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Integrative multi-omics analysis reveals the underlying toxicological mechanisms of enrofloxacin on the growth of wheat seedling roots

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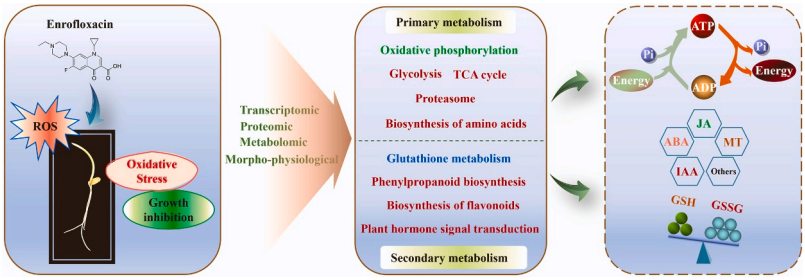
HIGHLIGHTS

- Enrofloxacin inhibited wheat growth and induced oxidative stress.
- Enrofloxacin causes dysfunction of the oxidative phosphorylation pathway.
- Enrofloxacin induces enhancement of the carbon metabolism pathway.
- The ubiquitin-independent proteasomal degradation pathway plays an important role.
- The imbalanced AsA-GSH cycle highlights the role of GST in ROS scavenging.

GRAPHICAL ABSTRACT



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ABSTRACT

The continuous release of antibiotics into agroecosystems has raised concerns about the potential negative effects of antibiotic residues on crops. In this study, the toxicological effects of enrofloxacin (ENR) on wheat seedlings were analyzed using a combination of morpho-physiological, transcriptomic, proteomic, and metabolomic approaches. ENR inhibited the growth of wheat (*Triticum aestivum* L.) roots and induced oxidative stress. In particular, ENR downregulated the oxidative phosphorylation pathway, while it enhanced glycolysis and the tricarboxylic acid cycle, thereby regulating the balance of intracellular energy metabolism. In addition, sustained exposure to excessive reactive oxygen species (ROS) resulted in an increase in reduced glutathione (GSH), a slight decrease in ascorbic acid (AsA), and a significant decrease in the ratio of GSH to oxidized glutathione (GSSG), which imbalanced the AsA-GSH cycle. In addition, the resulting increase in abnormal proteins triggered ubiquitin-independent proteasomal degradation pathways. Further, an increase in abscisic acid (ABA) and a decrease in jasmonic acid (JA) and its derivatives alleviated the inhibitory effect of ENR on the growth of wheat roots. In conclusion, direct damage and signaling by ROS, hormonal regulation, a decrease in the GSH to GSSG ratio, and insufficient energy supply were identified as key factors for the significant inhibition of wheat root growth under ENR stress.

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1. Introduction

Antibiotics are utilized to prevent or treat infections in both humans and animals or as growth-promoting feed additives for livestock and fish [1]. The environmental impact of this use of antibiotics has become a research hotspot because of the discharge of incomplete sewage during production, incomplete sewage purification, and the residual release caused by the low rates of absorption in humans and animals [2]. Antibiotics primarily enter agroecosystems through irrigation with wastewater and the use of livestock manure as fertilizer [3]. The concentration of antibiotics in soil can reach levels as high as mg/kg (dry weight) [4,1,5]. The release of antibiotic residues poses a threat to microbial activity in the environment, plant and animal growth, and even human health. However, the use of antibiotics is increasing globally and expected to continue to increase further [6].

The impact of antibiotics on plant growth has been documented for various crops, including alfalfa, mung bean, lettuce, carrots, cabbage, tomatoes, ryegrass, wheat, and rice [7-13]. Zhang et al. [14] showed that the main sites of concentration of antibiotics in the root system of maize seedlings exposed to various antibiotics, including tetracycline, sulfamethoxazole, and sulfathiazole, are the cell division zone and the elongation zone of the root tip. In wheat seedlings, the application of ciprofloxacin, enrofloxacin (ENR), and levofloxacin resulted in a significant decrease in the length of shoots and the fresh weight of shoots and roots. Additionally, the seedlings exhibited a significant oxidative stress response, as shown by the increase in malondialdehyde (MDA), total antioxidant capacity, and total phenolic content with increasing concentrations of antibiotics [15,16]. Mukhtar et al. [13] showed that the growth of rice is negatively affected by the continuous application of antibiotics, such as levofloxacin, ciprofloxacin, ampicillin, amoxicillin, and ofloxacin at 10 mg/kg, for 4 months. They also found that germination rate, seedling root/shoot length, seedling biomass, and vitality index were all reduced, which indicated that the antibiotics were substantially toxic to the plants. Furthermore, antibiotics can affect the metabolism of nitrogen in plants and impose varying effects depending on the concentration of exposure, which has been demonstrated in mung bean and rice [10,17].

Quinolones supply 17 % of the total consumption of antibiotics on the global market. They are more stable and migratory than other antibiotics, which leads to higher rates of detection in the environment [2, 18]. The root system of a plant absorbs fluoroquinolone antibiotics, which are then distributed throughout its tissues and organs [1]. ENR is the most commonly used quinolone antibiotic. Although progress has been made in studying its toxic effects on plants, research on the underlying mechanism of toxicity is still limited, particularly in wheat [15, 16]. Multi-omics combined analyses enable global studies at multiple molecular levels, thus, reducing the limitations and false positives of a single method of omics analysis through mutual validation [19]. Da Ros et al. [20] utilized a systems biology approach to examine the physio-metabolic adjustments and transcriptome remodeling in wheat during its adaptation to drought, heat, and salinity stresses as well as combinations of these factors. Their study unequivocally demonstrated the advantages of utilizing a multi-omics-based data source for gene discovery under complex environmental conditions. This approach expedites causal gene validation for crop resistance. Zhang et al. [21] comprehensively elucidated the molecular regulatory mechanisms of the tolerance to and accumulation of cadmium in wheat. They used both proteomic and metabolomic data to analyze the morphophysiological differences between wheat varieties that were sensitive and tolerant to cadmium. The growth of ryegrass seedlings roots was found to be significantly inhibited under tetracycline stress; combined transcriptomic, proteomic, and metabolomic analyses provided a comprehensive dataset of numerous cellular processes that were used to suggest a less demanding multi-omics strategy to study this biological phenomenon [22]. In conclusion, the combination of morpho-physiological indicators with multi-omics technologies provides an in-depth and effective research strategy to disclose the internal molecular mechanisms underlying various biological phenomena [23].

This study examined the mechanism of toxicity of ENR in wheat by examining the morphological, physiological, and molecular responses of common wheat to ENR stress. Both growth inhibition and physiological and biochemical indices, related to oxidative stress under ENR stress were measured, and a combined analysis of the transcriptome, proteome, and metabolome was performed. Particular attention was directed to the relationship between the changes in primary and secondary metabolism and the inhibition of wheat root growth. This study provides new insight for a deeper understanding of the toxicity mechanism of ENR, as well as a more reliable basis for assessments of its ecological risk.

2. Materials and methods

2.1. Experimental description

Bainong4199—a common wheat cultivar—was used as the research material. The wheat seeds were treated with 0.2 % NaClO for 15 min and then rinsed with deionized water. A total of 15 wheat seeds were then evenly spaced and incubated in Petri dishes (9 cm in diameter) lined with three layers of filter paper. Next, varying concentrations (0, 5, 10, 20, 40, and 80 mg/L) of ENR were added to the Petri dishes. They were placed in a growth incubator for 4 d under controlled conditions of 16:8 h (light: dark) at 25 ℃: 18 °C. Ten plants were randomly selected from each Petri dish, and their shoot length (cm) and primary seminal root length (cm), as well as their shoot and root fresh weights (g), were measured. In addition, the shoot and roots of wheat seedlings grown under ENR treatments (0, 5, 10, and 20 mg/L) were sampled for five biochemical indices, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), MDA, and ROS. To examine the impact of ENR on the growth of wheat roots, 10 mg/L was determined to be the optimal concentration for treating wheat seeds. After 4 d of growth under this concentration, the growth of the wheat roots was significantly inhibited, while the growth of the shoots remained uninhibited (Fig. S2A, B). To ensure a strong correlation between the datasets, the same root sample was used for all the omics analyses. The roots of wheat seedlings treated with concentrations of 0 and 10 mg/L of ENR were pooled, rapidly frozen in liquid nitrogen, and stored at −80 °C.

2.2. Determination of antioxidant status, ROS and phytohormone content

The concentration of ROS and the activities of SOD, POD, MDA, and CAT were assessed in fresh shoot and root tissues. Jin et al. [24] utilized the thiobarbituric acid method to measure the concentration of MDA at an absorbance of 532 nm. The nitrotetrazolium blue chloride reduction method was used to measure the activity of SOD, while the rate of guaiacol oxidation at 470 nm was used to measure the activity of POD [24]. The activity of CAT was measured as described by Rahnama and Ebrahimzadeh [25], where the breakdown of H_2O_2 was tracked at 240 nm. 2′,7′-dichlorodihydrofluorescein diacetate was used to measure the production of ROS described by Han et al. [26]. The contents of GSH, GSSG, AsA, dehydroascorbate (DHA), glutathione S-transferase (GST), ABA, indole-3-acetic acid (IAA), JA, and methyl jasmonate (MeJA) in the roots were measured using Mlbio Assay Kits (Kit Codes: ml094991, ml094995, ml076466, ml095080, ml076443, ml077235, ml147100, ml077234, and ml077337) according to the manufacturer's instructions (Mlbio, Shanghai, China) (See supplementary figures and supplementary experimental methods).

2.3. Transcriptome profiling

An RNAprep Pure Plant Kit (TianGen, Beijing, China) was used to extract the total RNA. A Hieff NGS Ultima Dual-mode mRNA Library Prep Kit (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China)

was used to create the sequencing libraries, which were subsequently sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to obtain 150 bp paired-end reads. The common wheat reference genome (*Triticum_aestivum*.iwgsc_refseqv2.1.genome.fa) was used for mapping, which was performed using Hisat2 tools [27]. The levels of gene expression were quantified using fragments per kilobase of feature per million mapped reads. A functional annotation was performed using DIAMOND software, and a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted using KOBAS 2.0 software [28,29]. Differentially expressed genes (DEGs) were defined as genes with false discovery rate < 0.01 and $|\log_2FC| > 1$.

2.4. Proteome profiling

After the wheat roots had been sampled and snap-frozen in liquid nitrogen, they were stored at −80 °C until further analysis. The proteome was analyzed using tandem mass labeling technology from Biomarker Technologies Co., Ltd. (Beijing, China), as described by Jian et al. [30]. The tandem mass spectrometry data were processed using Proteome Discoverer 1.4 [31]. Differentially expressed proteins (DEPs) were selected for further analysis based on statistical significance ($P < 0.05$) and fold change (> 1.2 or < 0.83).

2.5. Metabolome profiling

The metabolome was analyzed using a liquid chromatography-mass spectrometry system consisting of an Acquity I-Class PLUS (Waters, Milford, MA, USA) connected to a Xevo G2-XS QTof high-resolution mass spectrometer (Waters) as described by Wei et al. [32]. Based on the quadrupole-time-of-flight platform, the metabolomes of 12 samples were analyzed both qualitatively and quantitatively. The significance of variables in the projection (VIP) values of the model was calculated using multiple cross-validations. Differential metabolites were identified using a combination of a multiplicity of differences, *P*-values, and VIP values from the orthogonal projections to the latent structures-discriminant analysis model. A total of 12,719 peaks were detected in all the modes, and 3825 metabolites were annotated. Differentially accumulated metabolites (DAMs) were filtered by VIP ≥ 1 and $|\log_2FC| > 1$.

2.6. Statistical analysis

The phenotypic and physiological data were analyzed using SPSS 25.0 (IBM, Inc., Armonk, NY, USA). The figures were generated using Origin 2021(OriginLab, Northampton, MA, USA). The DEGs, DEPs, and DAMs were annotated and classified based on the Gene Ontology (GO) and KEGG databases.

3. Results and discussion

3.1. Growth inhibition

ENR has been reported to have a weaker effect on seed germination in cabbage, tomato, and wheat, even though its experimental concentrations are much higher than those in the soil [15,33]. We also found the same phenomenon in this study and showed that different concentrations (5, 10, 20, 40, and 80 mg/L) of ENR did not affect the germination of wheat seeds (Table 1). Previous studies on mung bean, ryegrass, wheat, and rice have shown that overexposure to antibiotics inhibits the elongation of their roots [8,10,11,13]. Similarly, the negative impacts of ENR have been documented in detail. Examples include the effects of low ENR concentrations (≥5 mg/L) on root length and fresh weight, and the effects of higher concentrations on shoot length and fresh weight (Li et al., 2022; [16]). In this study, the morphological characteristics of wheat, including the lengths of shoots and roots, as well as the fresh weight, were assessed in plants under ENR stress (Fig. S1, Table 1). At ENR concentrations of 5–80 mg/L, significant decreases in primary seminal root length (by 25.7–43.0 %) and root fresh weight (3.7–78.6 %) were obtained compared to the control. Additionally, the shoot length and fresh weight were also clearly reduced by 1.1–16.6 % and 0.7–15.6 %, respectively. An increase in the concentration of ENR gradually inhibited the growth of wheat seedlings, and the impact on the roots was greater than that on the shoots. The primary seminal root length and root fresh weight began to be significantly suppressed at 5 and 10 mg/L, respectively. In contrast, the shoot length and fresh weight were less affected and began to be significantly inhibited at concentrations of 40 mg/L and above (Table 1 and Fig. S2A and B). Compared with previous studies, the results were slightly different because of differences in the wheat materials, but the sequence of the apparent inhibition of tissue growth caused by ENR was consistent [15,16]. In addition to the apparent changes in morphological characteristics, the results of a principal component analysis (PCA) also showed that ENR stress profoundly affected the transcriptome, proteome, and metabolome of the roots of wheat seedlings (Fig. S3).

3.2. Oxidant stress and antioxidant status

In plants, ROS can be produced by apoplasts, chloroplasts, mitochondria, and POD, although chloroplasts are the primary organelles that produce ROS [34,35]. Low doses of ROS are involved in signaling, seed germination, plant growth, and responses to abiotic stress, but excessive ROS doses can be highly reactive and cytotoxic and cause oxidative stress in plants [36,34,37]. High-concentration antibiotic stress disrupts the balance between the production and clearance of ROS in plants; this leads to the accumulation of excessive ROS levels that attack the membrane system, thus damaging the structure and function of both chloroplasts and mitochondria [38]. Furthermore, ROS control the balance between cell proliferation and elongation in root growth through auxin-independent signaling [37]. This study clearly demonstrates that the levels of ROS in both the roots and shoots increase significantly in response to all the levels of ENR treatment, and increase further with higher treatment concentrations (Fig. 1A). Almost simultaneously, activation of antioxidant defense systems, including SOD, POD, and CAT, was also observed. SOD—the first defense line against oxidative stress—plays an important role in the maintenance of redox homeostasis in plant cells [39]. The SOD activity increased by 38.8–65.2 % and 36.0–64.6 % in the shoots and roots, respectively, and continued to increase with increasing concentrations of ENR (Fig. 1B). Similarly, the POD and CAT activities increased by 63.2–179.0 % and 57.0–157.2 % in the shoots and roots, respectively (Fig. 1C and D).

Table 1
Effects of enrofloxacin (ENR) on germination, and root and shoot growth in wheat.

Treatment (mg/L)	Primary seminal root length (cm)	Root fresh weight (mg)	Shoot length (cm)	Shoot fresh weight (mg)	Germination percentage (%)
Control	5.1 ± 0.2^{a}	23.9 ± 1.1^{a}	3.7 ± 0.1^{a}	35.0 ± 1.2^{a}	94.7 ± 0.8^{a}
5	3.8 ± 0.2^{b}	23.1 ± 1.4^{a}	3.6 ± 0.1^{a}	34.8 ± 1.4^{a}	96.7 ± 1.9^{a}
10	3.6 ± 0.2^{bc}	17.7 ± 0.9^{b}	3.6 ± 0.1^{a}	33.2 ± 1.1^{a}	96.0 ± 1.0^{a}
20	3.3 ± 0.1^{bc}	5.5 ± 0.5^{c}	3.5 ± 0.1^{ab}	32.3 ± 0.7^{ab}	95.3 ± 1.6^{a}
40	3.2 ± 0.2^{bc}	5.1 ± 0.5^{c}	3.3 ± 0.2^{bc}	30.2 ± 0.8^{b}	95.3 ± 1.2^{a}
80	2.9 ± 0.2^{c}	5.1 ± 0.2^{c}	3.1 ± 0.2^{c}	29.6 ± 2.3^{b}	94.7 ± 1.7^{a}

Means with different superscripts within each column imply significant differences ($p < 0.05$).

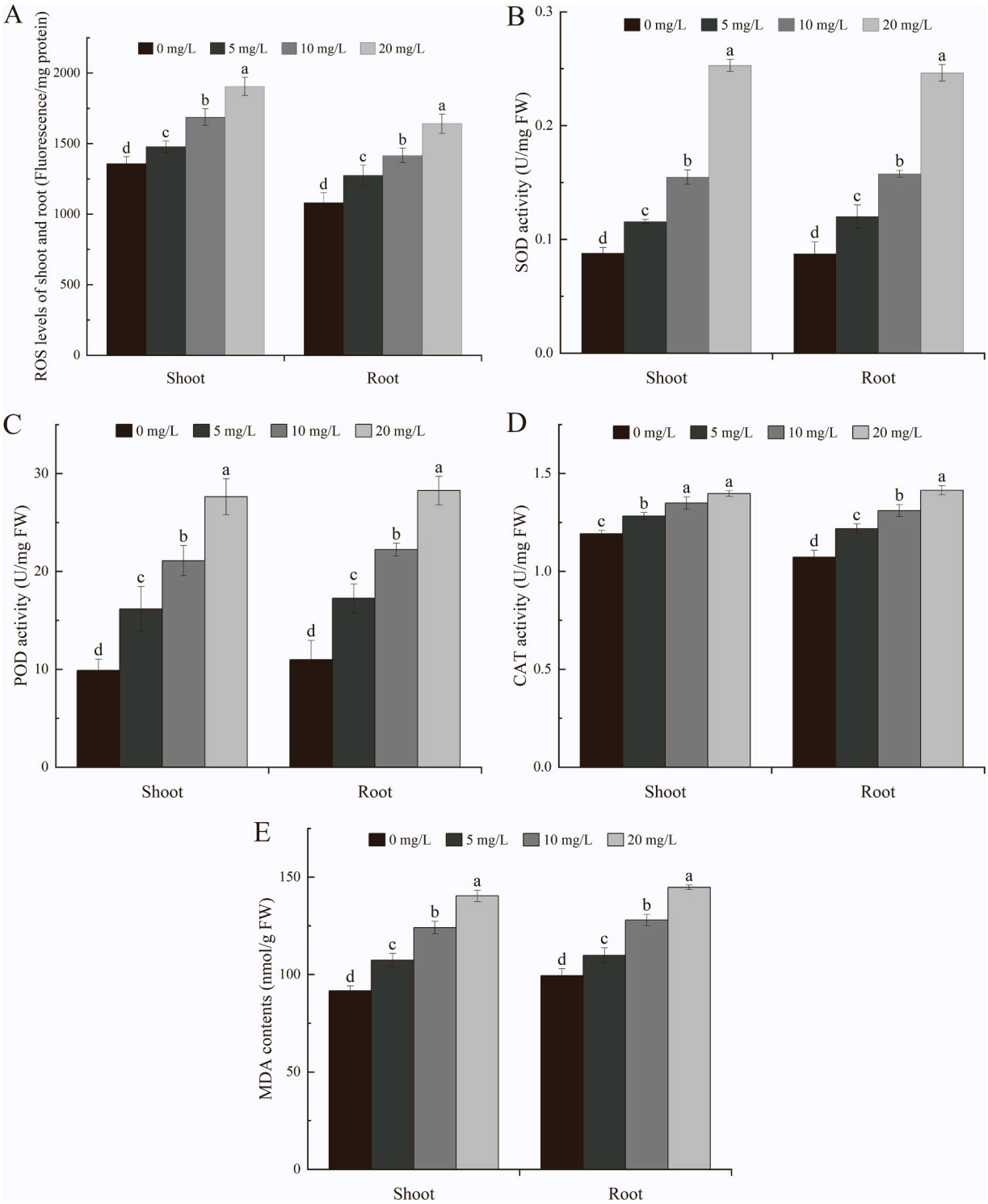

Fig. 1. Oxidant stress and antioxidant activities in the shoots and roots of wheat seedlings. (A) ROS level; (B) SOD activity; (C) POD activity; (D) CAT activity; and (E) MDA content. FW, fresh weight. Different superscript letters indicate statistical differences ($P < 0.05$).

These results are consistent with the results of Li et al. [15]. These results clearly demonstrate that the level of responses of antioxidant enzymes increased significantly under ENR stress, thereby improving the capacity of plants to scavenge ROS.

MDA is a widely accepted indicator of oxidative stress in cells since it reflects the degree of stress manifested to the cells [40]. In this study, the MDA content increased significantly in both the shoots (by 17.2–53.2 %) and roots (by 10.4–45.6 %) compared with the control (Fig. 1E). There was severe peroxidation of lipids under ENR stress, which indicated that the induced production of excess ROS exceeded the scavenging capacity of the antioxidant defense system. Therefore, the significant increase in MDA content provides evidence for the phytotoxicity of high doses of ENR toward wheat seedlings [15,16]. In summary, considering the versatility of ROS and the identified cytotoxicity of excessive levels of

ROS [35], it can be hypothesized that the levels of ROS are one of the reasons for the aberrant growth and development of the roots and shoots of wheat under ENR stress.

3.3. Transcriptomic analysis of the seedling roots in response to ENR stress

The transcriptome was sequenced to evaluate the effect of ENR treatment on the wheat roots. ENR significantly affected the transcriptome of the wheat roots, as demonstrated by the consistency of the heatmap results with those of the PCA (Fig. 2A and S3A). A total of 58,115 expressed genes were annotated, including 2759 novel genes assembled from reads that were not aligned to the reference genome (Table S1). A total of 3859 DEGs were identified and comprised 6.64 % of all genes; of these, 1757 genes were upregulated and 2102 were downregulated (Table S2, Figs. S4A and S5). A KEGG pathway analysis of the transcriptomics disclosed the significant enrichment of several DEGs in higher-ranked specific KEGG terms. These include glutathione metabolism, phenylpropanoid biosynthesis, steroid biosynthesis, starch and sucrose metabolism, flavonoid biosynthesis, isoflavonoid biosynthesis, phenylalanine metabolism, arginine and proline metabolism, flavone and flavonol biosynthesis, diterpenoid biosynthesis, zeatin biosynthesis, glycolysis/gluconeogenesis, and fatty acid metabolism in the wheat roots under ENR stress (Fig. 2B, Table S3). In conclusion, ENR imposes significant effects at the transcriptional level on many secondary metabolic pathways in wheat seedlings.

3.4. Proteomic analysis of the response of seedling roots to ENR stress

The results of the cluster heatmap were consistent with those of the PCA, which indicated that ENR significantly affected the wheat root proteome (Fig. 3A and S3B). A total of 3879 proteins were identified, and 3649 were functionally annotated (Table S4). Under ENR stress, 463 DEPs were screened; 244 were upregulated, and 219 were downregulated (Fig. S4B, Table S5).

A GO analysis was performed to further characterize the DEPs induced by ENR. A total of 246 biological processes terms, 102 molecular functions terms, and 272 cellular components terms were enriched. The top 20 terms of the GO enrichment analysis are presented in Fig. 3B and Table S6. Eight terms were enriched in the biological processes category and most of the proteins involved were upregulated. Among these, the glutathione metabolism process (GO:0006749), response to chemicals (GO:0042221), and glycolytic process (GO:0006096) had low *P*-values. Five cellular component terms (i.e., mitochondrion (GO:0005739), mitochondrial large ribosomal subunit (GO:0005762), mitochondrial respiratory chain complex I (GO:0005747), mitochondrial inner membrane (GO:0005743), and mitochondrial small ribosomal subunit (GO:0005763)) are located in mitochondria, which suggested that ENR significantly affects the energy metabolism of wheat. Seven terms were remarkably enriched in the molecular function category, including glutathione transferase activity (GO:0004364), monooxygenase activity (GO:0004497), and oxidoreductase activity, acting on paired donors (GO:0016705), which indicated that a protective mechanism had been initiated to alleviate the toxicity of ENR.

A KEGG enrichment analysis showed that upregulated DEPs were primarily enriched in carbon metabolism, glycolysis/gluconeogenesis, glutathione metabolism, proteasome, carbon fixation pathway in photosynthetic organisms, amino acid biosynthesis, and citrate cycle (Fig. 3C, Table S7). This pattern of enrichment suggests that the wheat seedlings regulated metabolic reprogramming to adapt to the stress induced by ENR. Most of the downregulated DEPs were significantly enriched in the oxidative phosphorylation (OXPHOS) pathway with the lowest *P*-value. This result is consistent with the cellular components category of GO enrichment analysis. This consistency indicates that the level of mitochondrial energy metabolism decreased under ENR stress, which is one of the reasons for the abnormal growth of the wheat roots.

3.5. ENR significantly alters the metabolome of the seedling roots

To obtain a better understanding of the effect of ENR on the metabolic activity of wheat seedlings, the wheat root metabolome was examined in both the presence and absence of ENR treatment using all the modes of the LC-MS system. The results of PCA clearly classified the treatment and control groups, which is consistent with the clustering results for DAMs as shown in the heatmap (Fig. 4A and S3C). Compared to the PCA, an OPLS-DA is the superior method to assess key information and accurately group a sample cohort. As shown in Fig. S6, both the R2Y and Q2Y values > 0.9, thus, providing strong evidence that the observed metabolomic differences are caused by the treatment with ENR. Thus, the VIP can be used as a screening condition for the DAMs. A total of 3825 metabolites were collected (Table S8), and 225 were downregulated (Fig. S4C, Table S9), while 435 were upregulated. A KEGG analysis identified 86 enriched pathways. Of these, the top 5 pathways that were the most reliable were isoquinoline alkaloid biosynthesis, biotin metabolism, flavone and flavonol biosynthesis, flavonoid biosynthesis, and ABC transporters (Fig. 4B, Table S10), which suggested that these pathways are crucial for the reaction to ENR stress.

Currently, hormones, ROS, and nutrition are the main themes studied in connection with the control of the development of plant roots. As shown by Arnao and Hernández-Ruiz [41], melatonin (MT) regulates hormone levels and responses to stress in plants. MT itself also possesses a strong antioxidant capacity [42], which is markedly elevated in response to ENR stress. In plants, auxin influences the development of tissues and organs as well as root extension and early cell division [43]. In this study, there was a substantial upregulation of IAA (Fig. S7). ABA is a stress hormone that responds to various stresses, and low levels have been shown to promote root development, while it is inhibited by high levels of this hormone [44]. Li et al. [15] found an increase in the levels of ABA under ENR stress, which was also observed in this study (Table S8, Fig. S7). This consistency suggests that enhancing the level of ABA is one of the strategies used by wheat roots to adapt to ENR stress. JA regulates both reproductive development and leaf senescence and inhibits primary root growth [45]. Significantly less JA accumulated under ENR stress, while the levels of MeJA and **Jasmonoyl-l-isoleucine** decreased only slightly (Table S8, Fig. S7). Additionally, JA is involved in the regulation of the production of diverse metabolites such as terpenoids and phytoalexins. In conclusion, the observed decrease of JA and its derivatives, along with the increase of low-level ABA, may mitigate the inhibition of root growth under ENR stress [15,46].

3.6. Multi-omics integration analysis of primary metabolism

Primary metabolism provides energy and intermediate products that are essential for the growth, development, and reproduction of plants [47]. In response to ENR stress, wheat seedlings can adjust their metabolism to adapt to this change in their environment and maintain basic metabolic functions that ensure survival and growth. A multi-omics analysis showed that compared to the control group, certain pathways, including carbon metabolism, OXPHOS, biosynthesis of amino acids, and proteasome, were highly enriched in the KEEG analysis. These pathways and their associated DEGs, DEPs, and DAMs may be crucial factors in the response of wheat to ENR stress and help it to both survive and grow.

3.6.1. Oxidative phosphorylation and carbon metabolism

The OXPHOS pathway is primarily active in the respiratory chain system located on the inner mitochondrial membrane. It is the principal pathway to produce the energy compound adenosine triphosphate (ATP) in cells, which plays a crucial role in the maintenance of metabolic homeostasis [48]. Exposure to stress can disrupt the balance between ROS production and ROS clearing systems in plants, which leads to the excessive production of ROS and oxidative damage of the mitochondrial membrane, which ultimately affects normal structure and function [49].

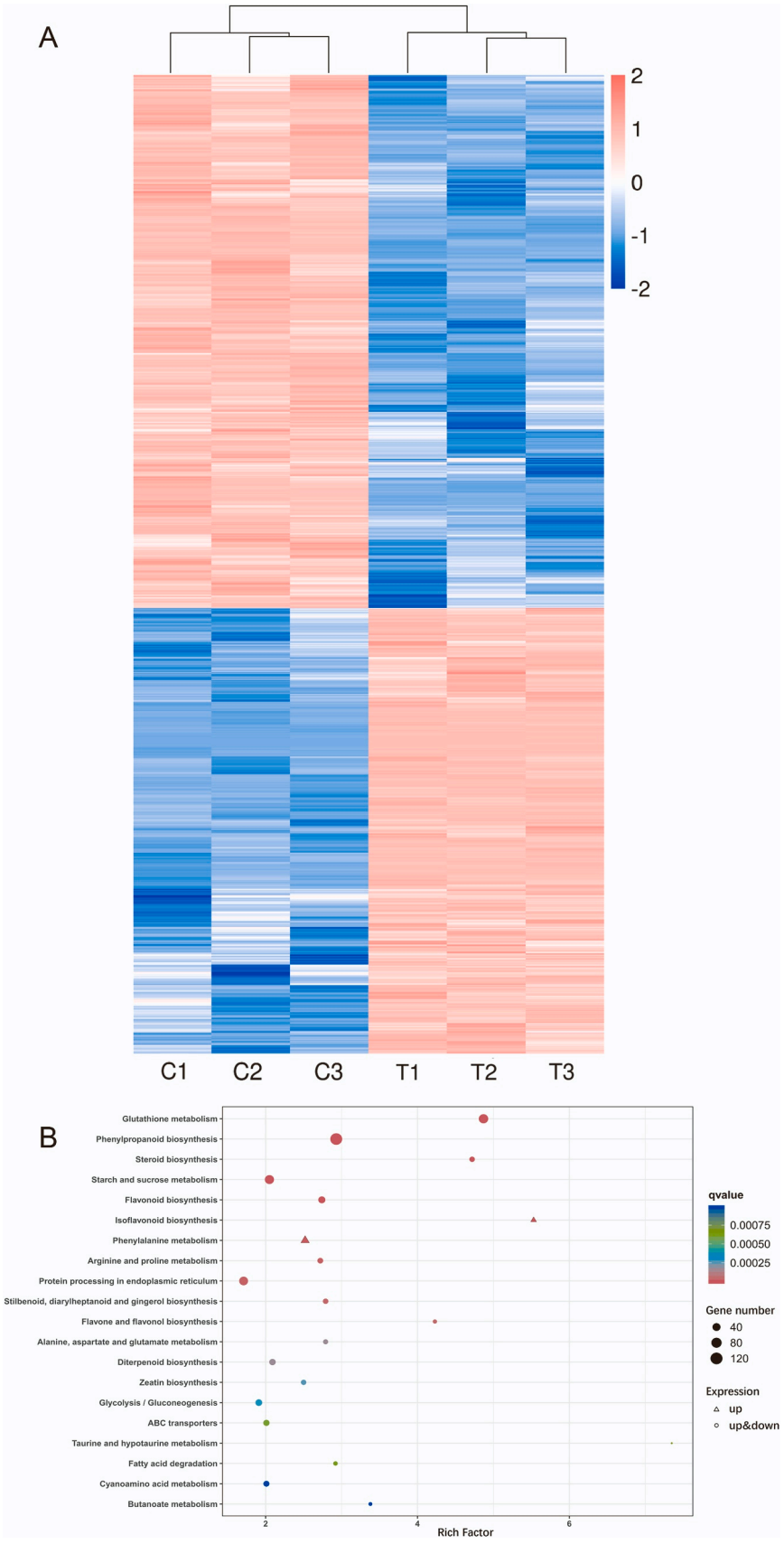

Fig. 2. Transcriptomic analysis. (A) The cluster heatmap of DEGs; (B) Diagram of the top 20 KEGG enrichment analysis.

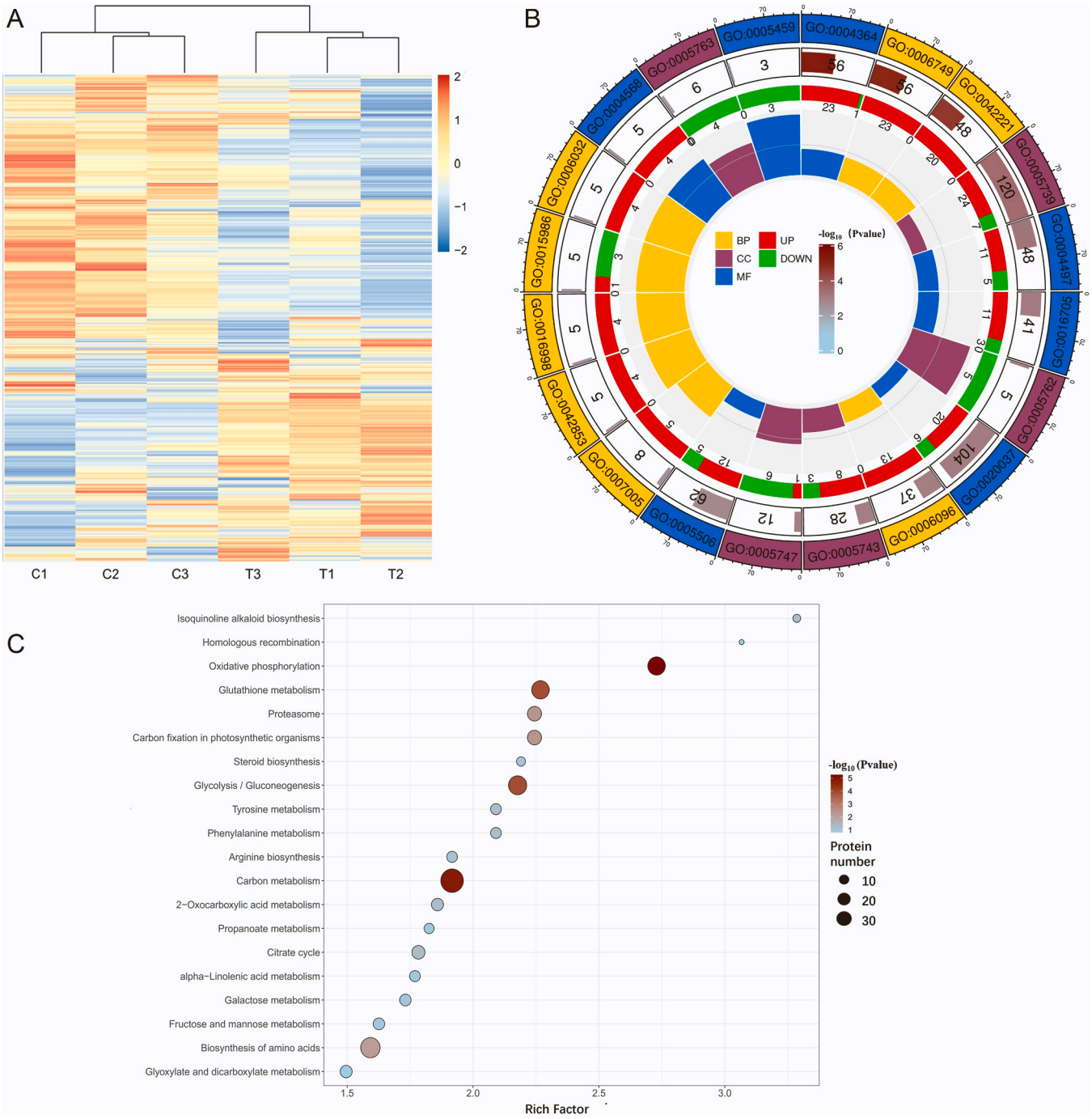

Fig. 3. Proteomic analysis. (A) The cluster heatmap of DEPs. (B) Diagram of the top 20 GO enrichment circles. The first circle represents GO terms; the coordinate scale for the quantity of proteins is located outside the first circle. The value of the second circle represents the number of proteins enriched to a specific GO term. The length of the bar increases proportionally with the number of proteins, while the color intensifies with the significance. The value of the third circle represents the number of DEPs enriched to specific GO terms, where red and green indicate upregulated and downregulated proteins, respectively. The fourth circle represents the proportion of DEPs enriched in this GO term to all proteins. (C) Diagram of top the 20 KEGG enrichment analysis.

An alternative oxidase is involved in the non-phosphorylated respiratory pathway, which plays a role in maintaining the redox balance of the mitochondrial electron transport chain and limiting the production of ROS [50]. As a marker protein for mitochondrial stress by ROS, a significant increase in the abundance of alternative oxidase protein was induced under ENR stress (Table S5). Correspondingly, most of the DEPs involved in OXPHOS were downregulated under ENR stress, which appeared to diminish the function of this pathway (Fig. 5, Table S4). Even worse, dysfunction of the respiratory chain components also leads to disturbed electron transport and elevated levels of ROS [51].

The results of this study clearly demonstrate that the enzyme succinate dehydrogenase (ubiquinone) flavoprotein subunit (EC 1.3.5.1) is upregulated despite a reduction in the product fumarate. This upregulation is assumed to be the result of MDA overproduction under high concentrations of ROS. MDA acts as a competitive inhibitor that directly competes with the substrate succinate for binding to the succinate dehydrogenase (ubiquinone) flavoprotein subunit, which results in some degree of inhibition of the response. The evidence provided herein

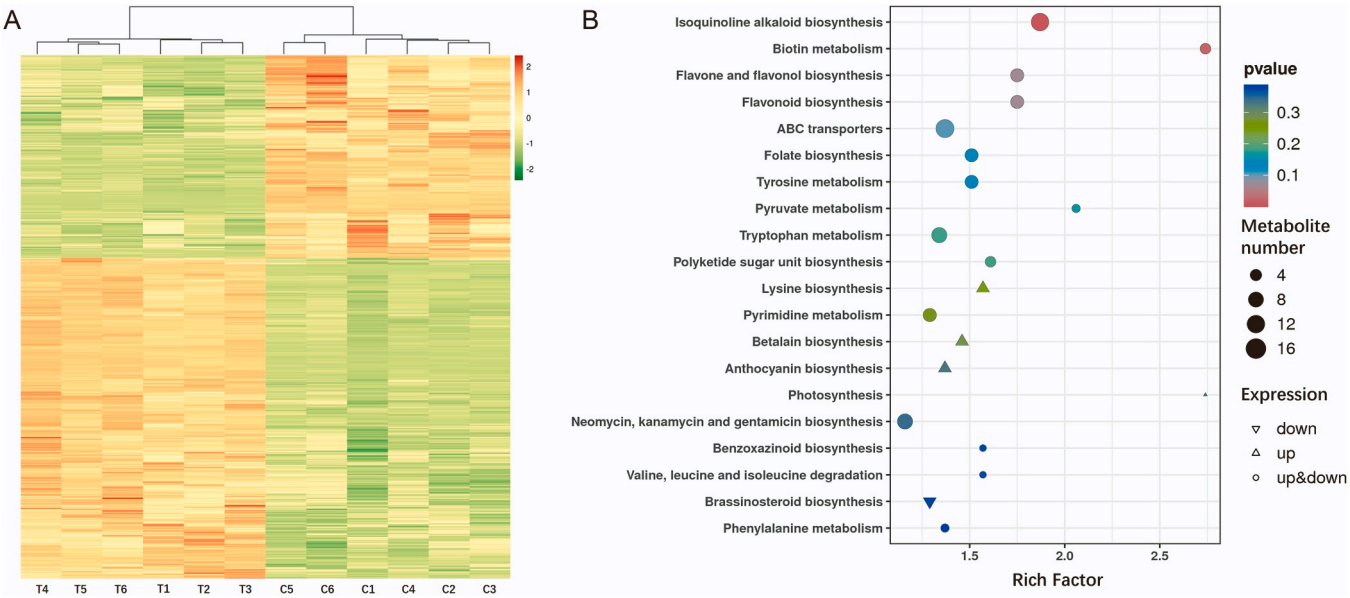

Fig. 4. Metabolomic analysis. (A) The cluster heatmap of DAMs; (B) Diagram of the top 20 KEGG enrichment analysis.

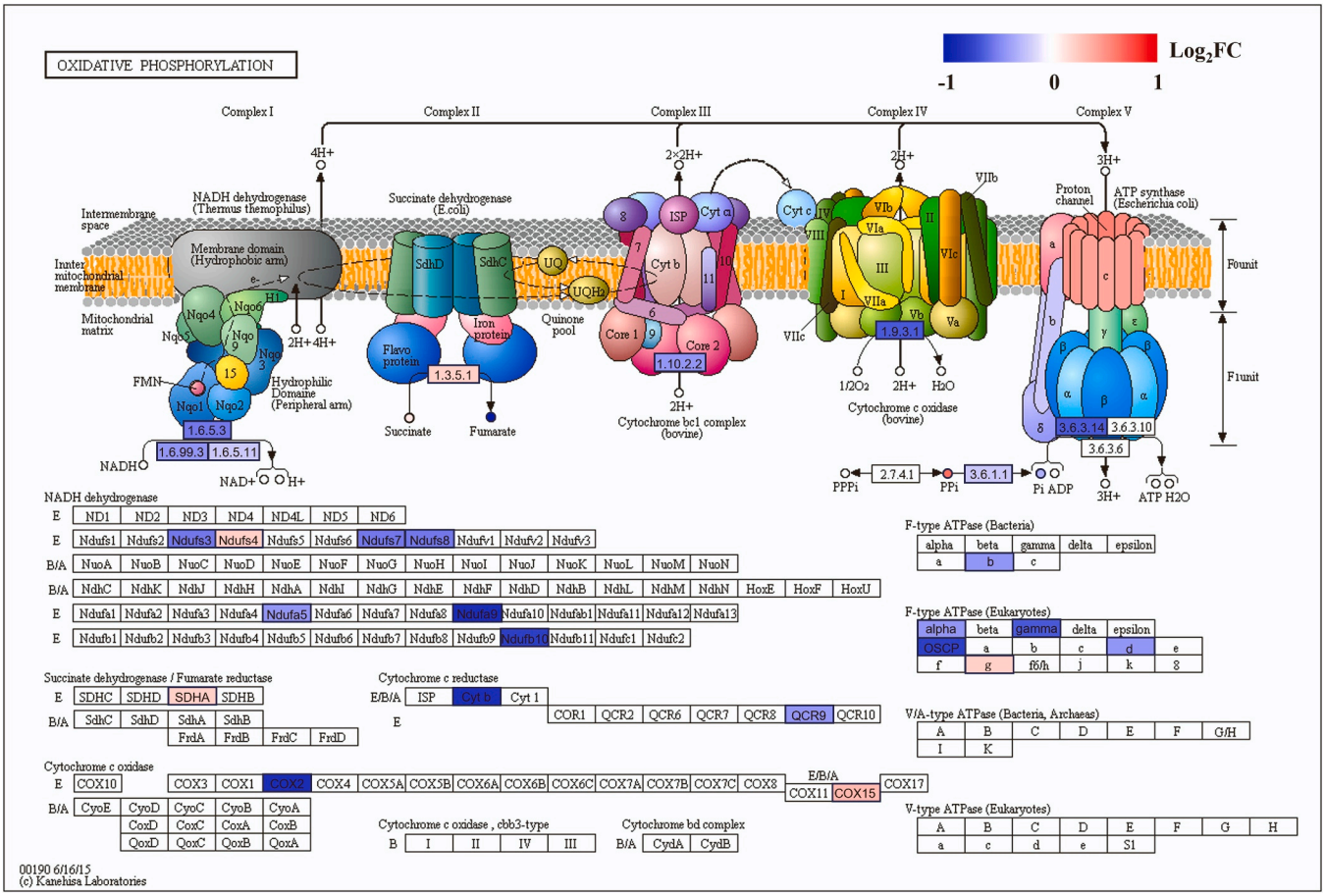

Fig. 5. Diagram of the oxidative phosphorylation pathway. The color block represents the value of $\log_2$FC(T/C). Red and blue gradients indicate proteins or metabolites that are upregulated or downregulated, respectively.

indicates that the normal function of the mitochondrial OXPHOS pathway is severely disrupted, which results in a decrease in the supply of ATP. As a result, it can be reasonably concluded that the stunting of root growth and development is caused by the toxicity of excess ROS and a reduction in the supply of ATP under ENR stress.

Carbon metabolism is a fundamental aspect of the growth and development of plants. It encompasses the metabolism of starch and sucrose, glycolysis, the tricarboxylic acid (TCA) cycle, and other

processes. These types of metabolism and processes provide the essential carbon skeleton and energy required for the synthesis of amino acids, proteins, and nucleic acids in nitrogen metabolism. ENR stress downregulates the OXPHOS metabolic pathway, thus leading to a decrease in energy supply. However, the abundance of protein in C metabolism, excluding pyruvate phosphate dikinase, is upregulated, which enhances the function of this metabolic pathway (Fig. 6 and Table S4). This enhancement implies an increase in the supply of energy substances through the substrate-level phosphorylation pathway. As a result, mitochondrial repair is favored and the supply of material to maintain mitochondrial function can be secured. The contents of the intermediate products D-fructose 6 P, cis-aconitate, succinate, and malate increased, which suggests a positive correlation between these intermediate products and proteins (hexokinase, aconitate hydratase, and succinyl-CoA synthetase alpha subunit; E4.2.1.2 A). However, the content of the intermediate products glyceraldehyde-3 P and isocitrate decreased, which implies a negative correlation with the corresponding proteins (fructose-bisphosphate aldolase and isocitrate dehydrogenase). It is worth noting that the interference by MDA greatly reduced the fumarate content, but the level of its downstream product, malate, was not affected by the reduction in fumarate accumulation. This result suggests that the regulatory strategy of the TCA cycle enhancement mechanism is in effect. These findings imply that during ENR stress, at least part of the intracellular synthesis of ATP tends to be redirected from OXPHOS to substrate-level phosphorylation. The cells themselves can adapt to changes in the metabolic environment through mutual coordination between glycolysis and OXPHOS to jointly maintain the balance required by the metabolism of intracellular energy [52].

3.6.2. Amino acid biosynthesis

Amino acids are vital macromolecules that play a crucial role in the construction of biological organisms. They are intricately linked to energy metabolism, protein synthesis, carbohydrate metabolism, hormone, and secondary metabolism, the carbon–nitrogen balance, and stress responses [53]. Under ENR stress, the five amino acids tryptophan, methionine, isoleucine, cysteine, and glutamine were significantly upregulated (Fig. 6 and Table S8). Tryptophan is a precursor of the biosynthesis of the secondary metabolites IAA and MT [54,55]. This study found an apparent accumulation of MT with strong antioxidant capacity under ENR stress, which was consistent with the observed increase in the content of tryptophan. MT is involved in the biosynthesis of polyamines, which play a role in the enhancement of the ability of cells to scavenge free radicals [42]. **Jasmonoyl-l-isoleucine** is one of the most prevalent active substances of the JA family [56]. Both cysteine and glutamine are involved in the biosynthesis of the antioxidant GSH [57]. The three amino acids threonine, alanine, and arginine were downregulated under ENR stress (Fig. 6 and Table S8). Arginine is an important storage and transport form of organic nitrogen in plants with a low C/N ratio (i.e., 1.5) [58]. Under ENR stress, the intracellular content of arginine was significantly reduced, and as a result, a relatively high intracellular C/N ratio was maintained, which improved the efficiency of nutrient utilization and promoted root growth. This may be one of the coordinated strategies used by wheat to adapt to the physiological and metabolic changes induced by ENR stress.

3.6.3. Proteasome

Under injury under stress, the accumulation of various abnormal or damaged proteins in cells is accelerated, thus affecting various types of intracellular metabolism and even disrupting the structural and functional integrity of the cells. In many cases, the damaged proteins can be repaired or refolded to some extent with the help of chaperones [59]. However, once the accumulation of abnormal proteins exceeds a certain threshold, the enhancement of protein degradation becomes an important strategy to address this crisis. Similar to protein translation and its post-translational processes, the degradation of protein plays a crucial role in regulating plant growth and development as well as maintaining cellular homeostasis [60]. Overall, the proteasome pathway proteins (except for DSS1) were significantly upregulated under ENR stress (Table S11). However, activation of the unfolded protein response and differential expression of the ubiquitination-associated proteins were not triggered upstream (Tables S11 and S12). In addition, several key proteins involved in protein processing in the endoplasmic reticulum pathway, such as the chaperone Bip, DnaJ homolog subfamily B member 11, and calreticulin, were significantly downregulated. These results strongly suggest that part of the excessive accumulation of abnormal or damaged proteins under ENR stress may be degraded by the proteasome in a non-ubiquitin-dependent manner [61]. These abnormal proteins are degraded to peptides by the proteasome or further degraded to individual amino acid residues for the synthesis of new proteins; thus, to some extent, the proteasome participates in the maintenance of intracellular protein homeostasis [62].

3.7. Multi-omics integration analysis of the secondary metabolism

Secondary metabolites are small molecular compounds that are not essential for the metabolism and growth of plants; however, the wide range and high diversity of secondary products provide key components of plant interactions with the environment, particularly in the adaptation to biotic and abiotic stress [63]. A multi-omics analysis showed that at least two of the DEGs, DAPs, and DAMs were simultaneously and significantly enriched in several pathways under ENR stress, including phenylpropanoid biosynthesis, flavonoids biosynthesis, and glutathione metabolism (Tables S3, S7, and S10). These pathways and the associated DEGs, DAPs, and DAMs may be the key factors for wheat to improve its adaptation to ENR stress, as described below (Figs. 8 and 9).

3.7.1. Biosynthesis of phenylpropanoids and flavonoids

As one of the major secondary pathways in plants, the phenylpropanoid pathway plays a critical antioxidative role in the resistance of plants to abiotic stresses [64]. Under ENR stress, the enzymatic proteins phenylalanine ammonia-lyase, shikimate O-hydroxycinnamoyltransferase, and serine carboxypeptidase-like 19 involved in the phenylpropanoid biosynthesis pathway were downregulated; the downstream metabolite of serine carboxypeptidase-like 19, sinapine, was expressed at a consistent level (Fig. 7, Tables S4 and S8). The enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase were upregulated, as was the downstream metabolite of cinnamyl-alcohol dehydrogenase, caffeyl alcohol. However, the downstream metabolite of cinnamoyl-CoA reductase, cinnamaldehyde, was downregulated. The metabolite caffeyl alcohol is the major compound used to synthesize catechyl lignin [65]. 5-O-Caffeoylshikimic acid is a potent antioxidant [66]. Based on these results, it can be hypothesized that in response to ENR stress, the downregulation of the two metabolites sinapine and cinnamaldehyde may be caused by a redirection of the metabolic flow in the phenylpropanoid pathway to enhance critical branching pathways, such as lignin biosynthesis and antioxidants.

Flavonoids are low molecular weight secondary metabolites synthesized by plants that are widely utilized in physiological activities including growth, development, and defense [67]. The general phenylpropanoid pathway includes the first three steps in the flavonoids biosynthesis pathways, which provide precursors for the metabolic pathways of different branches downstream. Examples include the biosynthesis of flavones and flavonols, anthocyanins, isoflavonoids, and flavonoids among others.

Under ENR stress, the metabolites chrysin, PHC 4′-O-glucoside, sakuranetin, and hesperetin in the flavonoid biosynthesis pathway were upregulated, while luteoforol, bracteatin 6-O-glucoside, and 4-O-Methylxanthohumol were downregulated (Fig. 7, Table S8). Chrysin, sakuranetin, and hesperetin have multiple biological and pharmacological effects, including antioxidant, anti-inflammatory, anticarcinogenic, and antiviral activities [68-70]. Anthocyanins are a branch of flavonoids that are involved in ROS scavenging and an improvement of the tolerance to

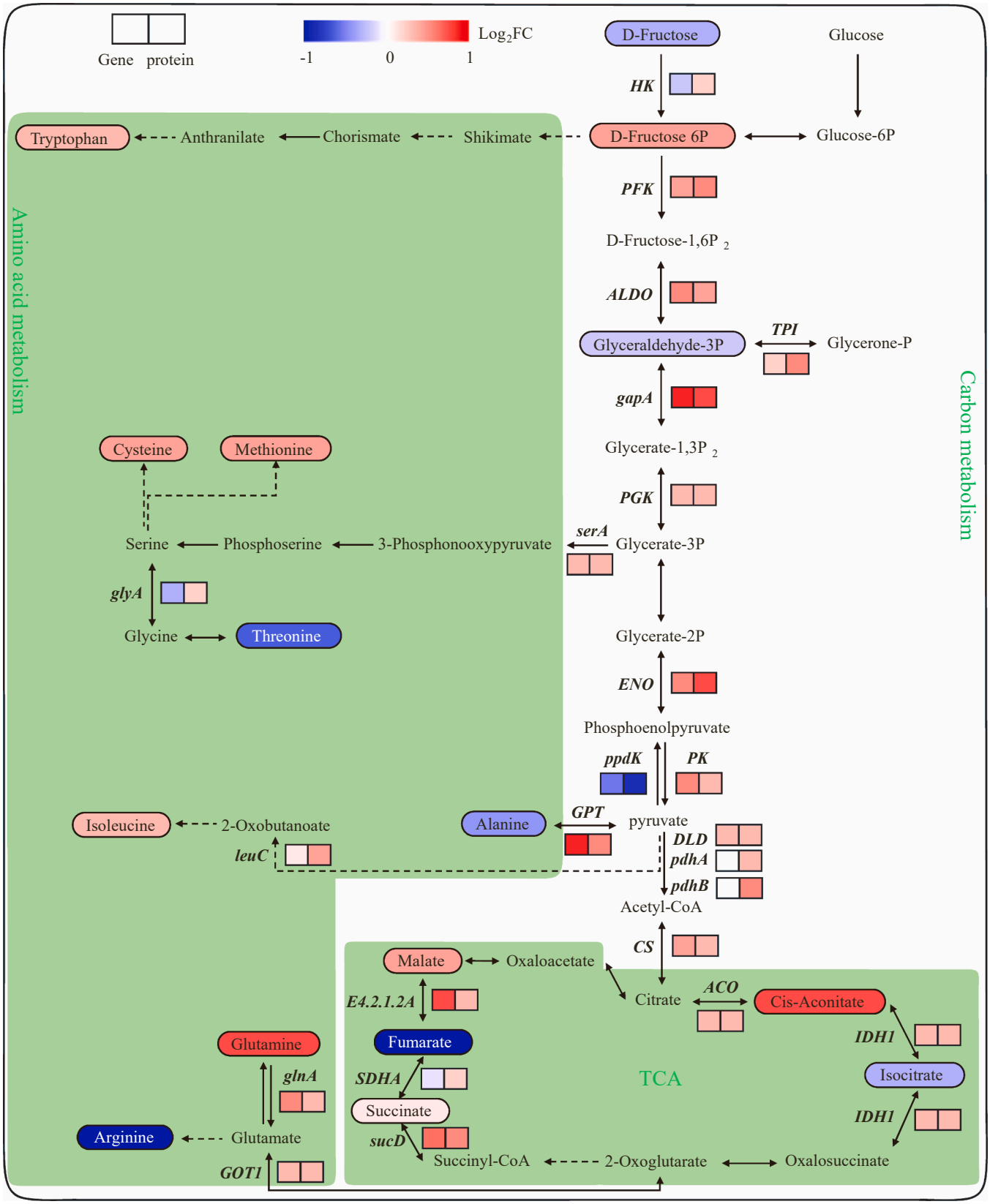

Fig. 6. Diagram of carbon metabolism and biosynthesis of amino acids pathway. The color block represents the value of log_2FC(T/C). Red and blue gradients indicate genes, proteins, or metabolites that are upregulated or downregulated, respectively. HK, hexokinase; PFK, 6-phosphofructokinase 1; ALDO, fructose-bisphosphate aldolase; TPI, triosephosphate isomerase; gapA, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; serA, D-3-phosphoglycerate dehydrogenase; glyA, glycine hydroxymethyltransferase; ENO, enolase; ppdK, pyruvate, orthophosphate dikinase; PK, pyruvate kinase; GPT, alanine transaminase; leuC, 3-isopropylmalate; DLD, dihydrolipoyl dehydrogenase; pdhA, pyruvate dehydrogenase E1 component subunit alpha; pdhB, pyruvate dehydrogenase E1 component subunit beta; CS, citrate synthase; ACO, aconitate hydratase; IDH1, isocitrate dehydrogenase; sucD, succinyl-CoA synthetase alpha subunit; SDHA, succinate dehydrogenase (ubiquinone) flavoprotein subunit; E4.2.1.2 A, fumarate hydratase; GOT1, aspartate aminotransferase; glnA, glutamine synthetase.

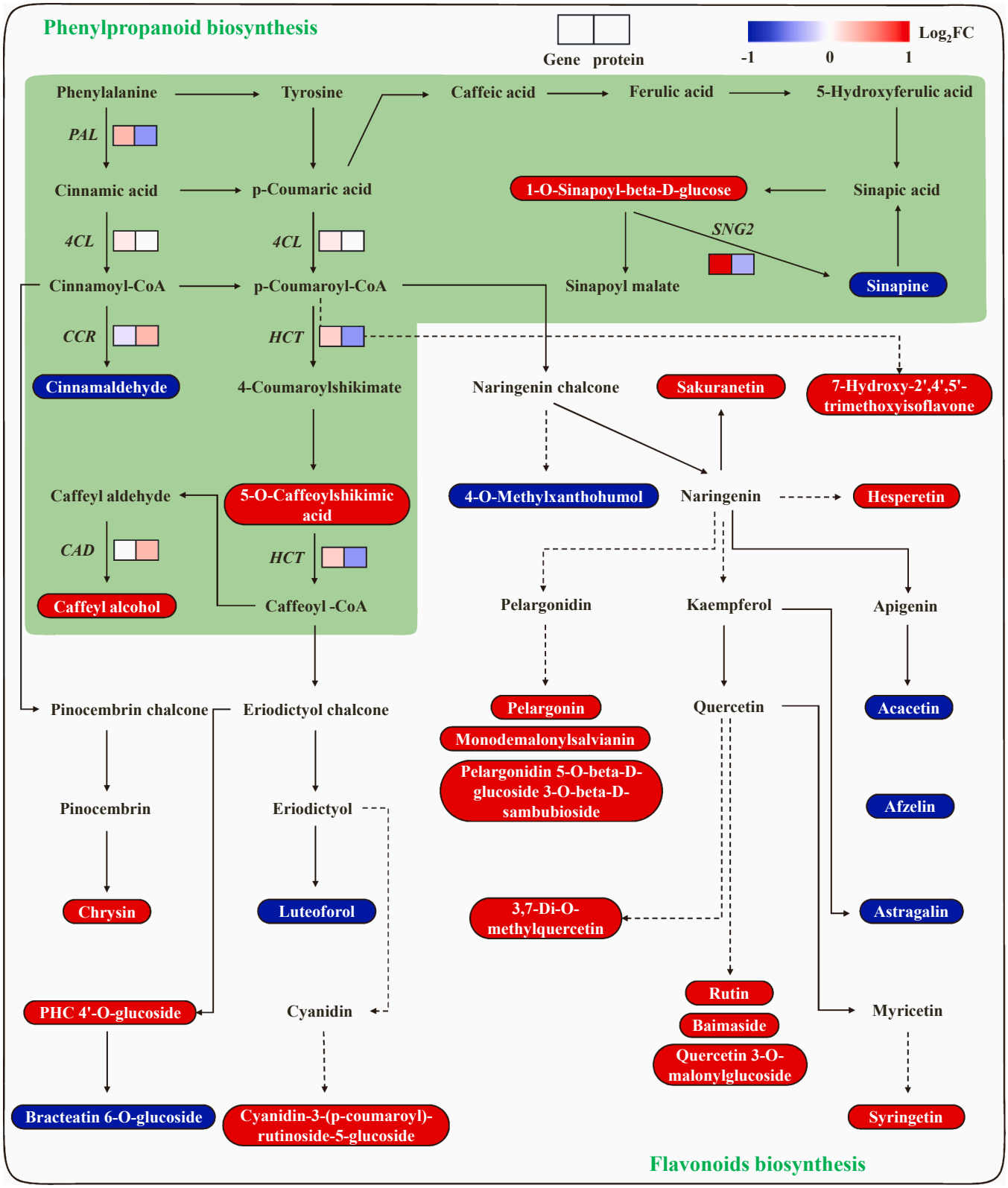

Fig. 7. Diagram of phenylpropanoid biosynthesis and flavonoids biosynthesis pathway. The color block represents the value of $\log_2$FC(T/C). Red and blue gradients, respectively, indicate genes, proteins, or metabolites that are upregulated or downregulated. PAL, phenylalanine ammonia-lyase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; HCT, shikimate O-hydroxycinnamoyltransferase; CAD, cinnamyl-alcohol dehydrogenase; SNG2, serine carboxypeptidase-like 19.

low levels of nitrogen [71]. The metabolites cyanidin-3-(p-coumaroyl)-rutinoside-5-glucoside, pelargonin, monodemalonylsalvianin, and pelargonidin 5-O-beta-D-glucoside 3-O-beta-D-sambubioside were significantly upregulated in the anthocyanin biosynthetic pathway. In the flavone and flavonol biosynthetic pathway, the metabolites acacetin, afzelin, and astragalin were significantly downregulated, while the metabolites syringetin, rutin, baimaside, quercetin 3-O-malonylglucoside, and 3,

7-Di-O-methylquercetin, which comprised most of the metabolites, were significantly upregulated. In addition, the metabolite 7-hydroxy-2′,4′, 5′-trimethoxyisoflavone in the isoflavonoid biosynthesis pathway was also upregulated.

Abiotic stress can alter the levels of endogenous hormones in plants. Plant hormones not only affect the expressions of nucleic acids, proteins, and enzymes but also control the synthesis of secondary metabolites such as flavonoids, terpenoids, and alkaloids, by directly regulating the expression of structural genes or transcription factors [72]. As shown in this study, ABA, JA, IAA, and MT were involved in the regulation of the flavonoid metabolism under ENR stress. In general, most of the metabolites that were significantly regulated had some antioxidant effect. During the process of resisting ENR toxicity, there are much higher numbers of upregulated antioxidant metabolites than the number of downregulated antioxidant metabolites (Fig. 7, Table S8). This represents an evolutionary adaptive response of wheat roots in response to adversity.

3.7.2. Glutathione metabolism

The antioxidant system in plants is a crucial defense mechanism that can effectively protect and reduce the damage caused by environmental stresses. In addition to SOD, POD, and CAT antioxidant proteases, the AsA-GSH cycle plays a vital role in detoxifying ROS. Glutathione is the primary small molecule non-sulfhydryl pool in all living organisms, including plants, where it is primarily present in its reduced form. Under normal growth conditions, the proportion of GSH exceeds 90 %, which is essential for its biological functions [73]. Glutathione reduces the levels of ROS through enzymatic reactions, such as the AsA-GSH cycle, glutathione peroxidase, and GST [74]. The results of this study clearly show that GSH was upregulated, while the changes in GSSG were more pronounced, thus, resulting in a change of the GSH/GSSG ratio from 5.7 in the control to 0.56 following ENR stress (Fig. 8 and S7, Table S8). This increase in the AsA/DHA ratio is based on a slight decrease in the content of AsA and a more dramatic downregulation of DHA, which results in a reduction in the ability of AsA to scavenge H_2O_2. It can be hypothesized that the reduction in production caused by the severe disruption of the OXPHOS pathway in the primary metabolism exceeds the compensatory effects of glycolysis and other pathways, thus, resulting in inadequate supply of ATP and reducing power. As a result, the reduction of GSSG to GSH and that of MDHA to AsA is affected. In this case, although glutathione reductase was slightly upregulated, there was a limitation in the rate of reduction of GSSG to GSH. In addition, the reduction of the DHA content and the weakening of the MDHAR-based catalytic reactions resulted in a reduction in the accumulation of AsA.

Both GXP and GST utilize GSH as a substrate. During ENR stress, the regulation of GXP did not change significantly, while GST was significantly upregulated (Fig. 8, and S7, Table S8). GST catalyzes the formation of conjugates between GSH and both hydrophobic and electrophilic substrates, which can then be isolated in vacuoles or transferred to extracellular vesicles, thereby degrading both endogenous and exogenous deleterious compounds [75]. In addition, GST also helps to transport antioxidant substances such as anthocyanins and flavonoids into the vacuoles, thus, playing a role in the reduction of oxidative stress [76,77]. In summary, the continuous oxidative stress imposed by ENR disrupted the AsA-GSH cycle and rendered it unable to operate at full capacity. The detoxification of ROS through the GSH-dependent GST pathway may play an important role under ENR stress.

It has been reported that under normal growth conditions, mutations in the glutathione reductase gene can lead to a significant accumulation of GSSG and a slight elevation in the content of GSH. As a result, the GSH/GSSG ratio is significantly decreased, which results in the severe stunting of root growth; however, once GSH is added in vitro, the growth of roots improves [78]. Further studies have demonstrated that the regulation of the growth in the root apical meristem is directly impacted by the redox state of glutathione. Additionally, the crosstalk between auxin and glutathione also suggests that GSH is crucial for regulating root structure in stressful environments [79,80]. This study obtained comparable results. Therefore, it can be hypothesized that one of the primary causes of the limited growth of wheat roots under ENR stress is the lower ratio of GSH to GSSG.

3.8. Relationship between patterns of protein and mRNA expression

The relationship of the patterns of expression between mRNAs and proteins was analyzed to uncover the effects that ENR treatment had on wheat roots. A total of 1562 proteins were matched to mRNA, and 481

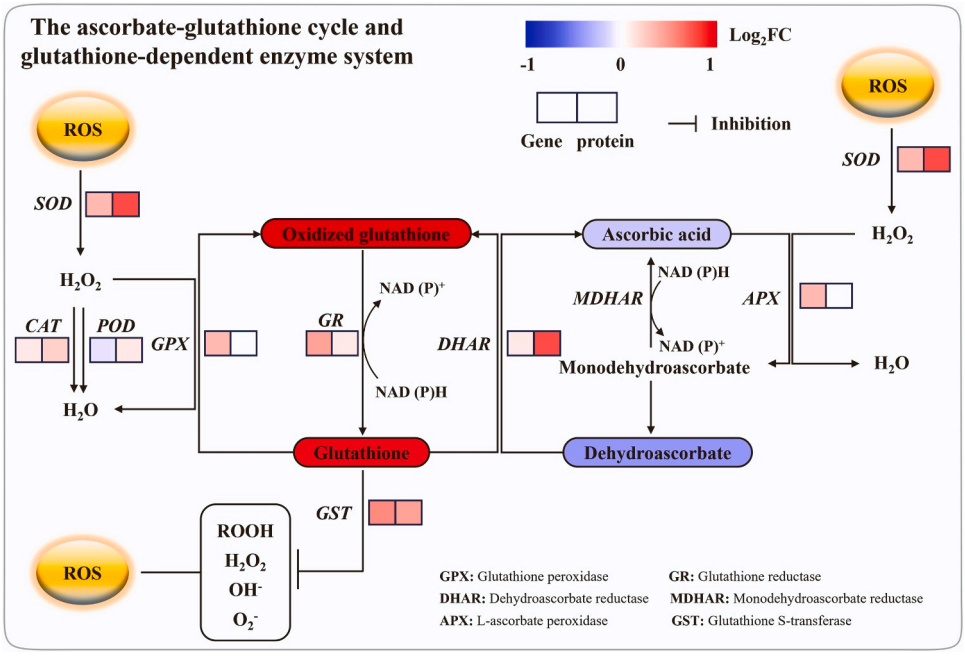

Fig. 8. The AsA-GSH cycle and glutathione-dependent enzyme system. The color block represents the value of $\log_2$(T/C). Red and blue gradients, respectively, indicate genes, proteins, or metabolites that are upregulated or downregulated.

(30.77 %), protein abundance and/or mRNA expression differences exceeded the threshold set in this study. For each pair of associated genes and proteins, the $\log_2$-fold change of protein abundance and gene expression was analyzed to obtain differential groupings of proteins and patterns of gene expression (Fig. 9 and Table S13). There was less protein in quadrants 1, 2, and 4 than related mRNA. However, in quadrants 3 and 7, mRNA was expressed in the same manner as the related proteins. There was generally no difference in the expression of proteins and mRNA in quadrant 5. The proteins in quadrants 6, 8, and 9 were more abundant than the related mRNA. Only 83 proteins (quadrant 3, 13.10 %; quadrant 7, 4.16 %) exhibited trends of expression that were consistent with those of the mRNA. The remaining genes and corresponding proteins displayed inconsistent or even opposing patterns of expression. This result suggests that the abundance of most proteins is not directly linked to their transcription and/or translation [22]. Previous research has shown that the transition from mRNA to protein formation undergoes a complex regulatory process. During this process, factors such as differential translation, protein degradation, contextual confounders, and pervasive protein-level buffering, all impact the production of proteins [81,82]. The results of a correlation analysis between multi-omics also showed lower levels of correlation between the relationships, with $R = 0.22$ ($p < 2.2 \times e^{-16}$) between the mRNAs and proteins, $R = 0.0013$ ($p = 0.17$) between the mRNAs and metabolites, and $R = 0.00016$ ($p = 0.95$) between the proteins and metabolites (Fig. S8). Some KEGG pathways were annotated utilizing data on nine-quadrant. In 2-oxocarboxylic acid metabolism, the biosynthesis of amino acids, carbon metabolism, oxidative phosphorylation, arginine biosynthesis, and purine metabolism pathways, and the correlation between mRNA and patterns of protein expression (R =0.88, 0.30, 0.42, 0.52, 0.87, 0.99) was much higher than the correlation between the transcriptomic and proteomic data (Fig. S9). It is suggested that in response to ENR, these processes and their associated metabolite alterations are driven, at least in part, by transcription and/or translation/synthesis.

4. Conclusions

This study showed that ENR stress inhibited root development in wheat seedlings and activated the oxidative stress defense system. In response to this phenomenon, a combination of transcriptomic, proteomic, and metabolomic analyses was used (providing the advantage of comprehensive and in-depth analysis) to explain the mechanism that underlies ENR toxicity in the roots of wheat seedlings (Fig. 10). A multi-omics analysis showed that ENR stress resulted in an impairment of the OXPHOS pathways and an imbalance in the AsA-GSH cycle. Cells enhanced the regulation of hormones, GSH-dependent GST-mediated ROS scavenging, ubiquitin-independent proteasomal degradation, glycolysis, amino acid biosynthesis, and flavonoids biosynthesis pathways to manage this quandary and thus, improve their adaptation to stress. A comprehensive analysis indicated that excessive levels of ROS, a low GSH/GSSG ratio, and an insufficient energy supply might be the main causes of the inhibition of root growth observed in wheat seedlings. A molecular regulatory network of genes, proteins, and metabolites in the response of wheat seedling roots to ENR toxicity is proposed, which provides a comprehensive framework for numerous cellular processes.

Environmental implication

Antibiotics are the residues of hazardous materials that are commonly found in agricultural soils at concentrations ranging from a few micrograms to several mg/kg, thus, posing a long-term risk to agricultural production and human health. For a long time, antibiotics have caused non-negligible environmental pollution, particularly in plant growth and development. This study aimed to determine the effects of typical antibiotic ENR on wheat seedlings and reveal its toxicological mechanism by combining transcriptomics, proteomics, and metabolomics. The findings will present novel insights into the environmental impacts of ENR and fresh views for the assessment of ecological risks.

CRediT authorship contribution statement

Haiyan Hu: Data curation. **Na Dong:** Data curation. **Gan Li:** Data curation. **Yuquan Wang:** Software, Data curation. **Weihua Ding:** Software, Data curation. **Xigui Hu:** Software, Data curation. **Dazhong Zhang:** Writing – review & editing, Software, Data curation. **Xiangdong Chen:** Writing – review & editing, Software, Methodology, Data

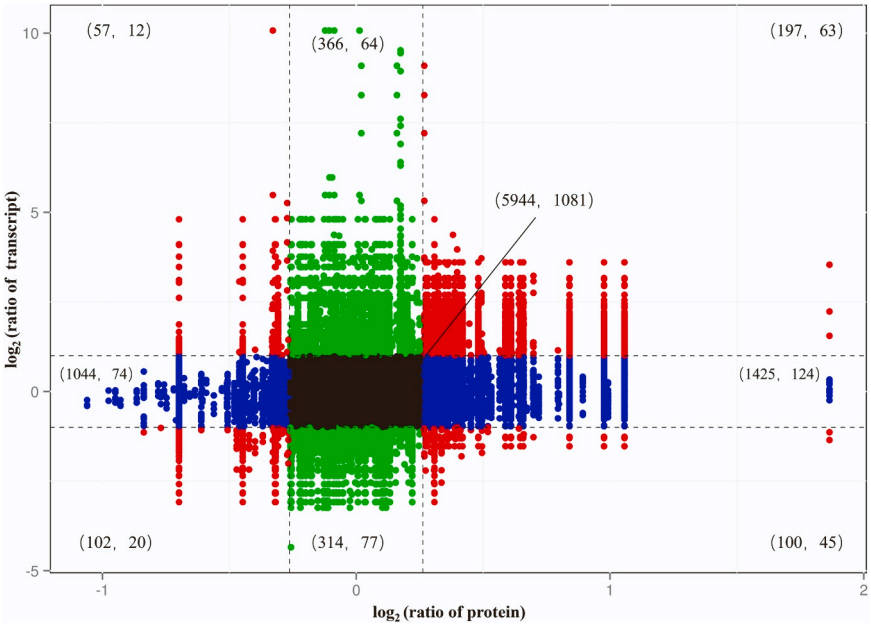

Fig. 9. Scatter plot of mRNA and protein 9-quadrant association analysis from $\log_2$FC (T/C). The number of genes and the matching number of proteins are enclosed in parentheses. Colored scatters present impacting > 1.2 or < 0.83 in proteins and/or > 2.0 or < 0.5 in mRNAs.

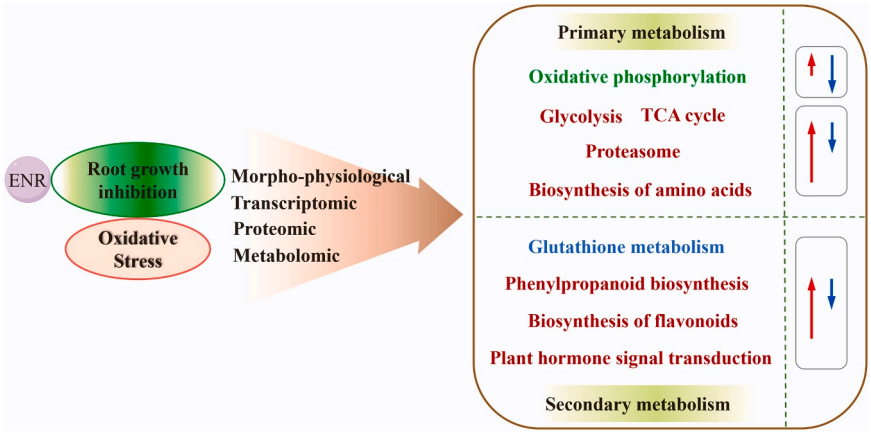

Fig. 10. Proposed model of enrofloxacin stress response in the wheat seedling roots. Within blocks, the red arrows are longer than the blue arrows, or the blue arrows are longer than the red arrows. This indicates that most of the differential proteins or metabolites are upregulated or downregulated, respectively.

curation. **Xiaojun Wu:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Zhengang Ru:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Tiezhu Hu:** Data curation.

Declaration of Competing Interest

The authors state that no known competing financial interest or personal relationship may have had any influence on any of the work disclosed in this study.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135303.

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