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Metabolomic and Transcriptomic Investigation of Metabolic Perturbations in *Oryza sativa* L. Triggered by Three Pesticides

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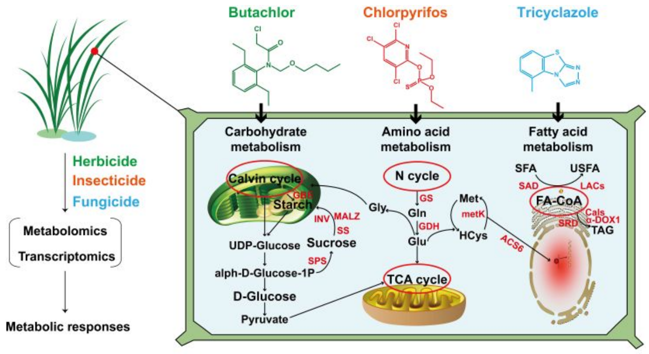
ABSTRACT

Inappropriate application of pesticide often triggers molecular alterations in crops, which inadvertently disturbs metabolites and finally affects crop quality. Therefore, understanding the mechanism of action of pesticides on crops is essential for evaluating the potential environmental impact of pesticides. Our findings indicated that three typical pesticides, including herbicide butachlor, insecticide chlorpyrifos and fungicide tricyclazole, induced the expression regulation of different key genes, exhibiting considerable distinction on metabolic responses in rice (*Oryza sativa* L.). Butachlor mainly affected five carbohydrate metabolism pathways (38.5%), and more than 48.0% of differentially expressed genes (DEGs) involved in starch and sucrose metabolism as well as photosynthesis, thereby disturbed the distribution of starch-sucrose. Chlorpyrifos dramatically affected six amino acid metabolism pathways (60.0%), and key DEGs mainly enriched in aspartate and glutamate metabolism, inducing an increase in free amino acid contents (up 29.02% of the control) and degradation of soluble proteins (down 48.72% of the control). Tricyclazole remarkably affected six fatty acid metabolism pathways (53.9%), and significantly up-regulated DEGs which primarily code oil bodies membrane proteins, resulted in the decline of saturated fatty acids (palmitic acid and stearic acid) and the rising of unsaturated fatty acids (linolenic acid and octadecadienoic acid). These findings provide a molecular-scale perspective on the response of crops to pesticides.

Keywords: Metabolite perturbation, Key genes, Herbicide, Insecticide, Fungicide

Graphical abstract



INTRODUCTION

Global usage of pesticides has increased exponentially over the past few decades, with widespread applications for crop protection.[1,2] However, the amount of applied pesticide that annually reaches the target organisms is commonly less than 1%. Other gigantic portion persists in soils due to off-target deposition or accumulates in crops via foliar absorption and soil-root migration.[3,4] China is the largest pesticide user worldwide, with the average annual amount being approximately 1.5- to 4-fold higher than the global average.[5] This high level of application inevitably causes pesticide residue accumulation in crops, thereby affecting food safety and quality.[6, 7] Although pesticides application is based on the evaluation of its visible phytotoxicity to nontarget crops, there are likely to be many non-visible and subtle effects on crop at the physiological, biochemical and molecular level.[8-11]

Assessment of these potential effects has received much research interest in recent years, with various studies on metabolites perturbation in crops following pesticide exposure, as these metabolites are real players in crop growth and stress responses. One study by Zhang et al.[7] showed that the organochlorine insecticides lindane and chlordecone altered at least 26 metabolites in maize roots, among which sucrose was down-regulated indicating fluctuations in starch and sugar metabolism. A similar experiment in lettuce (*Lactuca sativa* L.) exposed to the fungicide mancozeb exhibited changes in levels of ascorbate, sugar, lipid and nucleotide as well as amino acids.[12] Generally, the metabolic response of plants to pesticides stress is a highly complex process involving transcriptional regulation of multiple genes. These genes have been reported to perform fundamental structural and physiological functions, including transcription, translation, cellular communication and signaling, central metabolism, energy metabolism and stress responses.[13-15] Previous research has elucidated that the herbicide diclofop-methyl can induce citrate loss in the citric acid cycle (TCA cycle) in rice plant cells, by enhancing the activity and gene transcription of citrate synthase and reducing gene transcripts of citrate lyase.[11] Moreover, studies have shown a similar inhibition of acetylcholinesterase (AChE) in rice and animals, following exposure to the insecticide diazinon, with the subsequent accumulation of AChE mainly affecting the metabolism of osmolytes and TCA intermediates.[16] Furthermore, the azole fungicides have been reported to interfere with estrogen receptor α and inhibit steroid hormone biosynthesis in mammals,[17] although little research has been conducted on their impacts on crops. Overall, these studies implied that pesticides could induce metabolic responses and alter the expression of related genes in target or nontarget organisms. For all this, due to the rapid upgrade and vide varieties of pesticide species, the stress of pesticide on the regulatory mechanisms of gene expression in metabolic processes requires further exploration.

Rice is one of the most important cereal crops worldwide, serving as a staple food feeding nearly half of the global population.[7] It also frequently selects as a model plant in molecular biology research. Herbicide butachlor (BUT), insecticide chlorpyrifos (CPF) and fungicide tricyclazole (TRI) are commonly used in rice farms in China. Hence, we selected rice (*Oryza sativa* L.) as the representative crop, with the BUT, CPF and TRI applied as representative pesticides. The primary aim of this work is to unveil the complex mechanism of metabolites perturbations in rice exposed to pesticides at metabolic and transcriptional levels. The findings of this study provide a deeper understanding of how crops systematically respond to pesticide stress and therefore, helps improve environment risk assessments and the effective management of pesticide application schemes.

MATERIALS AND METHODS

Chemicals. Chlorpyrifos (480 g/L, emulsion), butachlor (600 g/L, emulsion) and tricyclazole (20%, wettable powder) were supplied by Iprochem in Shenzhen, China. The registration concentration of chlorpyrifos, butachlor and tricyclazole in China were 0.576-0.720 kg active ingredient/hectare (a.i./ha), 0.900-1.575 kg a.i./ha and 0.225-0.300 kg a.i./ha, respectively. Stock solutions were prepared by dissolving the three pesticides in deionized water. All other solvents and reagents used were of HPLC grade and analytical grade.

Rice Cultivation and Pesticide Treatment. *Oryza sativa* L. seeds were purchased from the Zhejiang Academy of Agricultural Sciences (Hangzhou, China). The plump rice seeds after screened were then sterilized with 3% (*v/v*) H_2O_2 for 30 min and rinsed three times with deionized water, before being soaked for 48 h at 25 °C. Next, the rice seeds were transferred to a seedling pots containing 5 000 mL of Hoagland nutrient solution and perlites. Cultivation was conducted in a plant growth chamber with a 14 h: 10 h light dark cycle, a constant temperatures of 25 °C, 70% relative humidity and a light intensity of 250 μmol/(photons m^2 s). *Oryza sativa* L. seedlings were harvested after 10 days and then transferred into colored vitreous pots containing 500 mL Hoagland nutrient solution. After cultivation for 30 d, tillering rice plants were sprayed pesticides using a Yangtse River 08 model sprayer with a nozzle of 1 mm diameter, with tap water being sprayed as a control. After exposure for 7 days, all rice plants were harvested and then tested. According to the Chinese agricultural trade standard (NY/T 788-2004), the highest treatment dose was recommended to be 1.5- to 2-fold higher than the maximum registered dose. Thus, the exposure concentrations of CFP were 0.576, 0.720 (registered dose), 1.080 (1.5-fold dose) and 1.440 (2.0-fold dose) kg a.i./ha. The exposure concentrations of BUT were 0.900, 1.574 (registered dose), 2.624 (1.5-fold dose) and 3.148 (2.0-fold dose) kg a.i./ha. The exposure concentrations of TRI were 0.225, 0.304 (registered dose), 0.455 (1.5-fold dose) and 0.607 (2.0-fold dose) kg a.i./ha.

Oxidative Damage and Antioxidant Enzyme Activities Assay. Rice leaves of control and pesticide-treated groups were ground in a mortar with 2 mL of ice-cold PBS buffer (0.05 M, pH 7.8). Samples were centrifugated at 3 000*g* for 15 min, the supernatants were collected for analysis of oxidative damage and antioxidant enzyme activities. The content of malondialdehyde (MDA) was determined via the thiobarbituric acid (TBA) test. The concentrations of reactive oxygen species (ROS) and phosphorylated histone H_2AX (γ-H_2AX) were analyzed using plant ROS and plant γ-H_2AX enzyme-linked immunosorbent assay (ELISA) kits (Meimian Biotechnology Co. Ltd., China), respectively. The activities of superoxide dismutase (SOD) and catalase (CAT) enzymes were assayed using the protocols described by Zhang et al.[18] Peroxidase (POD) activity was measured according to the method reported by Zaharieva at al.[19] Detailed description of the methods is provided in the Supporting Information Text S1.

Chlorophyll Fluorescence Analysis. Rice leaves of control and pesticide-treated groups were collected after light shading for 15 min. Chlorophyll fluorescence parameters were measured using a pulse-amplitude modulation (PAM) fluorometer (Walz, Germany). Detailed description of the methods is provided in the Supporting Information Text S2.

Transmission Electron Microscopy Detection. Transmission electron microscope (JEM-1230 microscope, JEOL Ltd., Tokyo, Japan) was employed to image the substructure of the leaf cell. Fresh rice leaves of control and pesticide-treated groups were fixed in osmic acid, wrapped with embedding medium, and sliced, and then observed with TEM. Detailed description of the methods is provided in the Supporting Information Text S3.

Starch and Soluble Sugar Determination. Soluble sugars in leaves were extracted and quantified by a modified version of the method reported by Xu et al.[20] About 0.1 g of fresh ground sample was extracted with 2 mL of 80% (v/v) ethanol at 80 °C for 30 min, followed by centrifugation at 3 000*g* for 10 min, with the process then repeated two more times. Then, the supernatants were combined for measurement of the soluble sugar content. Furthermore, the residues were evaporated in order to remove the ethanol, and then successively hydrolyzed in 9.2 mol/L and 4.6 mol/L perchloric acid. After centrifugation at 3 000*g*, the perchloric acid supernatants were collected for measurement of the starch contents. Finally, the determination of starch and soluble sugar concentrations were performed using the anthrone methods.[21]

Free Amino Acids and Soluble Proteins Determination. About 0.1 g of fresh leaves ground in liquid nitrogen was hydrolyzed in 5 mL 3% (w/v) of sulfosalicylic acid for 1 h, and then centrifuged at 10 000*g* for 15 min. The supernatant was filtered through a 0.22 μm filter membrane for the quantification of amino acids using an automatic amino acid analyzer L-8800 (Hitachi, Japan). The total soluble protein was extracted twice using ice-cold phosphate buffer (50 mM, pH 7.8). Protein concentration was quantified according to the Coomassie brilliant blue assay.[22]

Metabolomics Analysis. The method of metabolomic analyses of rice leaves was modified from the previously reported method by Li et al.[23] Metabolites were immediately determined via gas chromatography system (Agilent 7890B, USA) coupled with a quadrupole MS (Agilent 5977B, USA). Principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), analysis of variable importance in the projection (VIP) score and enrichment pathway were performed using the MetaboAnalyst 4.0 software (http://www.metaboanalyst.ca/). Detailed description of the methods is provided in the Supporting Information Text S4.

Transcriptome Analysis. Total RNA was isolated from frozen *Oryza sativa* L. leaves using TRIzol® Reagent (Plant RNA Purification Reagent) according to the manufacturer's instructions (Invitrogen) and genomic DNA was isolated using DNase I (TaKara). Then the quality and quantity of RNA was determined using a 2100 Bioanalyser (Agilent) and a ND-2000 (NanoDrop Technologies), respectively. RNA purification, reverse transcription, library construction and sequencing were performed according to the manufacturer's instructions (Illumina, San Diego, CA). The RNA-seq transcriptome library was prepared using a TruSeq™ RNA sample preparation kit (Illumina, San Diego, CA) using 1 μg of total RNA. The paired-end RNA-seq sequencing library was sequenced using an Illumina Novaseq 6000 (2×150 bp read length). Concentrations of RNA in the established library were assessed using an Qubit® RNA Assay kit in Qubit® 3.0 for preliminary quantification and then RNA samples were diluted to 1 ng/μL. Insert size was evaluated using an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), and only qualifying insert sizes (valid concentrate on > 10 nM) were accurately quantified using the StepOnePlus™ Real-Time PCR System. Cluster generation was performed via the cBot cluster generation system using HiSeq PE Cluster kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. The libraries were sequenced on an Illumina Hiseq 4000 platform and 150 bp paired-end reads in lengths were generated. Library preparation and RNA-Seqdata analysis were conducted by Majorbio Technology Co., Ltd. (Shanghai, China). To annotate the unigenes, several complementary publicly available protein databases (NR, NT, SwissProt, Uniprot, COG, Pfam, GO and KEGG) were utilized. Detailed description of the methods is provided in the Supporting Information Text S5.

Statistical Analysis. The results are presented as means ± SD (standard deviation). All experiments were performed in at least triplicates. The biochemical analysis was performed in six replicate samples for each treatment. The metabolite assay was performed in at least eight replicate samples, and the RNA-Seq assay was performed in triplicates for each treatment. The number of replicates used for each parameter is provided in the corresponding figure or table. A one-way analysis of variance (ANOVA) and student *t*-test were performed using GraphPad Prism (version 7.0, San Diego, CA, USA). Significant and highly significant differences were based on the probabilities of $p < 0.05$ and $p < 0.01$, respectively. The SD of the relative values was derived from the formula as follows: $SD = SD_{treatments} \times SD_{control}/V_{control}$.

RESULTS AND DISCUSSIONS

Physiological Response to Pesticides. MDA levels reflect the degree of cellular damage under conditions of oxidative stress. As shown in Figure S1, none of the three pesticides at standard-used concentration significantly affected MDA levels in rice leaves compared with unexposed control leaves ($p > 0.05$). However, exposure to pesticides at concentrations of 1.5-fold and 2-fold dose induced a significant increase in the MDA content ($p < 0.05$), indicating that the rice leaf cells were damaged. Moreover, the level of γ-H2AX, a highly sensitive biomarker for DNA double-strand breakage, showed no significant differences at any of the tested concentrations, suggesting that DNA remained undamaged by exposure to the pesticides. ROS level is another index that reflects cell damage. We found that exposure to all three pesticides at registered dose had no significant effect on ROS levels in *Oryza sativa* L. leaves ($p > 0.05$). However, exposure to high levels of BUT and TRI triggered excessive ROS generation. Generally, alterations in ROS levels are related to changes in activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) or peroxidase (POD), because these enzymes can scavenge and control ROS levels under oxidative stress conditions. In the present study, POD activity did not exhibit demonstrable changes following CPF exposure ($p > 0.05$), whereas it was elevated to 194.77% and 116.54% of the control following exposure to 2-fold dose of BUT and TRI exposure, respectively. Furthermore, SOD activity increased significantly with the increase in CPF exposure, while it was initially increased and then decreased with exposure to elevated concentrations of BUT and TRI. CAT activity exhibited a similar trend to SOD activity, with decline in SOD and CAT activity indicating that antioxidant enzymes could not scavenge ROS efficiently when exposed to 2-fold doses of BUT and TRI, resulting in excessive ROS accumulation.[24]

Moreover, changes in the *Oryza sativa* L. leaf cell ultrastructure were observed following exposure to all three pesticides. When exposed to the registered dose of CPF and BUT, rice chloroplasts appeared swollen and starch granules occupied a greater area of the chloroplast space as compared with the control (Figure S2a2, S2b2). Because of the extrusion by starch granules, the thylakoid grana were lessened and some of the links with stromal thylakoids disappeared. This finding is consistent with the results of previous studies on plant responses to abiotic stress, such as cold temperatures, high salinity, drought and etc.[25-27] Furthermore, exposure to 2-fold higher doses of CPF and BUT resulted in irregular cell shapes, chloroplast disappearance and starch granule disintegration (Figure S2a3, S2b3). Chloroplast injuries can inhibit the flow of energy and the conversion of soluble sugars to starch. Therefore, the observed chloroplast alterations suggest the likely inhibition of intracellular carbohydrate metabolism under CPF and BUT stress. Interestingly, TRI exposure induced the accumulation of oil bodies, affecting the lipid reserves and fatty acid composition, potentially indicating a hermetic stress-response.[28] However, membranes destruction and diminution of oil bodies occurred following treatment with high levels of TRI, indicating the lipid peroxidation (Figure S2c2, S2c3).

Chlorophyll fluorescence parameters are often used as indicators of photosynthesis efficiency. As shown in Figure S3, when the rice plants exposed to CPF and TRI, no evident differences were observed in F_v/F_m between the treatment groups and the control group, indicating that the photosynthetic efficiency of photosystem II (PSII) was unsusceptible to CPF and TRI stress. However, when the rice plants were exposed to 2-fold dose of BUT, PSII activity (ΦII) was significantly increased by 33.33% as compared with the controls, while non-photochemical quenching (NPQ) and non-photochemical quenching coefficient (qN) were significantly increased by

26.89% and 25.63%, respectively (p < 0.05), as compared with the controls. This suggests that PSII photochemical energy utilization was inhibited by BUT exposure. This finding also indicates that photosynthesis is highly sensitive to herbicides, which is consistent with the results of previously reported studies.[29]

Metabolic Response to Pesticides. Untargeted metabolomic analysis demonstrated substantial metabolic changes in *Oryza sativa* L. leaves based on the data for identified and quantified metabolites. Firstly, unsupervised principal components analysis (PCA) analysis was used to generate an overview of the clustering information among groups. The PCA score plot shows that the unexposed control group was clearly separated from BUT and TRI treatment groups along the first principle axis (PC1, 68.1% and 63.8%), meanwhile the unexposed control group was clearly separated from CPF treatment group along the second principle axis (PC2, 88.8%), indicating that all three pesticides markedly altered the metabolites composition in exposed rice leaves. Venn analysis further established that a total of 61, 79 and 92 common metabolites responded to BUT, CPF and TRI, respectively (Figure S4, $p < 0.05$). These metabolites mainly included in carbohydrates, amino acids, fatty acids, nucleotides, and secondary metabolites (polyols, glycosides and vitamins) ($p < 0.05$, Tables S1-S3, Supporting Information). Among them, fatty acids were only detected in groups exposed by BUT and TRI. Moreover, the metabolomics data was integrated to perform variable importance in projection (VIP) and metabolic pathway analysis using MetaboAnalyst 4.0 software (Figure S5). Enrichment analysis showed that the herbicide BUT significantly affected 5 metabolic pathways involved in carbohydrate metabolism, including the pentose phosphate pathway, glycolysis/gluconeogenesis, TCA cycle, galactose metabolism, starch and sucrose metabolism (38.5%, $p < 0.05$, fold enrichment > 2). Furthermore, the insecticide CPF significantly affected 6 metabolic pathways involved in amino acid metabolism, including arginine and proline metabolism, beta-alanine metabolism, glutamine metabolism, tyrosine metabolism, aspartate metabolism, glycine and serine metabolism (60.0%, $p < 0.05$, fold enrichment > 2), whereas the fungicide TRI significantly affected 7 metabolic pathways involved in fatty acid metabolism, including fatty acid biosynthesis, fatty acid metabolism, fatty acid elongation, glycolipid metabolism, linolenic acid and linoleic acid metabolism, oxidation of branched chain fatty acids (53.9%, $p < 0.05$, Figure 1).

Transcriptional Responses to Pesticides. To better understand the metabolic response mechanism of rice plants to pesticides stress, transcriptomic analysis was performed based on the RNA-Seq technique, to identify the genes expression in *Oryza sativa* L. Genes with a false discovery rate (FDR) of < 0.05 are considered to be differentially expressed genes (DEGs). Results showed that all three pesticides affected the gene transcription in rice leaves to varying degrees, with volcano plots visualizing the degree of variation of these DEGs based on red and green dots (Figure S6, S7)

To understand the biological significance of DEGs, we performed GO and KEGG enrichment analyses. As shown in Figure S8 and Figure S9, numerous DEGs were assigned to photosynthetic dark reactions in rice leaves under standard BUT dose exposure, whereas numerous DEGs were assigned to carbohydrates biosynthesis following 2-fold higher BUT dose exposure. This indicates that the herbicide BUT might influence carbohydrate synthesis by affecting the dark reactions of photosynthesis. In carbohydrate metabolism, the gene expressions of most enzymes were decreased, except for Os04g0664900 (beta-fructofuranosidase, INV) and Os06g0676700 (alpha-glucosidase, MALZ), which was consistent with the alterations in starch and sucrose concentrations observed following BUT exposure (Figure S10). Moreover, numerous DEGs were assigned to nitrogen metabolism in rice leaves exposed to standard doses of CPF, while DEGs were assigned to amino acid metabolism following exposure to 2-fold higher doses of CPF (Figure S9a and S9b, Table S5). This suggests that the insecticide CPF might influence amino acid biosynthesis by affecting nitrogen assimilation. The Furthermore, some DEGs involved in fatty acid elongation and alpha-linolenic acid metabolism were significantly affected by TRI exposure at standard doses, while DEGs involved in glycosphingolipids were significantly affected following 2-fold higher dose TRI exposure. Interestingly, following exposure to all three pesticides, DEGs in leaves were assigned to the mitogen-activated protein kinase (MAPK) signaling pathway in *Oryza sativa* L., indicating a stress-response of rice to pesticides.

Integrating Omics Results to Reveal Molecular Mechanisms underlying Plant

Responses to Pesticides. *Carbohydrates metabolism.* In the present study, the key carbohydrates, including sucrose, talose, xylitol and fructose, were significantly affected by pesticides exposure (Figure 2a and Figure S5). Particularly following the herbicide BUT exposure, starch and sucrose metabolism, the pentose phosphate pathway, glycolysis/gluconeogenesis and TCA cycle were remarkably enriched in *Oryza sativa* L. leaves (Figure 1). Herein, starch and sucrose metabolism is the main carbohydrate metabolism, occurring via two different pathways in *Oryza sativa* L. leaves.[30, 31] The first pathway involves starch synthesis, which takes place in chloroplasts. In this process, fructose-6-phosphate (Fruc-6P) which is produced via the Calvin cycle, is converted into glucose-6-phosphate (Gluc-6P) and then to starch, via the actions of ADP-glucose pyrophosphorylase (GLGC), starch branching enzyme (GBE), and granule-bound starch synthase (GLGA). The genes encoding GLGC (Os05g0580000) was up-regulated following herbicide BUT exposure. Because the elevated sucrose metabolism in downstream promoted the accumulation of sugar (e.g. Gluc-1P) in chloroplast, the GLGC gene up-regulated in order to transfer the increased Gluc-1P into ADP-glucose. However, The genes encoding GBE (Os04g0409200) and GLGA (Os10g0437600) were significantly down-regulated to 0.45-fold and 0.67-fold of the control following the registration concentration of BUT exposure, the gene encoding beta-amylase (Os10g0565200) was significantly down-regulated to 0.31-fold of the control under exposure in the registration concentration of BUT even undetected under exposure in high concentration of BUT, which indicated that the decomposition of starch was slower than the synthesis of starch. Thus, the increased metabolite in upstream and lower the decomposition of starch promoted the accumulation of starch in chloroplasts (Figure 2b). The second pathway occurs in the cytoplasm and involves the sucrose synthesis and decomposition. This process transports triose phosphate from chloroplasts to the cytoplasm, where it is converted to sucrose under the action of sucrose synthase (SS), beta-fructofuranosidase (INV) and sucrose-phosphate synthase (SPS) (Figure 2c). In the cytoplasm, sucrose can be further hydrolyzed into monosaccharides (e.g. glucose/UDP glucose and fructose) by INV and alpha-glucosidase (MALZ).[32] This process is considered to be the most efficient pathway for starch accumulation.[30] According to the results of the present study, the up-regulated expressions of INV, MALZ and SS genes promoted sucrose decomposition, which was consistent with the variations in soluble sugar contents in *Oryza sativa* L. leaves under exposure to BUT (Figure S10). It has been well established that decreased carbon flux through sucrose synthesis may lead to reduced export and higher accumulation of starch in leaves. When carbohydrate assimilation is compromised due to environmental stress, starch decomposition can reduce the adverse effects of stress-induced carbon depletion.[33, 34] Therefore, starch accumulation and decomposition is an effective stress response by *Oryza sativa* L. to pesticides. Because starch is the main nutrients of rice, the changes of this metabolite can affect the health and physiological state of rice, which finally affect the rice yield and quality.

Carbohydrates can be oxidized via the glycolysis pathway (EMP) and TCA cycle. Under abiotic stress conditions, EMP can often improve the adaptability of plants to their environment.[35] In general, the conversion of carbohydrates into pyruvic acid by the glycolysis pathway requires the catalysis of a variety of enzymes, among which phosphofructokinase (PFK), pyruvate kinase (PK) pyruvate dehydrogenase (PDHB) and pyruvate decarboxylase (PD) are some of the most important enzymes (Figure 2c). In the present study, it was found that the expressions of PFK, PK PDHB and PD genes in the EMP pathway were up-regulated following exposure to BUT, thus improving the tolerance of *Oryza sativa* L. to BUT exposure (Figure 2b). Enhancement of the EMP pathway can lead to higher ATP production in response to abiotic stress. The TCA cycle is central to cellular energy production, which works together with biosynthetic pathways to maintain the carbon balance in plants under stress conditions.[36] In this study, pesticide exposure stress increased the levels of vital TCA cycle intermediates in *Oryza sativa* L. leaves, such as malic acid (Figure 2c and Table S1). The alterations in these intermediates were regulated by isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDHB) and malate dehydrogenase (MDH), which perform major roles in the synthesis and degradation of isocitric acid, succinic acid, fumaric acid and malic acid. Among them, the gene Os04g0479200 and Os01g0654500 encoding IDH were up-regulated by 1.80 and 1.77-fold of the control in *Oryza sativa* L. leaves after 2-fold dose of BUT exposure, respectively. As well as the gene Os12g0632700 and Os08g0434300 encoding MDH were up-regulated by

1.67 and 2.80-fold of the control, respectively. Meanwhile, the gene Os08g0120000 encoding SDHB was up-regulated by 1.70-fold of the control. These results indicating the enhancement of TCA cycle (Table S4). Similar results have previously been reported, with the expression of genes involved in the TCA cycle increased in rice leaves after exposure to the herbicide imazethapyr.[24] These results indicated that crops can stimulate the TCA cycle to defend against the stress of pesticide exposure.

Amino acid metabolism. Metabolites involved in amino acid metabolism, including serine (Ser), glycine (Gly), threonine (Thr), were remarkably accumulated in *Oryza sativa* L. leaves after exposure to all three pesticides (Figure 3). Gly and Ser are two essential amino acids involved in the photorespiration process, their accumulations imply that the photorespiration process was enhanced in rice leaves under pesticide stress. It has been well established that cell carbon metabolism is involved in the balance between photosynthesis and respiration. The increase in photorespiration may result in net carbon loss, which supports previously reported findings that carbohydrate metabolism is inhibited by pesticides.[37] Moreover, exposure to CPF and BUT increased the accumulation of total free amino acids in rice leaves. Specially following the high level of CPF exposure, the total free amino acids increased by 29.02% of the control. Exposure to the insecticide CPF remarkably disturbed porphyrin metabolism, ammonia recycling, amino acid metabolism and urea cycle in *Oryza sativa* L. leaves. Here, aspartic acid (Asp), Ser, glutamic acid (Glu) and Gly were monitored as metabolic markers in response to CPF exposure, with the trend in their alterations showing a similar pattern to that of free amino acid (Figure 3a and Figure S10d). Glu is involved in nitrogen assimilation and transport within the plants, serving as the nitrogen donors in the biosynthesis of all essential amino acids and other nitrogen-containing compounds.[38] Nitrogen metabolism depends on three key enzymes, including glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate decarboxylase (GAD). In the present study, the expressions of gene for these enzymes were up-regulated (Os03g0223400, Os04g0543900, Os03g0720300, Os02g0650900), indicating that the nitrogen assimilation was enhanced by CPF stresses (Figure 3b). Besides, Asp has also been established as a transient marker of protein degradation. In the present study, a rise in Asp content was observed in rice leaves with CPF exposure, which companied by the degradation of soluble protein (down 48.72% of the control, Figure S10). Thus, the amino acid alterations observed under pesticides exposure may be associated with enhanced protein degradation.

Fatty acid metabolism. Fatty acids are critical components of cellular membranes. In the present study, fungicide TRI significantly affected the contents of saturated fatty acid (SFA) and unsaturated fatty acid (USFA) in rice leaves (Figure 4). Levels of the USFA linolenic acid (ALA, 18:3) and octadecadienoic acid (OA, 18:2) were significantly increased, exhibiting dose-dependent responses. In addition, levels of the SFA palmitic acid (PA, 16:0) and stearic acid (SA, 18:0) were significantly decreased (Figure 4a). This indicates that alteration of the cell membrane composition occurred under the stress of TRI. Herein, linolenic acid was found to be one of the main metabolic markers in response to TRI exposure, with increased levels of linolenic acid reported to cause the accumulation of MDA and induce membrane damage, which is in agreement with the results of the present study (Figure S1, S2). In general, the synthesis and degradation of fatty acids were regulated by a series of enzymes. Our results showed that genes of key enzymes, including 3-ketoacyl-CoA synthase (KCS, Os05g0574600), acyl-carrier-protein desaturase (SAD, Os01g0118300) and long-chain acyl-CoA synthetase (LACs, Os05g0132100), were significantly up-regulated in response to TRI exposure stress (Figure 4b). Among them, KCS and LACs mediate the prolongation of fatty acids chains, by activating SFA. The gene expression of these two enzymes were up-regulated, indicating that the elevated synthesis of very long chain fatty acids (VLCFAs) and decreased contents of SFA. Moreover, SAD is a dehydrogenase enzyme that plays a pivotal role in the transformation of SFA into USFA in lipid molecules. The upregulated expression of SAD gene can promote the synthesis of USFA, which is consistent with the observed variations in ALA and OA levels. Furthermore, the genes of key enzymes involved in lipids degradation, including alpha-steroid dehydrogenase (SRD), alpha-dioxygenase (α-DOX1), caleosin related protein (Cals) and alpha-sterol demethylase (CYP51), were significantly upregulated by TRI stress. It was interesting that all these enzymes were located in oil bodies (OBs) membrane (Figure 4c). As well known that OBs are intracellular organelles that store neutral lipids such as triacylglycerols and sterol esters, which are surrounded by some proteins such as oleosins, caleosins and steroleosin, embedded in the phospholipid monolayer.[39] Among these proteins, caleosins are involved in the regulation of OBs degradation. In this study, Cals gene (Os02g0734400) were up-regulated under TRI exposure stress, which was consistent with the reduction in lipid concentrations (glycerol and 2-palmitoylglycerol) and the increase in concentration of free fatty acids. Moreover, steroleosin are another abundant OB protein species, possibly related to the formation or degradation of oil bodies.[40] Our study shows that genes coding SRD (Os07g0162100) and CYP51 (Os05g0211100) were up-regulated by 2.21 and 4.42-fold with the TRI stress, which consistence with the declining contents of sterols storage (campesterol and stigmasterol) (Text S3). Furthermore, gene expression of α-DOX1, a fatty acid-metabolizing enzymatic protein, was up-regulated by 25.34-fold by exposure to high levels of TRI, indicating that the metabolic function of fatty acids was enhanced thereby resulted in the accumulation of free fatty acids in rice leaves.[41]

MAPK Signaling Cascade. Interestingly, all three assessed pesticides induced the MAPK cascades in the leaves of *Oryza sativa* L. (Figure S11). MAPK cascades play key roles in signal transduction in a various developmental processes, such as proliferation, differentiation, motility, cell cycle regulation, response to hormones response, and stress responses.[42,43] The cascades relay signals primarily through reversibly phosphorylated MAPKs, such as MAPK kinases (MAPKKs) and MAPK kinase kinases (MAPKKKs). MAPK, MAPKK, and MAPKKK are active in specific signal transduction pathways that affect diverse upstream receptors and downstream target components.[44] In our study, the expression of calmodulin gene (CaM, Os12g0132300) was upregulated by exposure to all three tested pesticides. CaM is a universal regulator for numerous proteins in all eukaryotic cells and is well established as a calcium-dependent modulator of enzyme activities, such as protein kinases and phosphatases, as well as other signaling proteins including membrane receptors, channel-forming and structural proteins.[45] Besides, CaM regulates downstream metabolism of carbohydrates, amino acids and fatty acids (Figure S11b). Therefore, CaM might be a key signaling protein for the regulation of metabolism under the stress of pesticide exposure. However, the comprehensive regulatory mechanisms of CaM should be further investigated in future studies.

ENVIRONMENTAL IMPLICATIONS

This study provides direct evidence for the molecular mechanism of metabolites perturbations in rice exposed to three pesticides. It was found that herbicide BUT, insecticide CPF and fungicide TRI triggered different metabolic responses through the regulation and interaction of rice genes and metabolites. The herbicide butachlor mainly affected carbohydrate metabolism, and most of DEGs involved in starch and sucrose metabolism as well as photosynthesis, thereby disturbed the distribution of starch-sucrose. The insecticide chlorpyrifos dramatically promoted amino acid metabolism, and key DEGs mainly enriched in aspartate and glutamate metabolism, inducing an increase in free amino acid contents and degradation of soluble proteins. The fungicide tricyclazole remarkably affected fatty acid metabolism, and significant up-regulated DEGs mainly coding oil bodies membrane proteins, resulted in declining of saturated fatty acids (palmitic acid, stearic acid) and rising of unsaturated fatty acids (linolenic acid, octadecadienoic acid). Because the main nutrients of rice are carbohydrates (starch), amino acids, proteins and fatty acids, knowledge on the changes of these metabolites can provide more information about the rice status (e.g. yield and quality) and can help elucidate how stressors affect the health and physiological state of rice. Overall, the present study provides a useful insight into the molecular mechanisms of different biological effects of pesticides on crops, and are useful toward efforts on environment risk assessments as well as on the management of applying pesticides.

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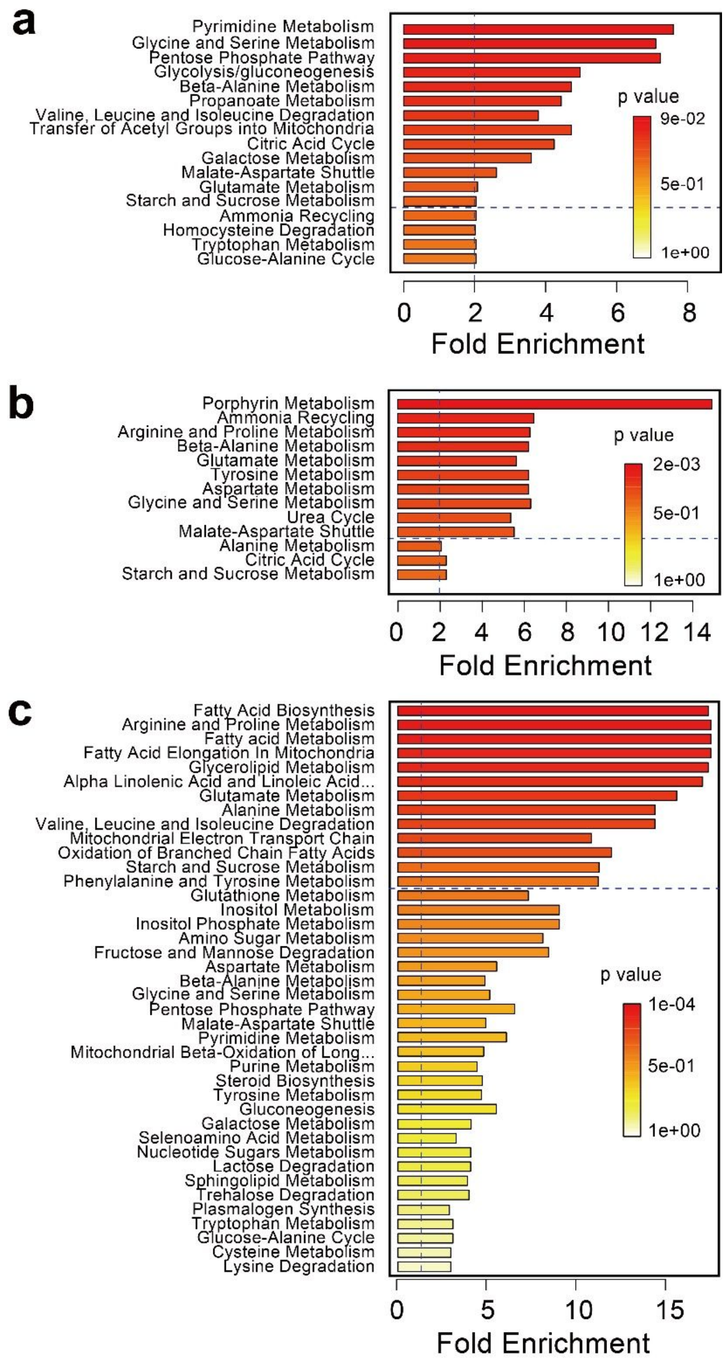

Figure 1 Enrichment analysis of changed metabolites in rice leaves exposed to BUT (a), CPF (b) and TRI (c). Results were from the analyses using the MetaboAnalyst 4.0 software. Every pillar represents a metabolic pathway, with red color indicating higher impact and yellow color indicating lower impact. The pathways with p-value < 0.05 and fold enrichment > 2 were determined to have significant changes.

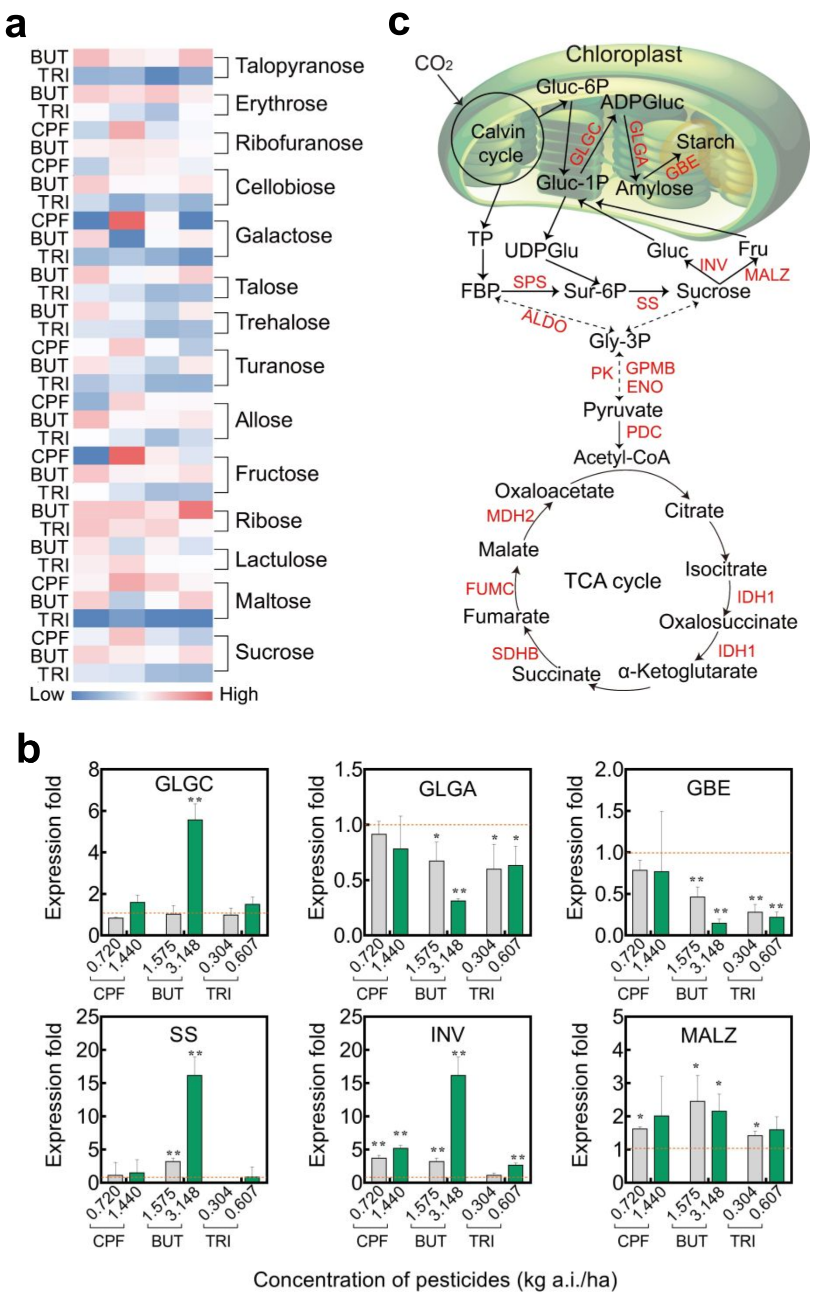

Figure 2 Carbohydrates metabolism in rice leaves with pesticide exposure. The contents of carbohydrates in leaves is displayed in the form of a heat map from low (blue) to high (red) as presented in the color scale (a). The genes encoding the key enzymes, including soluble starch synthase (GBE), starch branching enzyme (GBE), and granule-bound starch synthase (GLGA), sucrose synthase (SS), beta-fructofuranosidase (INV), sucrose-phosphate synthase (SPS) and

alpha-glucosidase (MALZ) (b). Other enzymes and their gene expressions are shown in Supporting Information Table S4. Schematic diagram carbohydrates metabolism in rice leaves (c). Enzymes involved in these pathways were marked in red. Error bars represent the standard deviation of three samples mean value. * and ** indicate that the values are significantly different compared with the control ($p < 0.05$ and $p < 0.01$).

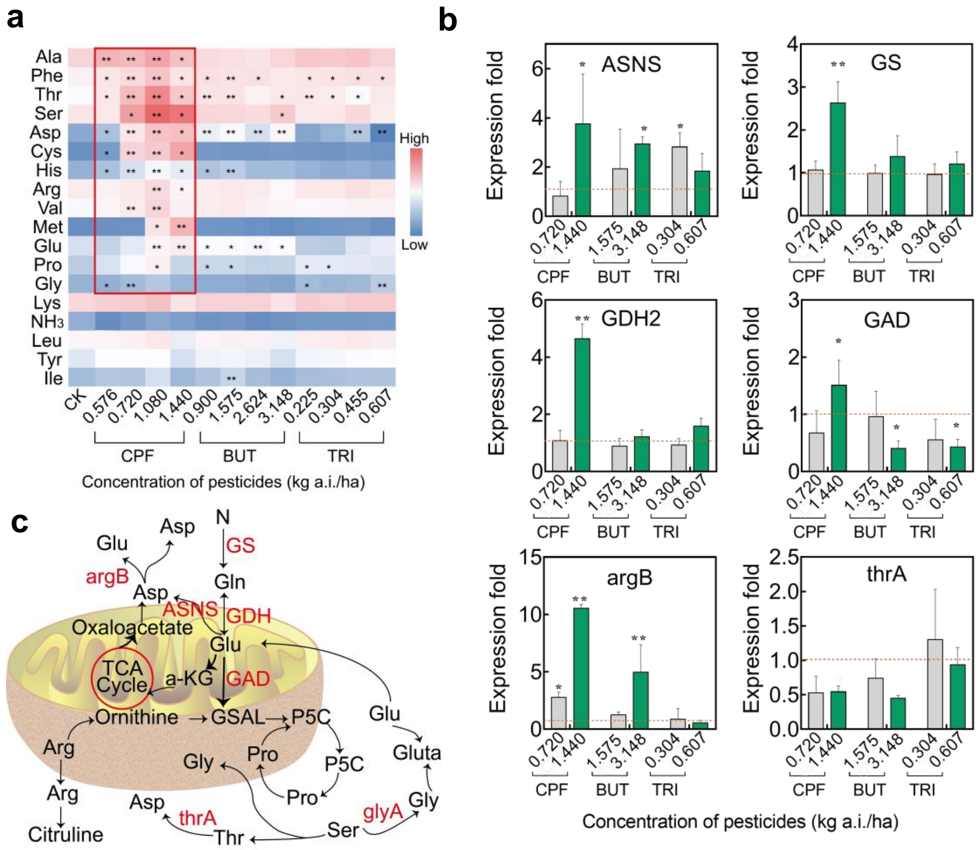

Figure 3 Amino acids metabolism in rice leaves with pesticide exposure. The contents of free amino acids in leaves is displayed in the form of a heat map from low (blue) to high (red) as presented in the color scale (a). The genes encoding the key enzymes, including glutamate dehydrogenase (GDH), glutamine synthetase (GS), glutamate decarboxylase (GAD), N-acetyl glutamate kinase 2 (argB), asparagine synthetase (ASNS) and homoserine dehydrogenase (thrA) (b). Other enzymes and their gene expressions are shown in Supporting Information Table S5. Schematic diagram amino acids metabolism in rice leaves (c). Enzymes involved in these pathways were marked in red. Error bars represent the standard deviation of three samples mean value. * and ** indicate that the values are significantly different compared with the control ($p < 0.05$ and $p < 0.01$).

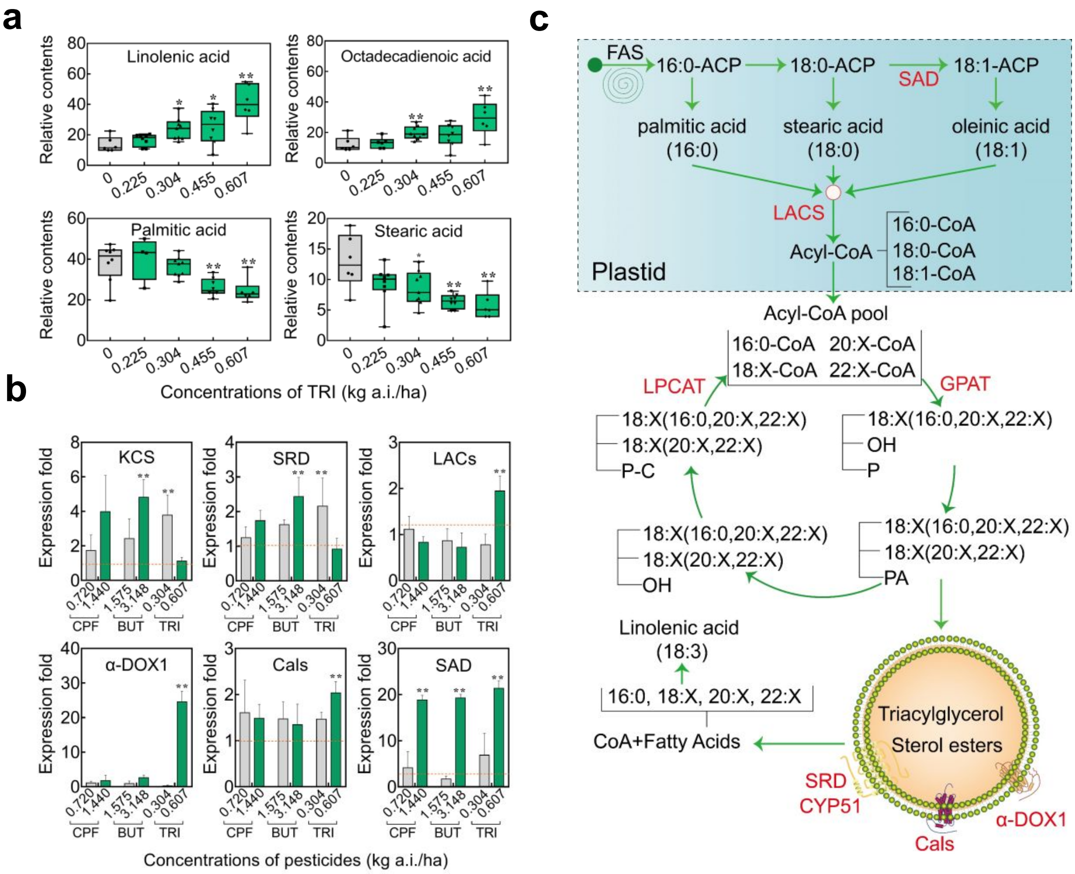

Figure 4 Fatty acids metabolism in rice leaves with pesticide exposure. Changes of the key fatty acids contents in rice leaves following the exposure to TRI (a). The genes encoding the key enzymes, including 3-ketoacyl-CoA synthase (KCS), Acyl-acyl-carrier-protein desaturase (SAD), long-chain acyl-CoA synthetase (LACs), alpha-steroid dehydrogenase (SRD), alpha-dioxygenase (α-DOX1), caleosin related protein (Cals) and alpha-sterol demethylase (CYP51). Other key gene expressions are shown in Supporting Information Table S6. The expressions of genes encoding these enzymes were shown (b). Changes in metabolites and gene expression levels mapped to fatty acids metabolism were shown (c). Error bars for fatty acids contents (a) represent the standard deviation of eight samples mean value. Error bars for gene expression (b) represent the standard deviation of three samples mean value. * and **

indicate that the values are significantly different compared with the control ($p < 0.05$ and $p < 0.01$).