

Physiological and metabolomic analyses provide insights into the cadmium tolerance of *Sophora tonkinensis*

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Abstract

Sophora tonkinensis is an important medicinal plant native to China and Vietnam, growing in stony mountains and calcareous or sandstone hill slopes. Cadmium negatively affects the quality of *S. tonkinensis*, indirectly threatening human health. To elucidate the physiological and metabolic mechanism of *S. tonkinensis* in response to Cd stress, we conducted a hydroponic greenhouse experiment and measured the growth parameters of *S. tonkinensis* treated with 0, 20, 40, 60, and 80 $\mu\text{mol L}^{-1}$ Cd. Furthermore, metabolite changes in *S. tonkinensis* roots were investigated using liquid chromatography–tandem mass spectrometry. We identified 380 differential metabolites, and significant differences were detected in the varieties and quantities of amino acids, organic acids, fatty acids, and ketones, which have significant impact on *S. tonkinensis* stress response to Cd, under different Cd concentrations. *S. tonkinensis* resisted the stress induced by 40 $\mu\text{mol L}^{-1}$ Cd by upregulating lysine, arginine, histidine, glycine, sarcosine, tryptophan, D-proline, DL-serine, cellobiose, linoleic, indole, cytosine, and genistein contents and downregulating betaine, leucine, citrate, oleic, trehalose, and daidzein contents. Linoleic, palmitic, citrate, maackiain, isomaltose, and cinnavalininate contents were upregulated, and L-phenylalanine, sarcosine, indole acetate, and cytosine were downregulated in response to 80 $\mu\text{mol L}^{-1}$ Cd stress. KEGG pathway enrichment analysis showed that the isoflavonoid biosynthesis pathway was significantly enriched, indicating its important role in the Cd resistance of *S. tonkinensis*. This study provides important information for understanding the tolerance and accumulation mechanisms of *S. tonkinensis* in response to Cd stress, which will contribute to the breeding of medicinal plant.

Background

Cd is a harmful environmental pollutant due to its high solubility, transmissibility, absorption, and toxicity. Cd accumulation in plants leads to various physiological and biochemical changes, inhibiting plant growth and development and even causing death [1]. Cd, which enters the human body through the food chain, can causes irreversible damage to people's health [2]. Cd was listed as a human carcinogen (group I) by the World Institute of Cancer in 1993 [3]. It poisons the gastrointestinal and respiratory tracts of people via Cd-contaminated water, food, and air [4–6].

Sophora tonkinensis Gagnep., belonging to the genus *Sophora* (Fabaceae), is native to China and Vietnam. The dried roots of *S. tonkinensis* have been used medicinally for over a thousand years, particularly for their therapeutic effects on pharyngitis and sore throat [7]. Over 200 compounds have been identified from *S. tonkinensis*, including flavonoids, alkaloids, and terpenoids. Alkaloids (including matrine, oxymatrine, and sophocarpine) [8] and flavonoids (including genistein, maackiain, and trifolirhizin) [9–12] are the main active ingredients of *S. tonkinensis*.

In China, *S. tonkinensis* is mainly distributed in the stony mountains, calcareous, or sandstone hill slopes of Hechi in Guangxi Province [13]. Located at the center of the karst zone in southwest China, Hechi's karst physiognomy is well-developed and concentrated [14]. Additionally, Hechi, an important mineral base in China, usually called the “land of nonferrous metals” [15]. Special hydrogeological conditions and frequent mining activities have led to high soil heavy metal content in Hechi, leading to serious environmental

problems for the agricultural industry and human health [16–18]. With the deterioration of Cd pollution in soil, Cd has become a major pollutant in *S. tonkinensis*, threatening human health. Therefore, understanding the molecular mechanism of Cd toxicity in *S. tonkinensis* is critical for producing high-quality, high-yielding crops and cultivating Cd-tolerant varieties. However, to our knowledge, the toxicity or detoxifying mechanism of Cd in *S. tonkinensis* remains unknown.

Plants adapt to abiotic stress by regulating self-metabolism; consequently, amino acids, fatty acids, and organic acids have been extensively studied [19–21]. Plants regulate the metabolic process of amino acids, organic acids, and fatty acids to cope with heavy metal toxicity; these metabolites vary with the plant variety or genotype [22, 23]. Differences exist in varieties and quantities of amino acids, organic acids, and fatty acids between different Cd stress [20, 24, 25]. Metabolomics has become the focus of research for its extensive use in various fields to elucidate organism metabolic processes and physiological and pathological change mechanisms [26–28]. Plant metabolomics can help us understand the basic plant metabolic mechanism under abiotic stress [29].

In this study, we investigated the metabolomics of *S. tonkinensis* under Cd stress using an untargeted metabolomics approach based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) in a hydroponic experiment. Further, we analyzed different metabolites and their metabolic pathways, revealing the underlying Cd toxicity or detoxification mechanisms of *S. tonkinensis*, which can provide reference for plant breeders.

Results

Changes in *S. tonkinensis* growth parameters under Cd Stress

After 10 d of Cd treatment, the growth parameters, including plant height, plant fresh weight, plant dry weight, root fresh weight, and root dry weight, were determined. No significant difference was observed in the plant dry weight, root fresh weight, and root dry weight between different Cd stress treatments (Table 1). However, the plant height increased, reached the maximum, and then decreased gradually with increasing Cd concentrations. Compared with T0, the plant fresh weight of T1, T2, T3, and T4 plants was inhibited by 15.6%, 7.2%, 7.4%, and 16.8%, respectively.

Table 1
The growth parameters of *S. tonkinensis* under different levels of Cd stress.

Treatments	Plant height (cm)	Plant fresh weight (g·plant ⁻¹)	Plant dry weight (g·plant ⁻¹)	Root fresh weight (g·plant ⁻¹)	Root dry weight (g·plant ⁻¹)
T0	8.548 ± 0.111 c	1.199 ± 0.095 a	0.217 ± 0.011 a	0.290 ± 0.014 a	0.033 ± 0.006 a
T1	9.070 ± 0.017 b	1.012 ± 0.055 b	0.198 ± 0.014 a	0.230 ± 0.017 a	0.028 ± 0.003 a
T2	9.663 ± 0.238 a	1.113 ± 0.039 ab	0.216 ± 0.010 a	0.261 ± 0.026 a	0.031 ± 0.004 a
T3	9.485 ± 0.203 a	1.110 ± 0.124 ab	0.211 ± 0.028 a	0.267 ± 0.029 a	0.031 ± 0.009 a
T4	8.535 ± 0.999 c	0.998 ± 0.083 b	0.198 ± 0.014 a	0.256 ± 0.026 a	0.024 ± 0.007 a

Data represent mean ± SD of three replicates. Different letters indicate significant differences among the treatments at $P < 0.05$ according to Duncan's multiple range test.

Cd induced cell death in *S. tonkinensis* roots

An NBT staining assay was performed to detect cell death or injury in the root tips of *S. tonkinensis* seedlings under Cd stress. The root tips of T2, T3, and T4 were more intensely stained than that of T0 (Fig. 1). Furthermore, the staining intensity of the root tips gradually deepened with increasing Cd concentrations, particularly in the meristem region. The results suggest that Cd stress induced cell death or injury in the root tips of *S. tonkinensis* seedlings and that the degree of cell death or injury increased with increasing Cd concentrations.

Metabolite detection

The plants from the T0, T2, and T4 treatments were selected for metabolomic analysis based on the growth parameter and NBT staining assay results. In total, 10,197 and 1,965 peaks were determined in POS and NEG, respectively. Finally, 1,170 metabolites, including 611 from POS and 496 from NEG, were detected in the *S. tonkinensis* roots (Fig. 2A), of which 655 were identified based on the MS2 library and classified into amino acids, peptides, and their analogs (22.44%), fatty acids and conjugates (5.50%), carbohydrates and carbohydrate conjugates (5.19%) (Fig. 2B).

Differences between metabolite groups

The score plot results of PCA showed that the T2/T0, T4/T0, and T4/T2 comparison group samples were separated by two principal components, corresponding to approximately 80.8%, 84.0%, and 74.8% of the variability, respectively (Fig. 3A-C). OPLS-DA was conducted to further analyze the data from T2/T0, T4/T0,

and T4/T2 comparison groups, with R2 and Q2 indicating the fit and the model predictive capability, respectively. The R2Y and Q2 values were 0.999 and 0.927 in T2/T0 comparison group, 0.996 and 0.965 in T4/T0 comparison group, and 0.999 and 0.923 in T4/T2 comparison group, respectively (Fig. 3D-F). These results showed an obvious distinction between the three groups with good model quality.

Differential metabolite (DM) screening

The multivariate and univariate results were integrated to acquire the criteria for screening the DMs in T2/T0, T4/T0, and T4/T2. The criteria included VIP > 1 and *P*-value < 0.05. In total, 380 DMs were identified (Table S1), of which 245 (137 downregulated, 108 upregulated), 231 (142 downregulated, 89 upregulated), and 252 (141 downregulated, 111 upregulated) DMs were detected in T2/T0, T4/T0, and T4/T2, respectively, and 76 DMs (16 upregulated, 32 downregulated) were common among the three comparison groups (Fig. 4).

Trends of DMs under different levels of Cd stress

Based on the molecular structures, the 380 different metabolites were classified into ten major groups: amino acids (17.9%), organic acids (15.8%), ketones (14.2%), fatty acids (12.4%), benzenes (11.3%), sugars (10.3%), alkalis (6.1%), nucleosides (3.1%), amides (2.1%), and others (6.8%). The T2 and T4 Cd treatments upregulated or downregulated the DMs of these ten major groups, and there were substantial differences in varieties and quantities (Fig. 5).

Trends of amino acids

In total, 53 (33 downregulated, 20 upregulated) and 52 (38 downregulated, 14 upregulated) different amino acids and their derivatives were identified in the T2/T0 and T4/T0 groups, respectively (Fig. 6B, C). Among them, 11 (accounting for 16.2%) and 37 (54.4%) were upregulated and downregulated, respectively, in both T2/T0 and T4/T0 groups. The upregulated amino acids included arginine, DL-proline, DL-tryptophan, D-asparagine, D-ornithine, and DL-asparagine; the latter three amino acids increased more in T4/T0, indicating that their content increased with increasing Cd content in *S. tonkinensis* roots. The downregulated amino acids included betaine, leucine, and γ -aminobutyric acid (GABA). In both groups, the downregulated metabolites outnumber the upregulated metabolites, resulting in a 17.8% and 16.2% decrease in the total relative content of amino acids and their derivatives in T2/T0 and T4/T0, respectively (Fig. 6A). The contents of lysine, histidine, D-proline, DL-serine, sarcosine, bestatin, and phenylalanine increased significantly under T2 Cd stress but decreased significantly under T4 Cd stress compared with T0.

Trends of fatty acids

In total, 41 (22 downregulated, 19 upregulated) and 46 (33 downregulated, 13 upregulated) different fatty acid were detected in the T2/T0 and T4/T0 groups, respectively (Fig. 6B, C). Compared with T0, nine fatty acids (19.1%) upregulated in both T2 and T4, including LPC 18:1, LPC 18:2, and dodecanedioic acid; in particular, dodecanedioic acid increased significantly in T4. In both T2 and T4, 18 fatty acids (38.3%) were downregulated, including cholic acid, citronellic acid, and azelaic acid. In both groups, the downregulated metabolites outnumber the upregulated metabolites, resulting in a 13.7% and 16.2% decrease in the total

relative content of fatty acids in T2/T0 and T4/T0, respectively (Fig. 6A). Of the remaining fatty acids, nine, including dienesol diacetate, exemestane, and hyocholic acid, increased significantly under T2, and two (21-deoxycortisol and shanzhiside) decreased under T2 but increased significantly under T4 compared with T0.

Trends of organic acids

We identified 25 (15 downregulated, 10 upregulated) and 21 (13 downregulated, 9 upregulated) organic acids in T2/T0 and T4/T0 comparative groups, respectively (Fig. 6B, C). Among them, 18 were downregulated (30.0%; including arginine, 2-cis 4-trans abscisic acid (ABA), dehydroascorbic acid (DHA), and salicylic acid (SA)), and 11 were upregulated (16.7%; including gentisic acid, barbituric acid, and ethylenediaminetetraacetic acid) in both groups. The number of downregulated organic acids was more than that of upregulated, and the total relative content of organic acids increased in T2 and decreased in T4, with no significant difference between them (Fig. 6A). Compared to T0, trans-ferulic acid, indole, and malonic acid contents increased significantly under T2 Cd stress but decreased significantly under T4 Cd stress. The contents of barbituric acid and cyclopentane hexanoic acid increased under both T2 and T4 Cd stress but increased substantially under T4, indicating that their content increased with increasing Cd concentrations in *S. tonkinensis* roots.

Differential metabolic pathway analysis

We performed a KEGG pathway enrichment analysis of the 380 DMs, obtaining 74 metabolic pathways (Table S2). As shown in Fig. 7, ABC transporters, isoflavonoid biosynthesis, and pyrimidine metabolism were significantly enriched in T2/T0. Isoflavonoid biosynthesis was significantly enriched in T4/T0. Starch and sucrose metabolism and tryptophan metabolism were significantly enriched in T4/T2 ($P < 0.05$) (Table 2, Table S3). Isoflavonoid biosynthesis was significantly enriched in both T2/T0 and T4/T0, including 11 DMs (Table 3). Furthermore, a metabolic map of the Cd stress resistance process of *S. tonkinensis* roots was developed based on the variation results of varieties and quantities of DMs and KEGG pathway enrichment analysis of DMs (Fig. 8).

Table 2
Significantly enriched metabolic pathways of *S. tonkinensis* under different Cd stress levels.

	KEGG	C_id (metabolites)
T2/T0	ABC transporters	C00009 (Phosphoric acid), C00089 (Sucrose), C00137 (Myo-inositol), C00140 (N-acetyl-d-glucosamine), C00185 (Cellobiose), C00212 (Adenosine), C00330 (Deoxyguanosine), C00387 (Guanosine), C00387 (Guanosine), C00475 (Cytidine), C00719 (Betaine), C00881 (2'-deoxycytidine), C00881 (Deoxycytidine), C01083 (Trehalose)
	Isoflavonoid biosynthesis	C00858 (Formononetin), C01562 (Calycosin), C06563 (Genistein), C10216 (Daidzein-7-O-glucoside), C10502 ((-)-Maackiain), C10509 (Formononetin-7-O-glucoside), C10538 ((-)-Maackiain-3-O-glucoside), C14536 (Glycitein)
	Pyrimidine metabolism	C00106 (Uracil), C00178 (Thymine), C00380 (Cytosine), C00429 (Dihydrouracil), C00475 (Cytidine), C00813 (Barbituric acid), C00881S (2'-deoxycytidine), C00881 (Deoxycytidine)
T4/T0	Isoflavonoid biosynthesis	C00858 (Formononetin), C01562 (Calycosin), C06563 (Genistein), C10208 (Daidzein), C10216 (Daidzin), C10502 ((-)-Maackiain), C10503 (Medicarpin), C14536 (Glycitein)
T4/T2	Starch and sucrose metabolism	C00089 (Sucrose), C00185 (Cellobiose), C00252 (Isomaltose), C01083 (Trehalose)
	Tryptophan metabolism	C00078 (Tryptophan), C00463 (Indole), C00780 (Serotonin), C00954 (Indoleacetate), C05640 (Cinnavalininate)

Table 3

Differential metabolites involved in the isoflavone biosynthesis pathway of *S. tonkinensis* under Cd stress.

metabolites	Rt (min)	mz	T2/T0			T4/T0			T4/T2		
			log2 FC	p	vip	log2 FC	p	vip	log2 FC	P	vip
Malonylglycitin	4.65	533.13	– 0.49	0.00	0.28	0.06	0.23	0.06	0.56	0.00	0.34
Daidzein	0.64	255.06	– 0.25	0.12	0.51	– 0.66	0.00	0.98	– 0.40	0.03	0.84
Daidzein 7-O-glucoside	4.16	417.12	– 0.79	0.01	0.40	– 0.81	0.01	0.38	– 0.02	0.93	0.02
Formononetin	0.34	269.08	– 0.93	0.01	0.40	– 1.11	0.01	0.38	– 0.18	0.93	0.02
Glycitein	0.37	285.07	– 0.79	0.00	2.72	– 0.83	0.00	2.67	– 0.10	0.04	0.71
(–)-Medicarpin	4.54	269.08	– 0.32	0.00	0.32	0.46	0.00	0.43	0.78	0.00	0.66
Formononetin-7-O-glucoside	3.28	431.14	– 0.51	0.02	0.42	– 0.12	0.58	0.10	0.39	0.11	0.37
(–)-Maackiain 3-O-glucoside	2.71	464.16	0.53	0.00	0.31	0.48	0.00	0.27	– 0.06	0.47	0.07
Calycosin	1.61	283.06	0.48	0.00	1.03	0.49	0.00	0.95	0.01	0.88	0.06
Genistein	4.82	269.02	1.66	0.00	0.90	1.75	0.00	0.89	0.09	0.57	0.15
(–)-Maackiain	4.22	285.07	0.34	0.30	0.64	0.86	0.03	1.42	0.53	0.15	1.25

Discussion

Amino acid changes under Cd stress

Plants often respond to abiotic stresses by promoting proline synthesis to regulate the concentration of permeants and increasing photosynthetic, enzymatic, and non-enzymatic antioxidant activities [30, 31]. Proline accumulation in plants is often positively correlated with the heavy metal content within a certain range [32]. Therefore, the proline level can be used to indicate the degree of metal toxicity in plants [32]. Furthermore, proline is involved in metal stress defense and plays a crucial part in metal binding [33]. The abundance of D-proline and DL-proline were upregulated in both T2 and T4 groups, indicating that proline was involved in the Cd stress response of *S. tonkinensis*. As a proline precursor, arginine accumulation may also promote D-proline synthesis in sagebrush [34]. Arginine plays a key role in plant stress resistance [35]. Herein, arginine was upregulated under T2 and T4 Cd stress, promoting proline synthesis to reduce antioxidant damage in *S. tonkinensis*.

Plants respond to various abiotic stresses by enriching lysine, serine, and arginine [36]. Heavy metal stress often leads to the production of large amounts of peroxides in plants. The chelation of lysine with zinc and iron can decrease heavy metal accumulation and increase the amino acid content and antioxidant activity in plants [37, 38]. Similar results were observed in this study; lysine and serine were upregulated under T2 Cd stress, indicating their involvement in Cd stress response and antioxidant damage reduction in *S. tonkinensis*.

Serine content was significantly increased under T2 Cd stress in *S. tonkinensis*. Serine have a significant impact on metabolism and signal transduction, directly or indirectly involving in the synthesis of nitrogenous bases (purine, pyrimidine, and thymidine), amino acids (cysteine, glycine, and tryptophan) [36, 39], phospholipids, sphingolipids, and choline [40, 41]. Choline is the precursor of the osmotic protective agent glycine betaine, and the change in its content is critical in plant resistance to Cd [31, 42]. However, betaine synthesis is inhibited under the combined stress of drought and salt in wheat seedlings, indicating that betaine does not play a major role in stress response [43]. Similar results were obtained in our study; betaine content gradually decreased as Cd concentration increased.

Under Cd stress, sarcosine content downregulates in cruciferous *Noccaea caerulea* [44] but significantly upregulates in aquatic *Elodea canadensis* [45], indicating that different species respond to Cd stress with different metabolites. Herein, sarcosine content was significantly upregulated under T2 Cd stress, suggesting an alternative detoxification mechanism for *S. tonkinensis*.

Tryptophan can improve Cd resistance by maintaining the levels of indole-3-acetic acid and promoting lignification in *Arabidopsis thaliana* [46]. Herein, tryptophan metabolism was significantly enriched, and tryptophan and indole-3-carboxaldehyde were upregulated under T2 and T4 Cd stress. It is hypothesized that tryptophan and indole-3-carboxaldehyde play pivotal regulatory roles during Cd defense responses in *S. tonkinensis*, and the mechanism remains to be investigated.

Role of organic acids under Cd stress

The accumulation of organic and amino acids can chelate heavy metal ions and scavenge reactive oxygen species (ROS), thereby reducing intracellular oxidative damage [21]. Heavy metals can be chelated with low molecular weight organic acids stored in vacuoles and transformed into low or non-toxic chelating states, significantly contributing to plant heavy metal stress resistance [47, 48]. For example, citric acid can alleviate Cd stress by reducing plants' Cd uptake [24]. Furthermore, amino acids (glycine and leucine) and organic acids (malonic acid, malic acid, citric acid, and oxalic acid) jointly influence the chelating ability of Cd [47]. In this study, glycine and malonic acid were significantly upregulated under T2 Cd stress, which may affect the Cd chelating ability of *S. tonkinensis*. Some plants synthesize phenolic acids in response to Cd and Zn stress, which have a strong free radical scavenging capacity and can alter Cd and Zn bioavailability under Cd and Zn stress. For example, cinnamic acid, which accumulates in response to Cd stress, is one of the most effective substances in combination with Cd [49]. Exogenous cinnamic acid alleviates salinity-induced stress in *Capsicum annuum* L. seedlings by upregulating antioxidant activities and proline accumulation [50]. In this study, trans-ferulic acid, a phenolic acid widely distributed in plants, accumulated significantly in

the T2 group, indicating that trans-ferulic acid accumulation under Cd stress improves the Cd detoxification ability in *S. tonkinensis*.

Role of fatty acids under Cd stress

Fatty acids and their derivatives play vital roles in enhancing stress tolerance through participating in kinds of defense pathways in plants[51]. The synthesis and release of unsaturated fatty acids is a plant defense mechanism against abiotic stresses[52]. *Salvia miltiorrhiza* downregulates unsaturated fatty acids (oleic acid, linoleic acid) and upregulates saturated fatty acids (stearic acid) under Cd stress[53]. However, the unsaturated fatty acid contents (linoleic, 13-hydroperoxy-9z,11e-octadecadienoic acid) increased with increasing Cd concentration in *S. tonkinensis*, consistent with previously reported results [54].

Phosphatidylcholine (PC) is involved in cell membrane synthesis [55]. PC is obtained by the addition of a fatty acyl group to lysophosphatidylcholine (LPC) [56, 57]. LPC 18:1 and LPC 18:2 increased under T2 and T4 Cd stress in *S. tonkinensis*. As PC precursors, LPC may help maintain cell homeostasis and cell membrane stability under Cd stress.

Role of flavonoids and isoflavones under Cd stress

Isoflavones are important secondary metabolites with strong oxidation resistance in plants, particularly those in free form, such as genistein and daidzein [58]. Genistein is an excellent antioxidant of the isoflavone family [59], which improves the resistance to fungal pathogens by reducing the H₂O₂ level and promoting phenolic substance accumulation in soybean [60]. Calycosin can be used as a salinity stress reliever, and it improves plant tolerance to NaCl [61]. Plants accumulate a large amount of calycosin in response to environmental stress, such as low temperature and UV [62, 63]. Maackiain plays an important role in the defense response of fungal pathogens through accumulating in large quantities in plants during fungal defense [64]. Chickpeas accumulate higher levels of maackiain and medicarpin in response to *Ascochyta rabiei* stress [65]. Medicarpin synthesis was induced in *Medicago sativa* L under Pb stress [66]. Cd stress causes isoflavone and derivatives accumulation in *Medicago truncatula* roots, such as formononetin, medicarpin, malonylononin, and 3-O-β-(6'-malonylglucoside) [67]. Isoflavonoid biosynthesis was enriched significantly, involving 14 metabolites in both T2/T0 and T4/T0 groups, of which genistein, calycosin, (–)-maackiain, and (–)-maackiain-7-O-glucoside were upregulated in T2/T0 and T4/T0 groups. Thus, isoflavonoid biosynthesis plays a vital role in the *S. tonkinensis* resistance to Cd stress. *S. tonkinensis* may activate the antioxidant mechanism by accumulating genistein, calycosin, and (–)-maackiain, thereby mitigating Cd stress-induced damage. However, the concentration of four isoflavones (daidzin-7-O-glucoside, formononetin, and glycitein) significantly decreased with increasing Cd content. The contents of these four isoflavones may be influenced by cadmium toxicity, and a negative correlation was observed between them.

Role of sugars under Cd stress

The cell wall is the first functional structure exposed to heavy metals. The synthesis and composition of cell wall could be severely influenced by heavy metals [68]. Cellobiose is the basic unit and decomposition product of the cell wall. Cellobiose was upregulated under T2 Cd stress, suggesting that *S. tonkinensis* reduced heavy metal absorption by accumulating cellobiose, which can promote cellulose synthesis. Sugars are essential for stress adaptation because they improve the osmotic pressure regulation ability of plants.

Isomaltose content increases in *Ginkgo biloba* L. under drought stress [69]. In *Physcomitrella patens*, the accumulation of several carbohydrates, including fructose, maltose, isomaltose, trehalose, and raffinose, was significantly increased under salt and cold stress [70]. In this study, the abundance of isomaltose and sucrose increased significantly under T4 Cd stress, indicating that their accumulation helps improve Cd stress defense as an osmotic agent under high Cd concentrations in *S. tonkinensis*.

Role of ATP-binding cassette (ABC) transporters under Cd stress

ABC transporters has regulatory effects on the response to biotic and abiotic stresses [71]. The selective transport of heavy metals in plant relies on the coordination of the transport system. In plants, Cd binds to glutathione (GSH) and phytochelatin via mercaptan ligands to form Cd complexes, which were then transported by transporters to vacuoles or cells by ATP extracellular membrane pumping to reduce Cd toxicity-induced damage [72]. *OsABCC9* in rice regulates Cd tolerance and accumulation by chelating Cd into root vacuoles [73]. *AtABCC3* reduces Cd toxicity-induced damage in *Arabidopsis* by transferring Cd from the cytoplasm into vacuoles for isolation [74]. *PtoABCG36* acts as a cadmium extrusion pump, involving in improment of Cd tolerance by decreasing Cd content in plant [75]. Ten metabolites were significantly enriched in ABC transporters in the T2/T0 comparison group (Fig. 8). Cellobiose and N-acetylglucosamine were upregulated, whereas a-glucoside, trehalose, and maltose were downregulated in ABC transporters under T2 Cd stress. Cellobiose and N-acetylglucosamine are important cell structural components [76, 77]. *S. tonkinensis* may maintain the normal morphology and basic function of cells to relieve Cd toxicity-induced damage in plants by upregulating cellobiose and N-acetylglucosamine contents.

Conclusions

This study disclosed the physiological and metabolic responses of *S. tonkinensis* under different Cd concentrations using LC-MS/MS. Cd inhibits the plant height and plant fresh weight of *S. tonkinensis*, and cell damage in root tips is aggravated with increasing Cd content. In total, 245 and 231 significant DMs were identified from *S. tonkinensis* in T2/T0 and T4/T0 comparison groups, respectively. The metabolic analysis indicated these metabolites, mainly including amino acids, organic acids, fatty acids, and ketones, caused substantial variations in varieties and quantities in *S. tonkinensis* roots when exposed to different Cd concentrations. The mechanism of alleviating Cd damage is primarily related to ABC transporters, isoflavonoid biosynthesis, pyrimidine metabolism, tryptophan metabolism, and starch and sucrose metabolism owing to their involvement in the synthesis of the related DMs. The isoflavonoid biosynthesis pathway was significantly enriched in both T2/T0 and T4/T0 groups; the significant DMs of this pathway may have alleviated the accumulation of or tolerance response to Cd in *S. tonkinensis*. These findings will help understand the tolerance and accumulation mechanisms of *S. tonkinensis* in response to Cd stress and promote medicinal plant breeding.

Materials and methods

Plant material and treatment

Mature *S. tonkinensis* seeds were collected from cultivated *S. tonkinensis* plant grown in Jinchengjiang district, Guangxi, China, with permission granted by the owner. The plant materials were formally identified by Professor Kunhua Wei and have been deposited in the Germplasm Repository of the Guangxi Botanical Garden of Medicinal Plants, Nanning, China, with voucher number YYZW20210011. Seeds were surface sterilized with 75% ethanol, washed with distilled water, sown evenly in pots containing a soil and vermiculite mixture (2:1, w/w), and incubated in the dark at 25°C for approximately 7 d. After germination, the seedlings were cultivated at 28°C/25°C for 14/10 h (light/dark). Subsequently, 30-day-old seedlings with the same development status were transferred to a plastic box filled with 2 L of 0.5×Hoagland nutrient solution. After 7 d, the seedlings were treated with 0 (T0, control, CK), 20 (T1), 40 (T2), 60 (T3), and 80 (T4) $\mu\text{mol L}^{-1}$ CdCl_2 with 0.5×Hoagland nutrient solution for 10 d.

Determination of agronomic characters

S. tonkinensis seedlings were softly pulled out, and roots were washed carefully after 10 d of Cd stress. The plant height, fresh weights of plant and root were measured. Dry weights of plant and root were measured using a weighing balance. The roots of the remaining seedlings were collected, immediately frozen in liquid nitrogen, and stored at -80°C .

Nitro blue tetrazolium (NBT) staining

NBT (50 mg) was fully dissolved in 100 mL Tris buffer (pH 7.4). *S. tonkinensis* roots were dyed in NBT solution at 25°C for 2 h, then washed with distilled water to remove the excess dye. Finally, the root tips were observed using an electron microscope (Axioscope 5; Suzhou, China).

Metabolome data collection and analysis

The cooled samples were ground into subdivisions. Metabolites were extracted with methanol : acetonitrile : H_2O with a volume ratio of 2 : 2 : 1, and then centrifuged at low temperature for 15 min, and the supernatant was vacuum dried. The samples were redissolved in 100 μL acetonitrile / water with a volume ratio of 1 : 1 and analyzed by LC-MS.

LC-MS/MS analysis

The metabolites were separated using Agilent 1290 Infinity LC ultra-high performance liquid chromatography (UHPLC) HILIC column (Thermo Fisher, Germany), the samples were placed in an automatic sampler at 4°C throughout the analysis. The separated samples were detected by Q Exactive TM HF-X (Thermo Fisher, Germany). The scanning range is m/z 100–1500 and the primary and secondary spectra of samples were obtained using an AB Triple TOF 6600 mass spectrometer.

Data processing and metabolite identification

Detected peak signals were filtered through Compound Discoverer 3.1, aligned, and quantified. The main parameters were set to default. The molecular formula was predicted by peak intensity normalization method. Peak comparison was performed using mz Cloud (<https://www.mzcloud.org/>), and qualitative and

quantitative corrections were performed using mz Vault and Mass List database. Statistical analysis was performed using R (version 3.4.3), Python (version 2.7.6), and CentOS (release 6.6).

Data quality control

The detection of metabolites was carried out in Positive (POS) and negative (NEG) ion modes, respectively, providing a higher metabolite coverage and better detection effect. Quality control (QC) samples are usually used when metabolomics is based on mass spectrometry [78], Mass Bank, Metlin, MoNA, and other public databases combined with self-built secondary mass spectrometry database matching for material annotation.

Data Analysis

Data were normalized using z-score and hierarchically clustered using the R package pheatmap [79]. The heatmap displayed the expression patterns of metabolites in all samples. Variability in metabolite composition and abundance between samples were quantified by correlation analysis between samples using R and by drawing heatmaps using the R package heatmap (v1.0.12) [79]. The similarity of metabolite composition and abundance between samples increases as the correlation coefficient approaches 1. Principle component analysis (PCA) (<http://www.r-project.org/>) was performed using R language gmodels (v2.18.1) [80]. Results are more reliable when QC distribution is denser. Partial least squares discriminant analysis (PLS-DA) was used for data dimension reduction to better distinguish the metabolomics profile of two groups [81]. PLS-DA was applied in comparison groups using R package ropls [82] (<http://www.r-project.org/>). OPLS-DA was applied in comparison groups using R package gmodels [83] (<http://www.r-project.org/>).

Statistical analysis

The data concerning the agronomic characters are presented as the means $\pm$ standard error (SE) of three replicates. One-way variance analysis (ANOVA) was performed via SPSS version 16 (SPSS, Inc., Chicago, IL, USA) software. The means were analyzed via the least significant difference (LSD) test at $P \leq 0.05$ for statistical significance.

Abbreviations

S.tonkinensis

Sophora tonkinensis

KEGG

Kyoto Encyclopedia of Genes and Genomes

LSD

least significant difference

LC-MS/MS

liquid chromatograph–tandem mass spectrometry

NBT

Nitro blue tetrazolium

POS
positive
NEG
negative
PCA
Principle component analysis
OPLS-DA
Orthogonal projections to latent structures discriminant analysis
DM
Differential metabolite
SE
standard error.

Declarations

Supplementary Information

Additional file 1: Table S1. 380 differential metabolites of *S.tonkinensis* under cadmium stress

Additional file 2: Table S2. KEGG pathway enrichment analysis of the 380 differential metabolites

Additional file 3: Table S3. Metabolic pathways of *S.tonkinensis* under different Cd stress

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Authors' contributions

Hao Chen conducted the experiments. Hao Chen and Su-Hua Huang analysed the results and wrote the manuscript. Ying Liang, Shuang-Shuang Qin, Chang-qian Quan, Gui-Li Wei, assisted in the experimental procedures. Dan-Feng Tang revised the manuscript. Kun-Hua Wei made suggestions for the writing of the manuscript. Fan Wei designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable. The authors declared that permission to collect *Sophora tonkinensis* seeds had been obtained, and experimental research works on the plants described in this paper comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors report no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

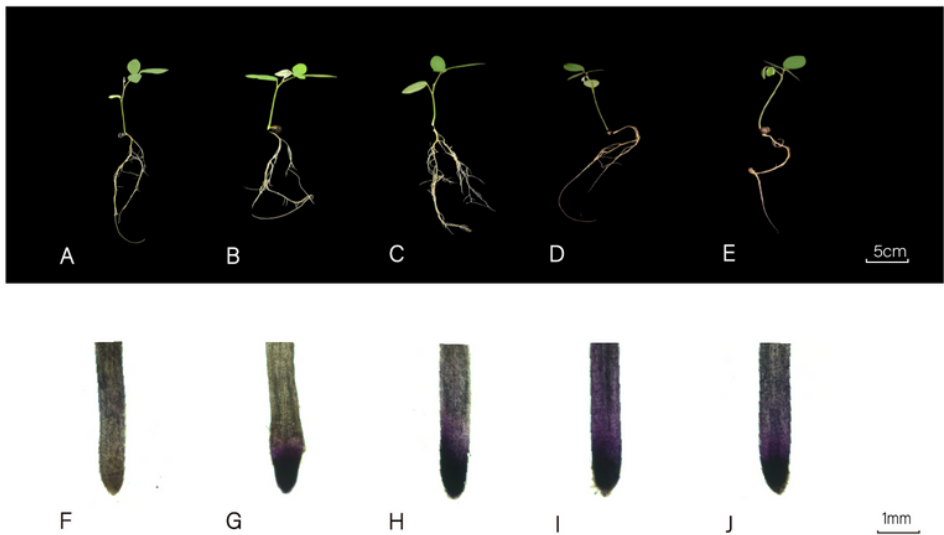


Figure 1

Plants and NBT-stained root tips of *S. tonkinensis* at different Cd stress levels. (A, F) T0, (B, G) T1, (C, H)T2, (D, I) T3, (E, J) T4.

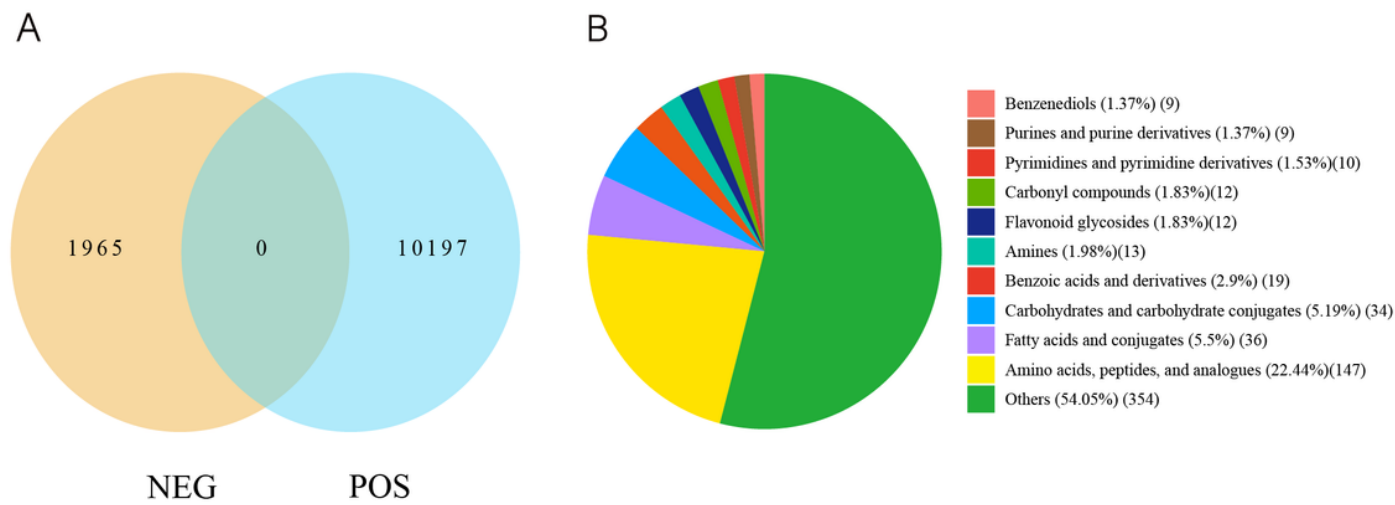


Figure 2

(A) Metabolites determined in the positive (POS) and negative (NEG) ionization modes. (B) Top 10 SubClass categories of the metabolites from the MS2 library.

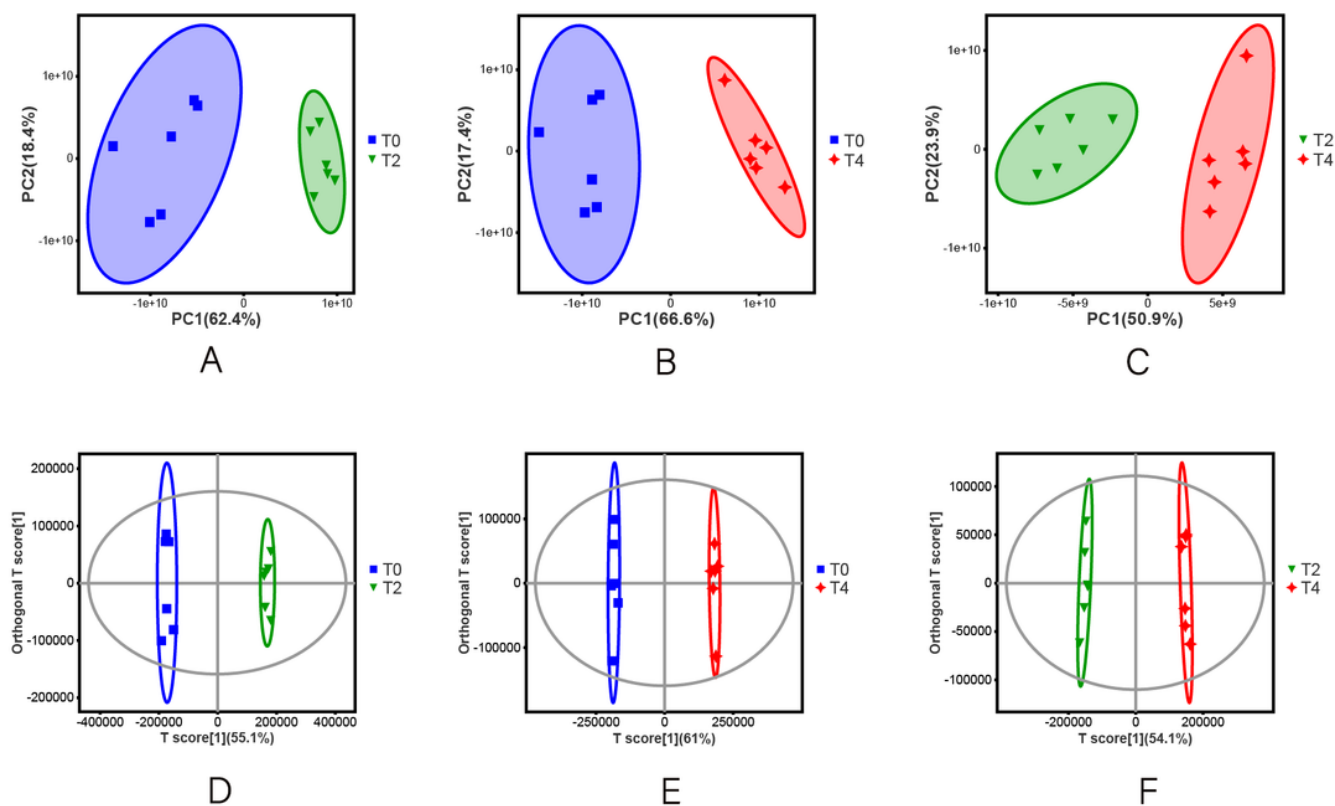


Figure 3

PCA and OPLS-DA of metabolite data in *S. tonkinensis* roots under different Cd stress levels. **(A-C)** PCA score plots of metabolite data obtained from LC-MS/MS in *S. tonkinensis* roots of T2/T0, T4/T0, and T4/T2. PC1, first principal component; PC2, second principal component. **(D-F)** OPLS-DA score plots of metabolite data obtained from LC-MS/MS in *S. tonkinensis* roots of T2/T0, T4/T0, and T4/T2.

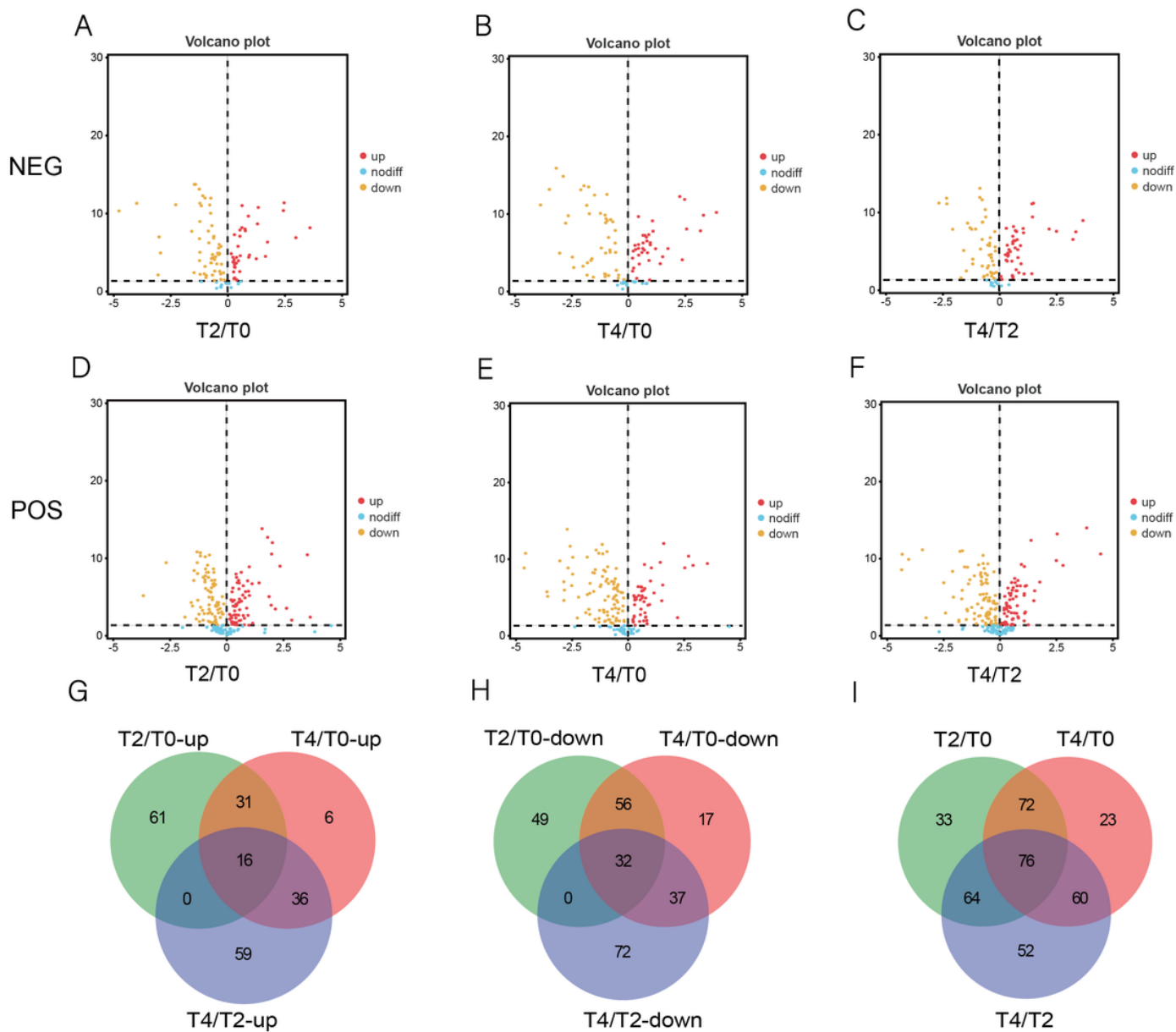
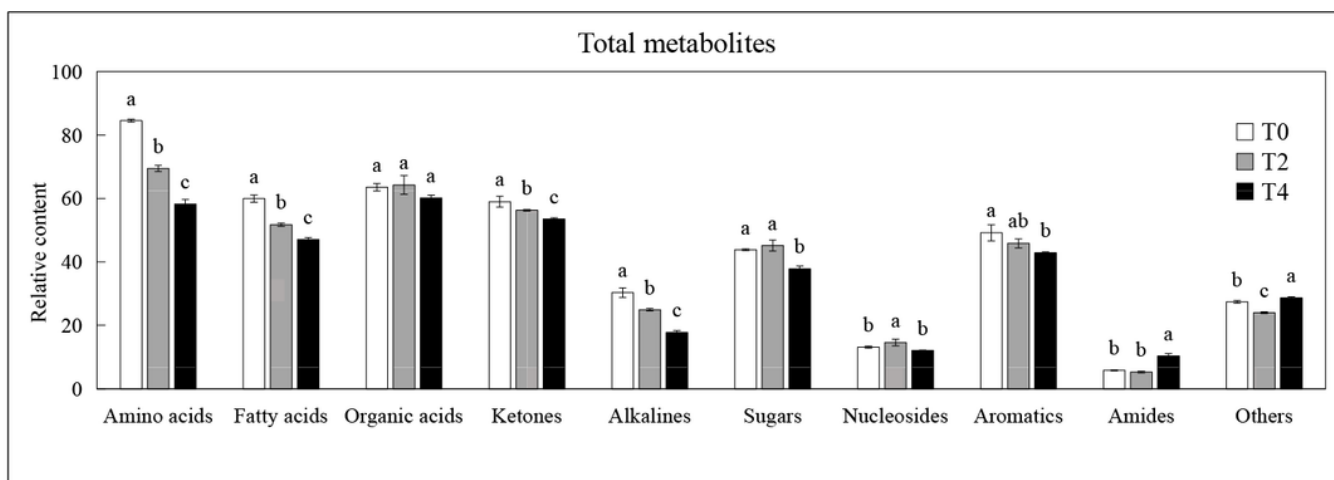


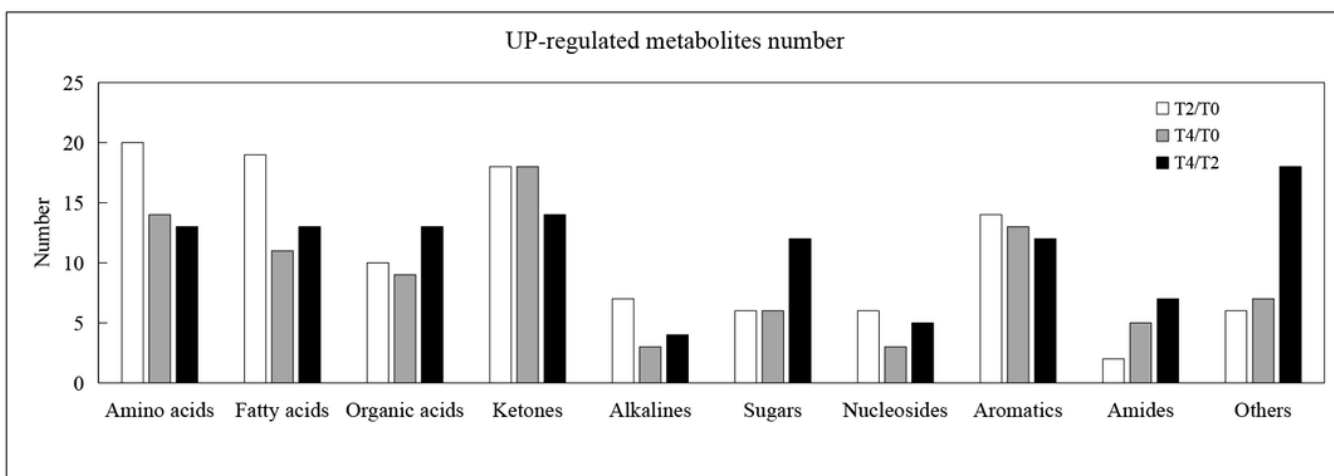
Figure 4

Variations in the metabolites in *S. tonkinensis* roots under different Cd stress levels. **(A–F)** Differential metabolites of T2/T0, T4/T0, and T4/T2 in the positive and negative ionization modes. **(G–I)** Changes in the total number of upregulated, downregulated, and both upregulated and downregulated metabolites in *S. tonkinensis* roots with different levels of Cd treatment.

A



B



C

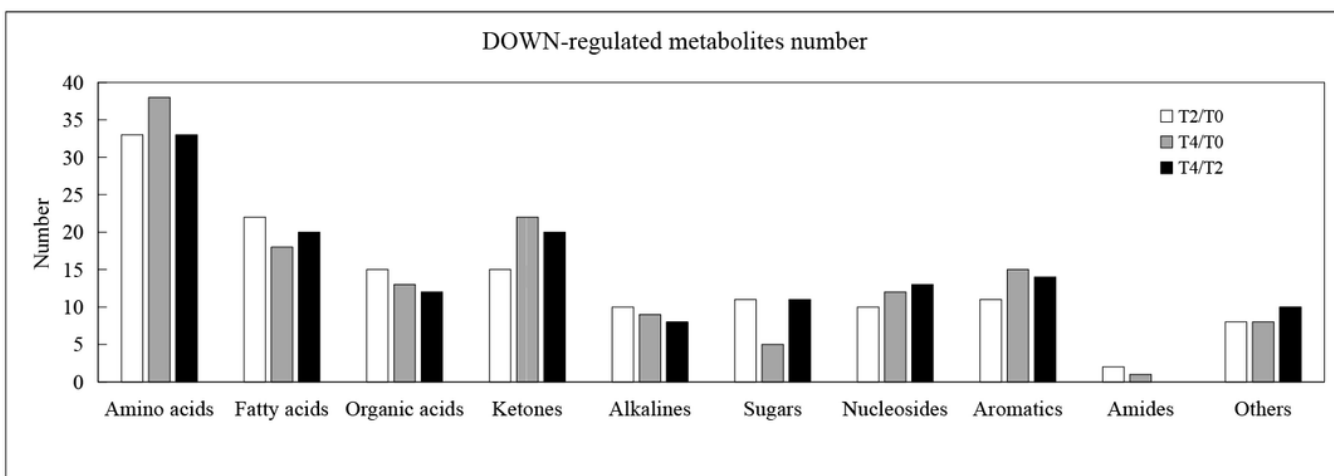


Figure 6

(A) Total relative contents of amino acids, fatty acids, organic acids, ketones, alkalis, sugars, nucleosides, aromatics, amides, and other components in *S. tonkinensis* roots. Different lowercase letters on the columns of the same metabolite indicate significant differences within each treatment in the roots ($P < 0.05$). Vertical bars above the columns indicate the standard error of each mean (mean $\pm$ SE). **(B–C)** The number of upregulated and downregulated metabolites in *S. tonkinensis* roots in T2/T0, T4/T0, and T4/T2.

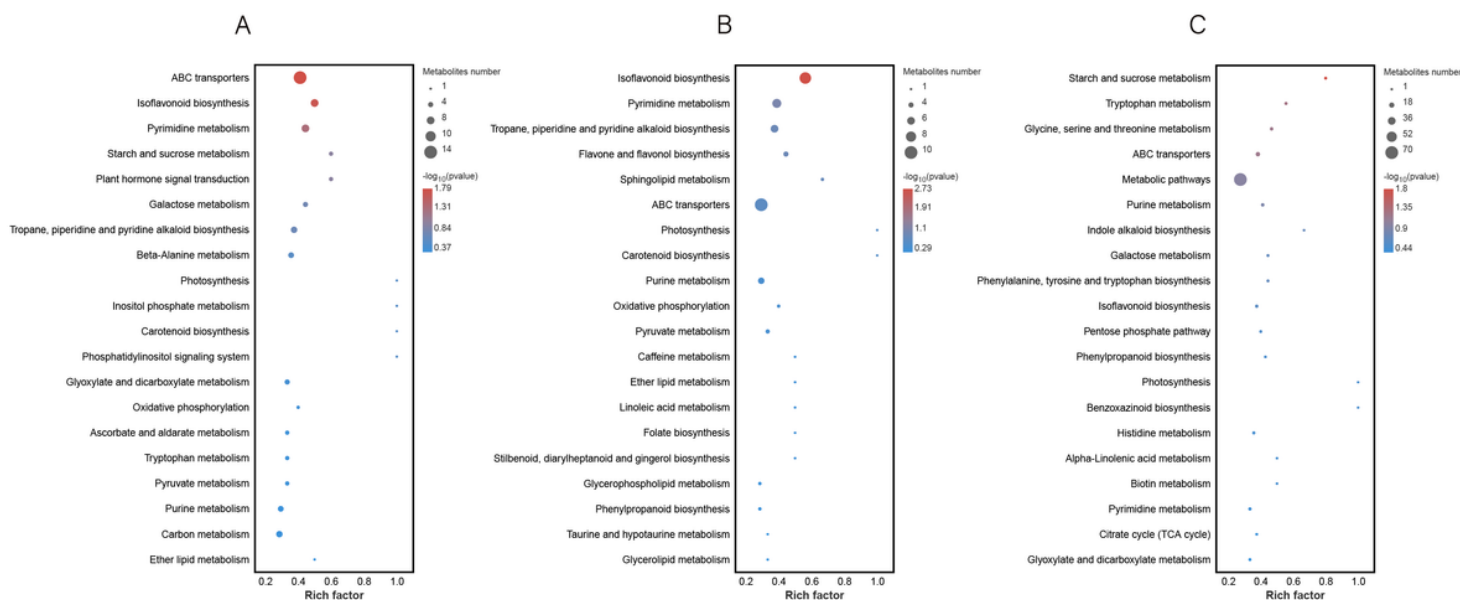


Figure 7

KEGG enrichment analysis of differential metabolic pathways in *S. tonkinensis* roots. **(A–C)** Differential metabolic pathways enrichment in T2/T0, T4/T0, and T4/T2. The vertical coordinate represents the name of the metabolic pathway, and the horizontal coordinate represents the enrichment factor (Rich factor, number of significantly different metabolites/total number of metabolites in the pathway). The greater the Rich factor, the greater the degree of enrichment. The size and color of the bubble represent the metabolite number hits and $-\log_{10}(P)$ values for the corresponding pathway.

