicine derived from plants in their original state) play important roles in disease treatment and life extension and make significant contributions to global pharmaceutical industries [9, 10]. As reported, some traditional medicinal plants contain unsafe Cd concentrations (e.g., *Plantago*

*asiatica*, *Gardenia jasminoides*, *Lonicera japonica*, etc.), lowering the efficacy and quality of phytomedicines and threatening human health [11-15]. Accordingly, heavy metal contamination is one restriction of the sustainable development of the traditional Chinese medicine (TCM) industry and the export bottlenecks of TCMs [16, 17].

*Salvia miltiorrhiza* is a perennial plant and widely distributed in north and south China [18]. Commonly used in TCM, it has dry roots and rhizomes (called Danshen in China) containing tanshinone, salvianolic acid, rosemary and other chemical components [19, 20]. Nowadays, it has been widely used to treat cerebrovascular and cardiovascular diseases, hyperlipidemia and acute ischemic stroke in China and other Asian countries [21, 22]. With the increasing need for Danshen, the planting area of *S. miltiorrhiza* has expanded year by year in China. *S. miltiorrhiza* from some production areas in China contained Cd concentration above the limits set out by the Chinese Pharmacopoeia Commission [11, 23, 24].

*S. miltiorrhiza* is a significant medicinal plant. Cd could debase the quality of *S. miltiorrhiza* by inhibiting the accumulation of active ingredients [25]. However, little is known as relates to the effect mechanisms of Cd pollution for the synthesis of active ingredients in *S. miltiorrhiza*. In this study, *S. miltiorrhiza* was used as samples to conduct a pot-culture experiment. The queries to be addressed are: (1) How does Cd stress affect the physiological characteristics and the metabolite profiles of *S. miltiorrhiza*? (2) How does Cd affect the synthesis of active ingredients in *S. miltiorrhiza* based on metabolisms and physiologies? The study would assist with understanding how *S. miltiorrhiza* responds to Cd stress physiologically and metabolically.

# Methods

### Soil preparation

The soil used in the pot experiment were collected from the topsoil (0-20 cm) in Shennong Garden of the Jiangxi University of Chinese Medicine in Jiangxi, China (E 115°44'24", N 28°41'24"). The soil was air-dried, sieved to remove gravels through a 4 mm mesh sieve, and then mixed thoroughly. The basic physicochemical properties (total nitrogen (N), available N, total phosphorus (P), available P, total potassium (K), available K and total Cd concentration, organic matter content, and pH value) (Table 1) of the soil were measured following the methods of Wen et al. [52]. The total Cd concentration was 0.95 mg $kg^{-1}$, less than the critical value (1.0 mg $kg^{-1}$) of the soil to ensure normal plant growth in agriculture and forestry production.

The appropriate contents of cadmium chloride hemi (pentahydrate) ($CdCl_2 \cdot 2.5H_2O$, Sinopharm Chemical Reagent Co., Ltd., China) were thoroughly blended with soil to obtain final concentrations of 25, 50 and 100 mg Cd per kilogram of soil, respectively. The selected Cd concentrations were consistent with that of previous studies of Wang et al. [53] and Zhang et al. [25]. Then the soils with or without Cd treatment were transplanted into plastic pots (16 cm in diameter × 17 cm in height) and watered every 5 d with pure water. Before use, they were equilibrated for 30 d.

## Plant material and Cd treatment

*S. miltiorrhiza* seedlings were purchased from *S. miltiorrhiza* planting areas (Shangdong, China). The formal identification of the samples was carried out by Assoc Prof. Xiaoyun Wang. Cultivated in Shennong garden for 30 d, the seedlings were transferred into appropriate pots containing 2.5 kg soil. Each pot contained two seedlings. A pot was taken as one replicate, and three replicates were performed for each treatment. In the experiment, seedlings without Cd treatment were used as the control group (CK), and seedlings with 25, 50, 100 mg $kg^{-1}$ Cd treatment were named TR, TS, and TT, respectively. Each seedling presented uniform growth. After being watered once per week for 4 weeks, plant samples were harvested for further study.

In order to perform LC-MS and physiological analyses, root samples from each pot were selected to make a composite root sample, simultaneously frozen in dry ice and stored at -80 °C. Leaves and remaining roots from each pot were collected to make a composite leaf sample and root sample, respectively, to perform Cd concentration analysis. Then both samples were dried in the oven at 60 °C for 4 d. Voucher specimens (NO. DS-001) were deposited in a public herbarium in Research Center for Traditional Chinese Medicine Resources and Ethnic Minority Medicine of Jiangxi University of Chinese Medicine.

## Determination of Cd concentration

The oven-dried samples were ground to fine powder, passed through a 2-mm sieve, and then digested with a mixture of nitric acid ($HNO_3$) and hydrogen peroxide ($H_2O_2$) (3:1, v/v) in the Teflon tanks using an electric heating board at 160 ℃ thoroughly. The Cd concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Scientific, USA).

## Physiological analysis

The ninhydrin colorimetry was used to determine the proline content [54, 55]. A total of 0.05 g fresh root samples were pooled in the centrifugal tube with 5 mL 3% sulfosalicylic acid, extracted in the boiling water bath for 10 min, and shaken frequently. The extraction was filtered into a volumetric flask. Then the volume was constant to 25 mL with distilled water. A total of 2 mL extraction solution mixed with 2 mL glacial acetic acid and 2 mL acidic ninhydrin was added in a centrifuge tube. The mixture was treated with the boiling water bath for 30 min. After cooling down to room temperature, 4 mL toluene was added to the mixture and shaken thoroughly. When they were stratified during standing, the absorbance of the upper solution was measured at 520 nm with an ultraviolet spectrophotometer (UV-8000, Metash, China).

The content of MDA and activities of POD, SOD and CAT were detected by assay kits (Suzhou Keming, China). A total of 0.1 g fresh samples and 1 mL solutions (1:10, v/v) were grounded in a water bath and centrifuged at 8000 × g and 4 ℃ for 10 min. According to the manufacturer's instructions, supernatant absorbances were measured at 532 nm and 600 nm to assess MDA content and at 470 nm, 240 nm and 560 nm to evaluate the activities of POD, CAT and SOD, respectively.

## Metabolite analysis

The mothed of metabolites extraction was referred to Wang et al. [56]. A total of 25 mg fresh samples were placed into an EP tube with 500 μL extract solution (methanol:water = 3:1 (v/v), with the isotopically-labeled internal standard mixture), homogenized at 35 Hz for 4 min and sonicated for 5 min with an ice-water bath. The homogenization and sonication cycle were conducted 3 times. The samples were incubated for 1 h at -40 ℃ and centrifuged at 12000 rpm and 4 ℃ for 15 min. Subsequently, the resulting supernatant was transplanted into a fresh glass vial for further analysis. Besides, the quality control (QC) sample was formed by mixing an equal aliquot of the supernatants from all samples [57].

Combining a UHPLC system (Vanquish, Thermo Fisher Scientific, US) and Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo, US), LC-MS/MS analyses were performed to analyze sample metabolites using a Waters ACQUITY UPLC HSS T3 (2.1 mm × 100 mm, 1.8 μm; Waters, USA). Mobile phase A was water containing 5 mmol $L^{-1}$ ammonium acetate and 5 mmol $L^{-1}$ ammonia hydroxide, while mobile phase B was acetonitrile. The mobile phase elution procedure was set as follows: the concentration of mobile phase A was 5% in 0-5 min, 35% at 7 min, increased to 60% holding for 1 min, then decreased to 5% holding for 3 min; the mobile phase B was 95% in 0-5 min, 75% at 5 min, decreased to 20% holding for 1 min, then increased until 95% holding for 3 min. The auto-sampler temperature was 4 ℃, the injection volume was 3 μL, and the flow rate was 0.5 mL $min^{-1}$. The QE HFX mass spectrometer was conducted to acquire MS/MS spectra based on information-dependent acquisition mode in controlling the acquisition software (Xcalibur, Thermo, USA). In the mode, the acquisition software continuously evaluated the full scan MS spectrum. The ESI source conditions were conducted as follows: The flow rates of sheath gas and Aux gas were 30 Arb and 10 Arb, respectively; the capillary temperature was 350 ℃; the full MS resolution was 60000; the MS/MS resolution was 7500; the collision energy was 10/30/60 in NCE mod; the spray voltage was 4.0 kV (positive) or -3.8 kV (negative).

After the original data was converted into mzXML format using the software ProteoWizard (https://proteowizard.sourceforge.io/), the R package XCMS (version 3.2) was used for the peak recognition, extraction, alignment, and integration. The preprocesses of the original data included the following: 1) Data filtering. The filtering standard was to remove the data with no definite substance name or no spectrum comparison similarity. 2) Missing values processing. Substances of more than 50% missing in comparisons were filtered directly, and substances of less than 50% missing were performed the imputation of missing values using the k-nearest neighbor (KNN) algorithm. 3) Normalization. The internal standard (IS) or total ion current (TIC) of each sample was used for the normalization. A total of 1111 and 305 peaks of the original data were retained for positive and negative ion modes, respectively. The excel sheets, including the name of peak and sample, and the standard data of normalized peak area were obtained for further data analysis.

## Statistical analysis

Firstly, the transfer factor (TF) and bio-concentration factor (BCF) of Cd were calculated as follows [26-28]:

TF = Cd concentration of aboveground parts (mg $kg^{-1}$) / Cd concentration of roots (mg $kg^{-1}$)

BCF = Cd concentration in the tissues / Cd concentration in soils

Secondly, the multivariate ordination principal component analysis (PCA) was conducted to reveal the overall distribution of samples. The supervised partial least squares discrimination analysis (PLS-DA) was utilized to assess the differences in roots between the control and Cd treatment groups. The PLS-DA model was validated by permutation tests (200) or ANOVA of the cross-validated residuals (CV-ANOVA). A Q2 value of above 0.5 and an R2 value of above 0.7 for the permutation test or *p* value of below 0.05 for the cross-validated residuals (CV-ANOVA) denoted the highly significant model [58]. The variable importance in projection (VIP) was crucial for explaining the data of the PLS-DA model. Metabolites with a *p* value of below 0.05 and a VIP of above 1.0 were filtered out as differential metabolites, playing larger roles in distinguishing roots between the control and Cd addition groups.

Thirdly, the pathway analysis of all the metabolites was conducted by the software MetaboAnalyst 4.0 (http://www. metaboanalyst.ca/faces/ModuleView.xhtml). Based on the pathway impact value of above 0.1, the potential metabolic target pathways were obtained [59]. Thirdly, the Venn diagram was obtained using the software Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/) to present the overlap of all the named metabolites in the control and Cd-treatment groups. Subsequently, t-test and correlation analyses were conducted to evaluate the variability of identical metabolites in samples and relationships between Cd concentration in tissues (leaves and roots) and Cd concentration in soils with SPSS 18.0 (SPSS Inc., USA), respectively. The PCA and PLS-DA models were performed by SIMCA-P version 14.1 (Umetrics, Sweden). All data were log10-transformed before analysis.

### Availability of data

All analyzed data are included in this article and its Supplementary Files.

# Results

### Cd concentration in leaves and roots

With the increase of soil Cd concentration, Cd concentrations in leaves and roots increased significantly ($p < 0.05$) (Figures 1A, 1B, 1C). When soil Cd concentration was 100 mg $kg^{-1}$, Cd concentrations in leaves and roots reached the maximum (15.53 mg $kg^{-1}$ for leaves, and 16.66 mg $kg^{-1}$ for roots), and the max-concentrations of Cd in leaves and roots were 99.68% and 99.70% higher than these of the control group, respectively (Figures 1A, 1B).

Besides, with the soil Cd concentration rising, the TF and BCF of *S. miltiorrhiza* changed significantly ($p < 0.05$). The max-TF presented in roots of the control group (Table 2). As Cd concentration went up, the BCF

of *S. miltiorrhiza* seedlings decreased firstly, then increased, finally decreased significantly ($p < 0.05$). The max-BCF also appeared in plants of the control group (Table 2). With soil Cd concentration rising, the BCFs in roots rose. The BCFs of tissue samples with 100 mg $kg^{-1}$ Cd treatment were higher than those in other groups ($p < 0.05$) (Table 2). All TFs and BCFs were below 1 except the TF in *S. miltiorrhiza* of the control group (Table 2).

## Physiological characteristic in roots

The proline content in roots went up significantly at first and then went down significantly with soil Cd concentration increasing ($p < 0.05$). The proline content in 50 mg $kg^{-1}$ Cd stress group was higher than these of other groups ($p < 0.05$) (Figure 2A). However, as soil Cd concentration increased, the MDA content declined (Figure 2B). Furthermore, the activities of POD and CAT in roots increased and then decreased significantly with soil Cd concentration rising ($p < 0.05$) (Figures 2C, 2D). The maximal activities of POD and CAT both appeared in roots under 50 mg $kg^{-1}$ Cd stress (Figures 2C, 2D). However, the SOD activity of roots revealed no significant differences in different levels of Cd-contaminated soils (Figure 2E).

## Metabolomic changes in roots

A total of 305 metabolites were identified, as showed in Table S1 (Additional file 1). Most of them were C-containing and N-containing metabolites (e.g., amino acids, organic acids, fatty acids, etc.). The relative contents of these metabolites of the control group differed from those in Cd-treated groups (Figures 4, 5; Additional file 4: Figure S1; Additional file 1: Table S1). Besides, a metabolic map was obtained according to pathway analysis results, involving all the identified metabolites (Figure 5). The target pathways (with the PI value of above 0.1) were presented in Table S3 (Additional file 3), coupled with amino acid metabolism and some secondary metabolism as the central metabolic pathways (Additional file 2: Table S2). Some of them were involved in the synthesis of active ingredients (Figure 5).

## Differential metabolites in roots

According to the result of PCA analysis, root samples in the control and Cd treatment groups were clearly distinguished in the pot experiment (Figure 3A). The results of the significant PLS-DA model (with VIP of above 1) and the t-test (with a $p$ value of below 0.05) (Figures 3B, 3C, 3D; Additional file 5: Figure S2) facilitated the identification of differential metabolites, with 175 differential metabolites for the discrimination of the control group and 25 mg $kg^{-1}$ Cd-stressed group, 161 for the discrimination of the control group and 50 mg $kg^{-1}$ Cd-stressed group, and 192 for the discrimination of the control group and 100 mg $kg^{-1}$ Cd-stressed group (Figure 4; Additional file 1: Table S1; Additional file 2: Table S2). Most of the same differential metabolites identified in all the groups were amino acids and organic acids (Figure 4D). The major contributors from these metabolites were covered in the synthesis of salvianolic acids (e.g., D-Gln, L-Asp, L-Phe, L-Tyr and RA) or involved in the tanshinone synthesis (GGPP) (Figures 4, 5; Additional file 3: Table S3). With soil Cd concentration rising, the relative contents of L-Phe and L-Tyr

increased firstly and then decreased significantly ($p < 0.05$) (Figures 5A, 5B), but relative contents of D-Gln and L-Asp declined and then rose significantly ($p < 0.05$) (Figures 5C, 5D). The maximum relative contents of L-Phe and L-Tyr were shown in roots with 100 mg $kg^{-1}$ Cd treatment (Figures 5A, 5B). Besides, with the increase of soil Cd concentration, the relative content of GGPP declined and then rose significantly ($p < 0.05$) (Figure 5E), and the relative content of RA increased significantly ($p < 0.05$) (Figure 5F).

# Discussion

### Accumulation characteristics of Cd in S. miltiorrhiza

The study revealed that *S. miltiorrhiza* was a non-Cd-hyperaccumulator with a substantial part of Cd enriched in roots. Although in the pot experiment, Cd concentration in leaves was lower than it in roots under each level of soil Cd treatment, the Cd concentration in the two tissues was much smaller than 100 mg $kg^{-1}$ (the threshold value for the Cd hyperaccumulator in soils of 100 mg $kg^{-1}$ Cd (Fig. 1). TF could reveal the ability to enrich and transport heavy metals from roots to aboveground parts in plants [26, 27]. BCF could reflect the enrichment ability of plants [28]. All the TFs and BCFs in Cd addition groups were below 1 (Table 2). The enrichment characteristics of Cd in *S. miltiorrhiza* differed from the Cd-hyperaccumulators, which could enrich at least 100 mg $kg^{-1}$ Cd in soils of 100 mg $kg^{-1}$ Cd or more, with the TF and the seedling BCF of above 1 under different levels of Cd stress [7, 26, 29].

Simultaneously, proline and MDA could reflect the degree of being compelled by heavy metals in plants, and antioxidant enzymes could effectively remove free radicals, protecting cells from oxidative stress [30, 31]. In the study, with soil Cd concentration rising, activities of POD and CAT and proline content increased and then apparently declined in roots ($p < 0.05$) (Fig. 2). The change differed from those in hyperaccumulator *Phytolacca americana* under Cd stress [32]. This might be because as soil Cd concentration rose, *S. miltiorrhiza* absorbed much Cd in roots, part of which was transferred into leaves, increasing leaf Cd concentration (Fig. 1A). As soil-added Cd levels increased, the dilution of root Cd was enhanced along with the remarkable increase of TF and BCFs (Table 2; Fig. 1) [33]. Similarly, with the content of added Cd rising, Cd concentration in roots and leaves of *Murraya paniculate*, *Catharanthus roseus*, and *Loropetalum chinense* increased, and all the roots could accumulate more Cd than the leaves [34].

Other Cd was accumulated in roots, increasing root Cd concentration. Cd stress led to the accumulation of reactive oxygen species and peroxidation of membrane lipid in non-Cd-hyperaccumulating plants [35]. Then these plants carried out the synergism of antioxidant enzymes (e.g., POD, CAT and SOD) (Fig. 2). In current studies, with Cd concentration rising, activities of POD and CAT increased and then decreased significantly ($p < 0.05$), although the SOD activity presented no significant change (Fig. 2). To many non-Cd-hyperaccumulators, due to low Cd tolerance, the activities of antioxidant enzymes would decrease with the increasing Cd level. However, they might still remarkably clean the active oxygen free radical and lower the lipid peroxidation of plants, resulting in the lower MDA content of plants under Cd stress than

that in Cd-contaminated plants (Fig. 2) [36]. The change trends differed from those of activities of antioxidant enzymes of *P. americana* under Cd stress conditions, but showed the same change as *Daucus carota* [32, 37].

### Effect of Cd on the synthesis of phenolic acids in S. miltiorrhiza

Cd stress enhanced the synthesis of phenolic acids (especially RA) in *S. miltiorrhiza* roots, which were boosted with soil Cd increasing. In the study, the major contributors in distinguishing the samples in different levels of Cd stress groups included D-Gln, L-Phe, L-Tyr and RA (Fig. 4; Additional file 2: Table S2). Firstly, amino acids could participate in the detoxification of plants, such as ion transport, nitrogen metabolism and secondary metabolism [38, 39]. Among them, Gln was the first type of organic nitrogen (N) compounds converted from N absorbed by plants from soils and participated in various life activities, such as amino acid metabolism [40, 41], and Asp acted as the amino donor of amino acid metabolism, nucleotide metabolism and TCA cycle [42]. Hence, with soil Cd concentration from 0 to 50 mg $kg^{-1}$, the relative contents of both D-Gln and L-Asp reduced (Fig. 5). It might be used to deal with the increase of the contents of some amino acids (e.g., L-Phe, L-Tyr, L-Asp). The situation might be caused by the enhanced activities of POD and CAT, which could regulate Cd stress resistance in plants as reported above (Figs. 2, 5). However, as Cd concentration increased from 50 to 100 mg $kg^{-1}$, the homeostasis might be broken with the activities of POD and CAT significantly decreasing ($p < 0.05$) (Fig. 2), resulting in the increase of relative contents of D-Gln and L-Asp and reducing relative contents of L-Phe and L-Tyr (Fig. 5; Additional file 2: Table S2).

Secondly, as Ellis and Towers [43] verified, L-Phe and L-Tyr were the precursors of the biosynthetic pathway of RA, an important phenolic acid in *S. miltiorrhiza* [44, 45]. Therefore, as soil Cd concentration changed from 0 to 50 mg $kg^{-1}$, relative contents of RA, L-Phe and L-Tyr increased significantly ($p < 0.05$) (Fig. 5). However, with soil Cd increasing from 50 to 100 mg $kg^{-1}$, the relative content of RA also increased markedly ($p < 0.05$), and relative contents of both L-Phe and L-Tyr declined significantly ($p < 0.05$) (Fig. 5). This might be caused by that, although both the relative contents of L-Phe and L-Tyr declined as soil Cd concentration ranged from 50 to 100 mg $kg^{-1}$, relative contents of both two amino acids in 100 mg $kg^{-1}$ Cd-stressed group were more than twice of these in the control group (Fig. 5). The accumulated L-Phe and L-Tyr could also promote the synthesis of RA in *S. miltiorrhiza* roots. Conversely, Strejckova et al. [46] and Zhang et al. [25] proved that Cd stress restrained the synthesis of RA in *Scenedesmus quadricauda* and *S. miltiorrhiza*, respectively. The differences might be resulted from that the tolerance and response to Cd varied greatly among plants of different species, varieties or populations [47].

### Effect of Cd on the synthesis of tanshinones in S. miltiorrhiza

Cd stress could suppress the synthesis of tanshinones. As reported above, GGPP was one of the major contributors mainly in distinguishing samples of the control and Cd-stressed groups (Additional file 2: Table S2). GGPP was the critical precursor of synthesizing tanshinones, parts of the main active

ingredients of *S. miltiorrhiza* roots [48]. In the present study, with soil Cd concentration rising, the relative content of GGPP significantly decreased and then increased ($p < 0.05$) (Fig. 5E). Consistent with the study findings, Zhang et al. [25] revealed that the accumulation of GGPP was higher in the control group than that of the Cd-stressed group (Fig. 5E). This might be caused by the comprehensive regulation of antioxidant enzymes and GGPPS genes.

On the one hand, antioxidant enzymes could scavenge reactive oxygen free radicals produced by Cd stress [49]. As reported above, the comprehensive regulation of antioxidant enzymes regulated the resistance of *S. miltiorrhiza* roots to different levels of Cd stress. This might lead to the variation of the relative content of GGPP with soil Cd concentration changing (Figs. 2, 5E). On the other hand, Cd stress could influence the expression of genes in *S. miltiorrhiza* roots [50]. Ali et al. [51] discovered that Cd could down-regulate the expression of GGPPS genes, exhibiting a wide range of responses to abiotic stresses, and suppressed the activities of GGPP synthases in *S. miltiorrhiza* roots. This led to the higher relative content of GGPP in *S. miltiorrhiza* roots than that of each Cd added group (Fig. 5E).

# Conclusion

Cd is one of the major heavy metal pollutants in the environment, threatening human health by affecting the safety and quality of medicinal plants. This study characterized the associated roles of physiological and metabolic regulation in synthesizing active ingredients in *S. miltiorrhiza* at different levels of Cd contamination. Firstly, in all groups, the Cd concentration in leaves was lower than that in roots, with the TF and BCFs in Cd addition groups below 1. Besides, with the concentration of Cd rising, the proline content increased and then declined significantly with MDA content decreasing; Activities of POD and CAT rose and then went down significantly, with SOD activity showing no significant differences. This verified that *S. miltiorrhiza* was a non-Cd-hyperaccumulator and primarily enriched Cd in roots, with proline, POD and CAT playing vital roles in resisting Cd stress to roots. Secondly, amino acids (especially D-Gln, L-Try, L-Asp and L-Phe) and organic acids (especially GGPP and RA) were major contributors in significantly distinguishing the samples of different levels of Cd. With soil Cd concentration rising, relative contents of D-Gln and L-Asp declined and then increased; conversely, relative contents of L-Phe and L-Try first rose and then declined. This illustrated that Cd treatment enhanced the synthesis of RA through regulating the metabolism of amino acids (especially D-Gln, L-Try, L-Asp and L-Phe) but suppressed the synthesis of tanshinones via decreasing the content of GGPP. Besides, this progress was related to the comprehensive regulation of antioxidant enzymes (especially POD and CAT). The findings revealed how the synthesis of active ingredients in *S. miltiorrhiza* resists different levels of Cd stress via regulating metabolism and physiology.

# Declarations


### Acknowledgments

We are grateful for the support by Nanjing Genepioneer Biotechnologies Co. Ltd.

## Funding

This work was financially supported by the Scientific Research Foundation for Doctor of the Jiangxi University of Chinese Medicine (2020BSZR011) and the National Key R&D Program of China (2019YFC1712302).

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### Contributions

J.Y. and X.W. designed the research and undertook the formal identification of the samples; J.Y. performed the research and wrote the paper; H.F. and X.W. revised the paper.

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## Ethics declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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# Tables

Table 1 Basic physicochemical properties of the pristine soil.

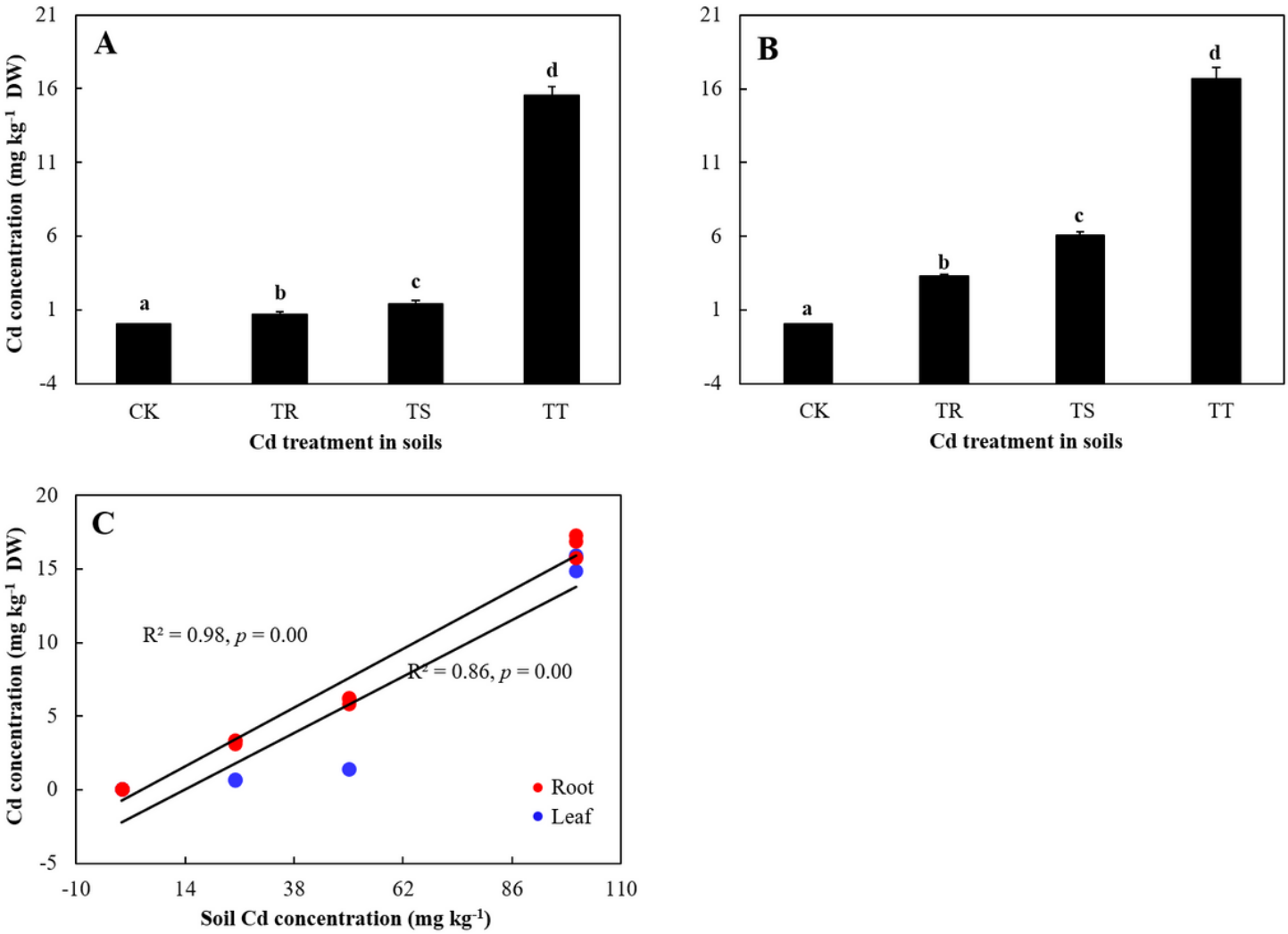
| Physicochemical properties (wt.%) | Pristine soil |
|---|---|
| Total P (g $kg^{-1}$) | 0.28 ± 0.00 |
| Total K (g $kg^{-1}$) | 27.15 ± 0.82 |
| Total N (g $kg^{-1}$) | 0.30 ± 0.02 |
| Available P (g $kg^{-1}$) | 0.01 ± 0.00 |
| Available K (g $kg^{-1}$) | 0.08 ± 0.00 |
| Available N (g $kg^{-1}$) | 0.01 ± 0.00 |
| Organic matter (g $kg^{-1}$) | 1.97 ± 0.07 |
| Cd (mg $kg^{-1}$) | 0.94 ± 0.04 |
| Ph | 4.60 ± 0.36 |

Table 2 The transfer factor (TF) and bioconcentration factors (BCF) of *S. miltiorrhiza* under different levels of Cd stress.

| Cd treatment | TF | BCF of roots | BCF of leaves | BCF of the seedlings |
|---|---|---|---|---|
| CK | 1.0085 ± 0.0433a | 0.0532±0.0022a | 0.0536±0.0026a | 0.9531±0.0041a |
| TR | 0.2116 ± 0.0111b | 0.1316 ± 0.0056b | 0.0278 ± 0.0009b | 0.4320 ± 0.0205b |
| TS | 0.2344 ± 0.0065b | 0.1219 ± 0.0040b | 0.0284 ± 0.0004b | 0.6462 ± 0.0230c |
| TT | 0.9338 ± 0.0624a | 0.1666 ± 0.0078c | 0.1553 ± 0.0060c | 0.5215 ± 0.0236d |

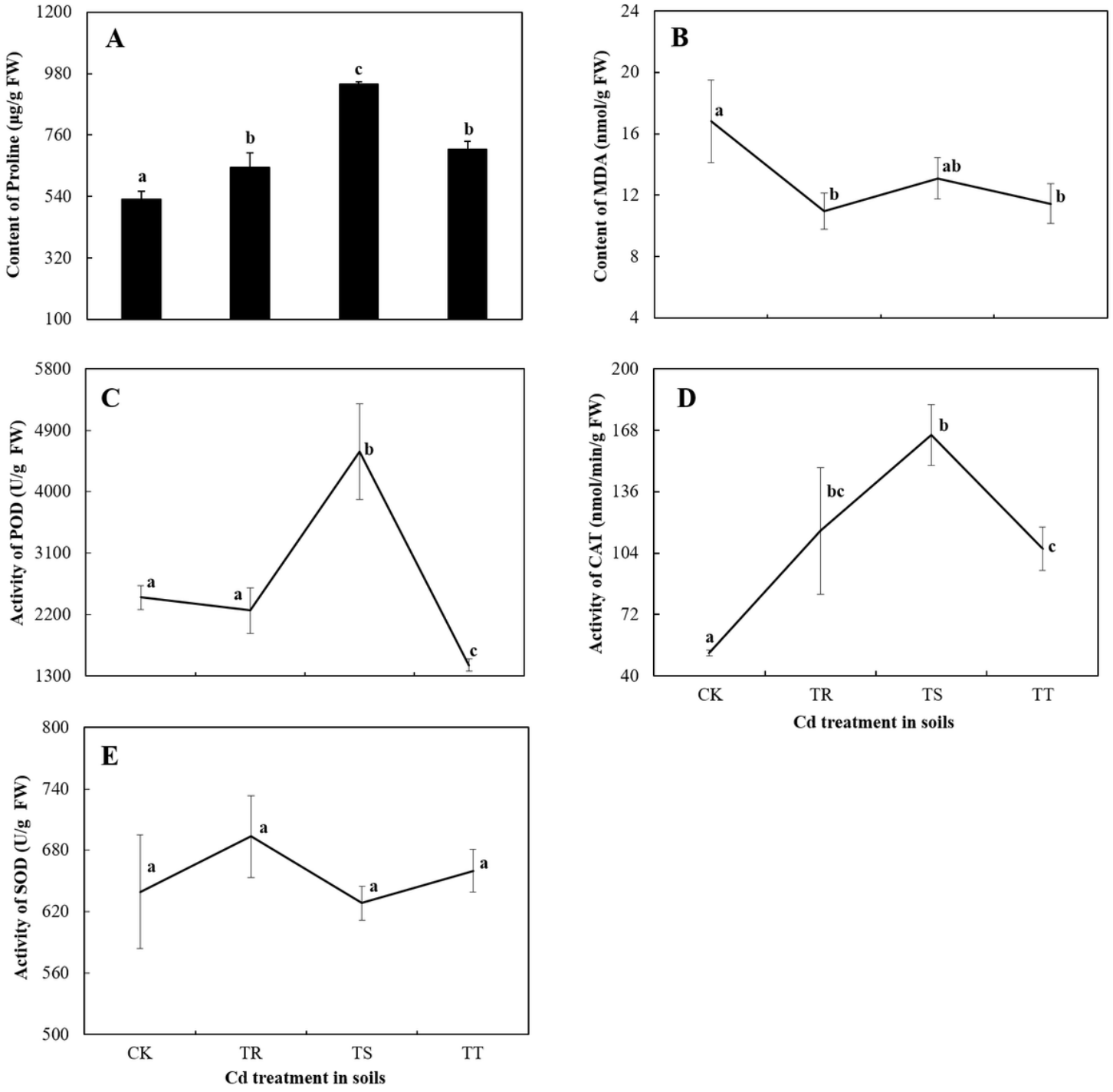
CK, TR, TS and TT stand for roots in the control, 25 mg $kg^{-1}$ Cd, 50 mg $kg^{-1}$ Cd, and 100 mg $kg^{-1}$ Cd group, respectively (the same below); The data with different little letters in same column show significant difference ($p < 0.05$).

# Figures



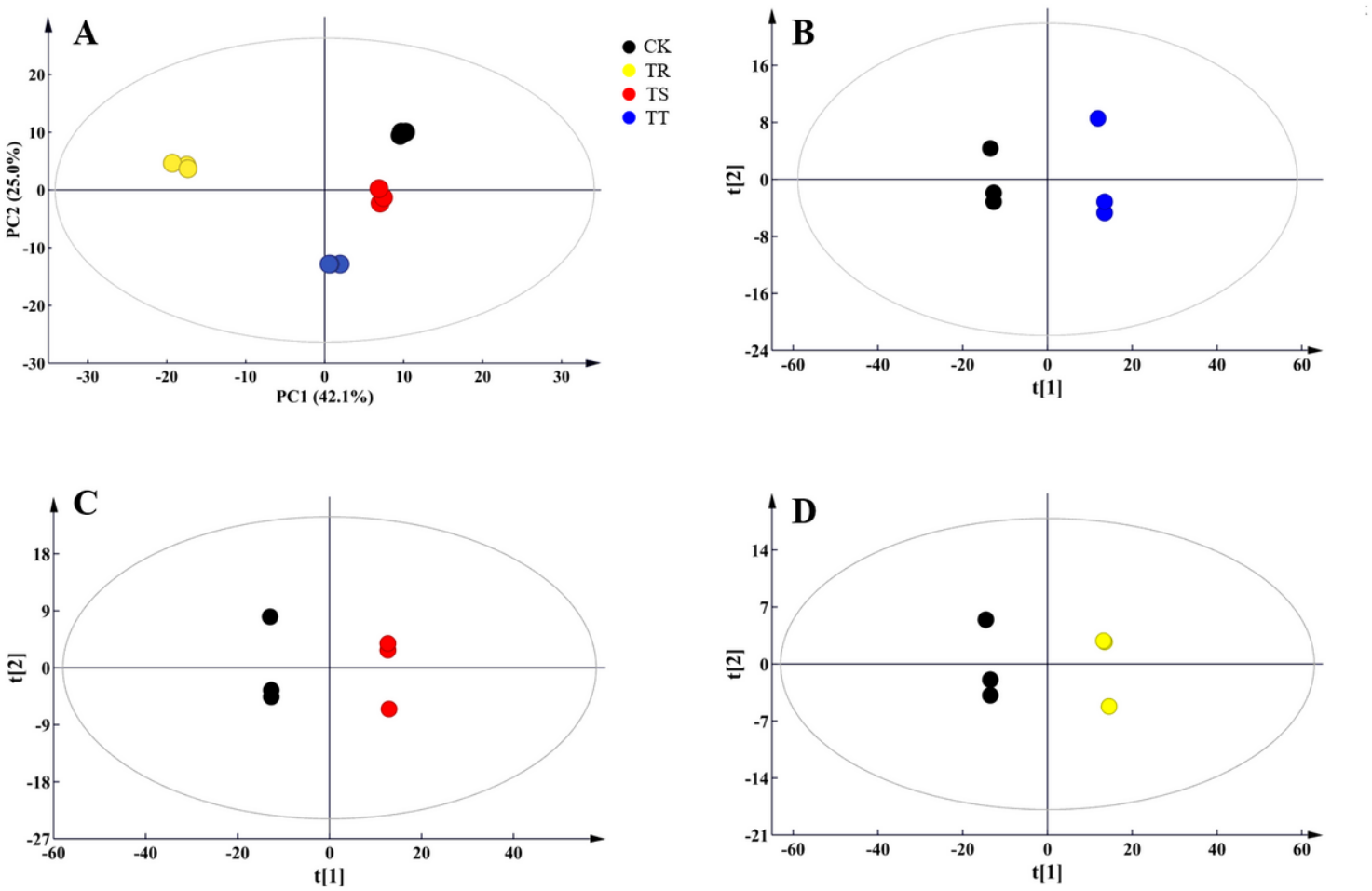

## Figure 1

Enrichment characteristics of Cd in leaves and roots of S. miltiorrhiza with different levels of soil Cd stress. A and B stand for Cd concentration in S. miltiorrhiza leaves and roots with different levels of soil Cd stress, respectively; C stands for relationships of Cd concentration in leaves and roots and soil Cd concentration, respectively.



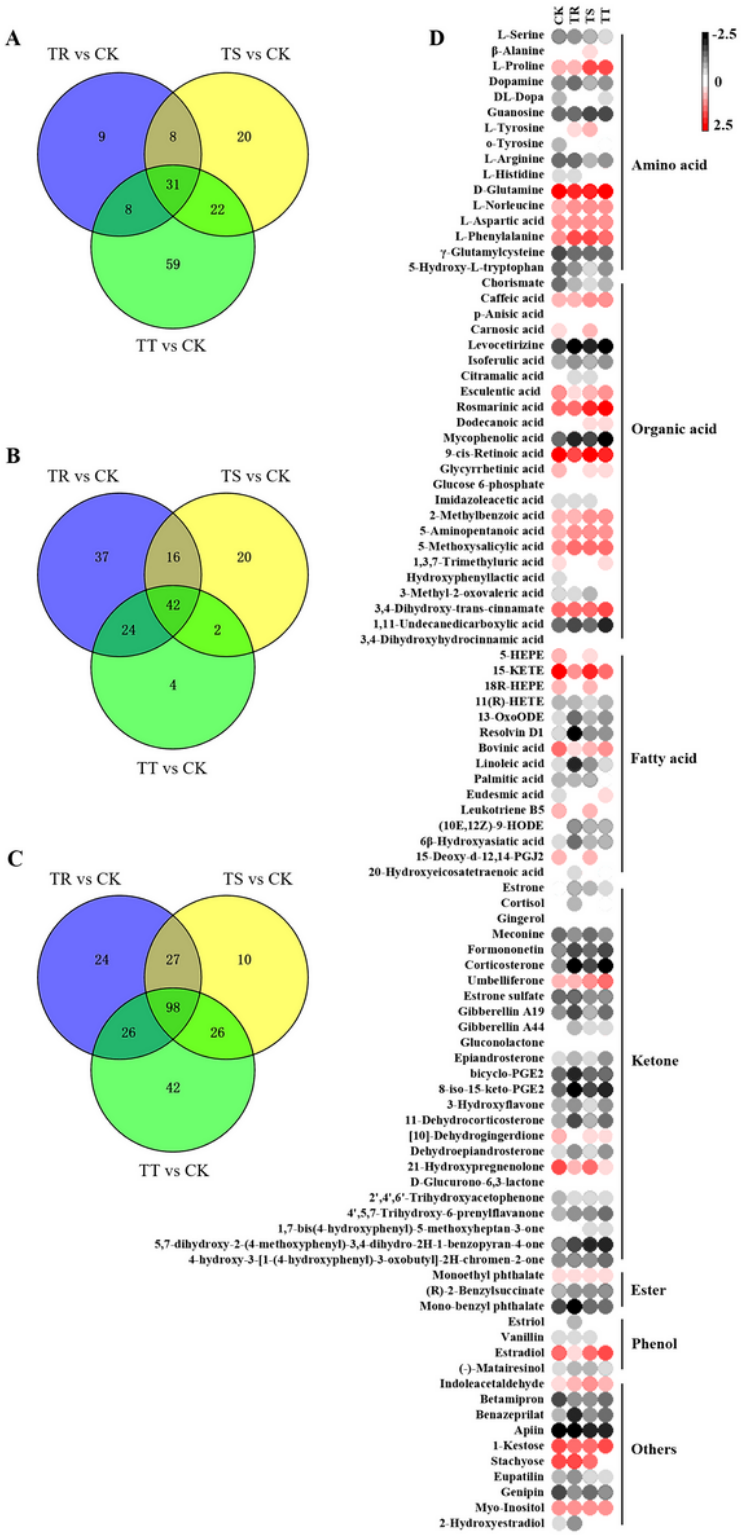

## Figure 2

Physiological characteristics of S. miltiorrhiza roots with different levels of soil Cd stress. A and B stand for effect of Cd on the content of proline (A) and MDA (B) in S. miltiorrhiza roots, respectively. C, D and E stand for effect of Cd on the activities of POD, CAT and SOD in S. miltiorrhiza roots, respectively.



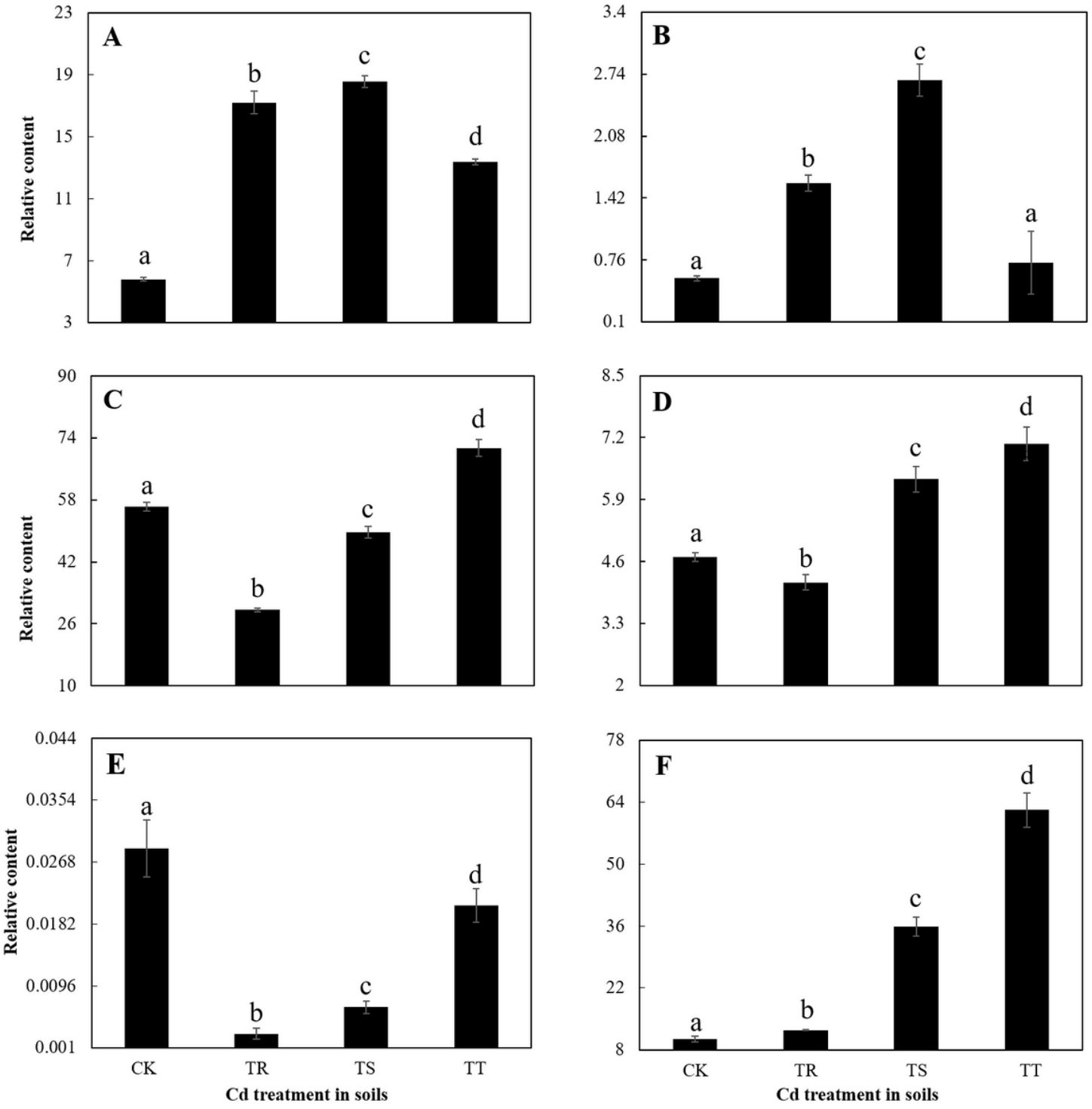

## Figure 3

Changes of metabolites in S. miltiorrhiza roots under different levels of Cd stress. A, The score plots of PCA for metabolomic data from S. miltiorrhiza roots under different levels of Cd stress. PC1, the first principal component; PC2, the second principal component. The ellipse indicates the Hotelling'sT2 (95%); B, C and D stand for score plots of PLS-DA for metabolomic data from S. miltiorrhiza roots of the control and the 25 mg kg-1 (B), 50 mg kg-1 (C), and 100 mg kg-1 (D) Cd group, respectively.




## Figure 4

Variation of the differential metabolites in S. miltiorrhiza roots under different levels of Cd stress. A, B and C stand for overlap of the up-differential metabolites, down-differential metabolites and all the differential metabolites of S. miltiorrhiza roots in response to different Cd stress, respectively; D, Heatmap analysis of the same differential metabolites in S. miltiorrhiza roots in response to different Cd stress.




## Figure 5

Relative content of L-Phe (A), and L-Try (B) D-Gln (C), L-Asp (D), GGPP(E), RA (F) of S. miltiorrhiza roots under different levels of Cd stress.

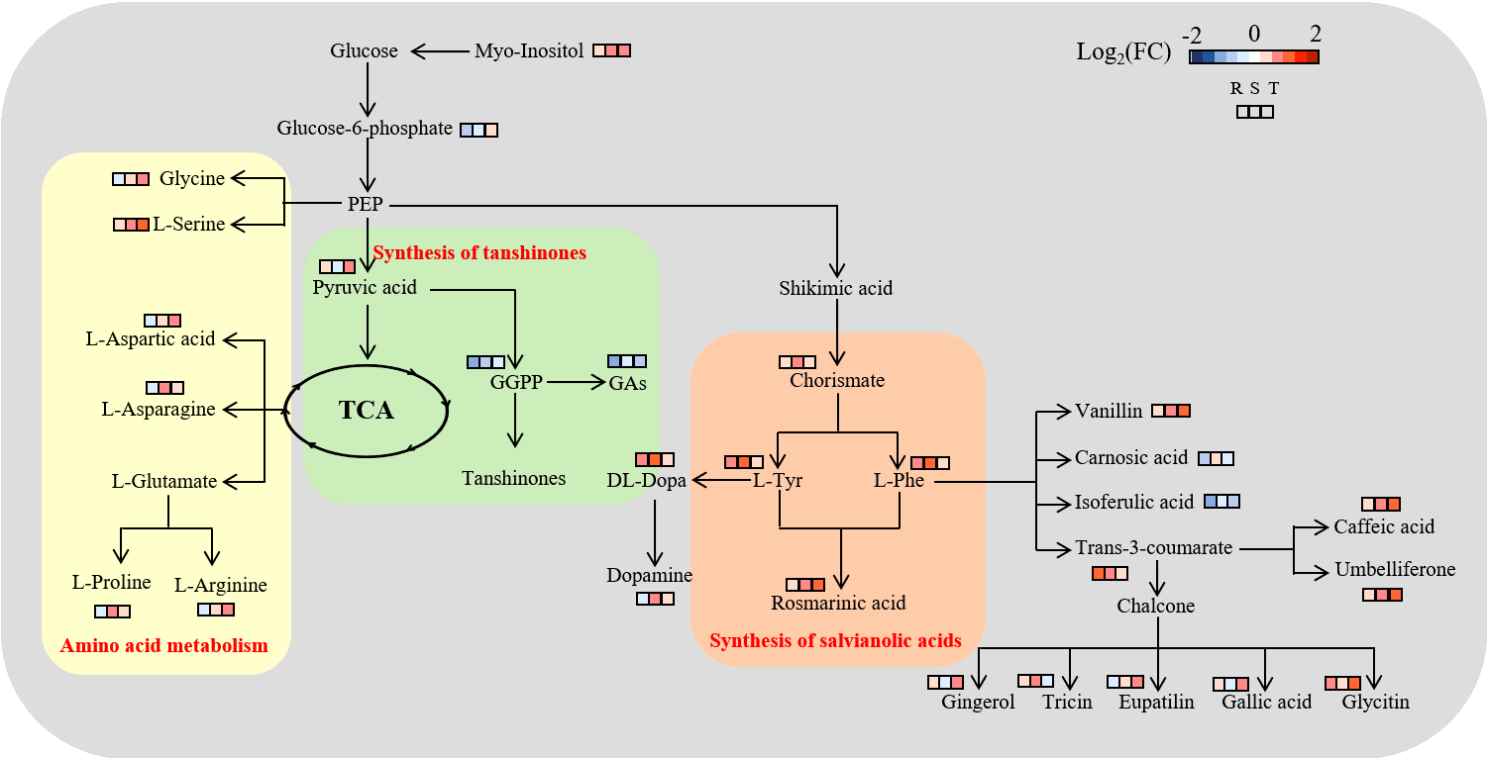



**Figure 6**

Schematic diagram of the metabolic pathways under different levels of Cd stress. L-Tyr, L-Phe, GGPP and GAs stand for L-Tyrosine, L-Phenylalanine, geranylgeranyl-PP and gibberellins, respectively; Metabolisms in orange, blue and red backgrounds were amino acid metabolism, synthesis of tanshinones, and synthesis of salvianolic acids, respectively; log2(FC) stands for an estimate of the log2-transformed ratio of the relative content of metabolites in roots of the Cd stress group to that of the control group; R, S and T stand for an estimate of the log2-transformed ratio of the relative content of metabolites in roots of the 25 mg kg-1, 50 mg kg-1 and 100 mg kg-1 Cd stress group to that of the control group, respectively.

# Supplementary Files

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