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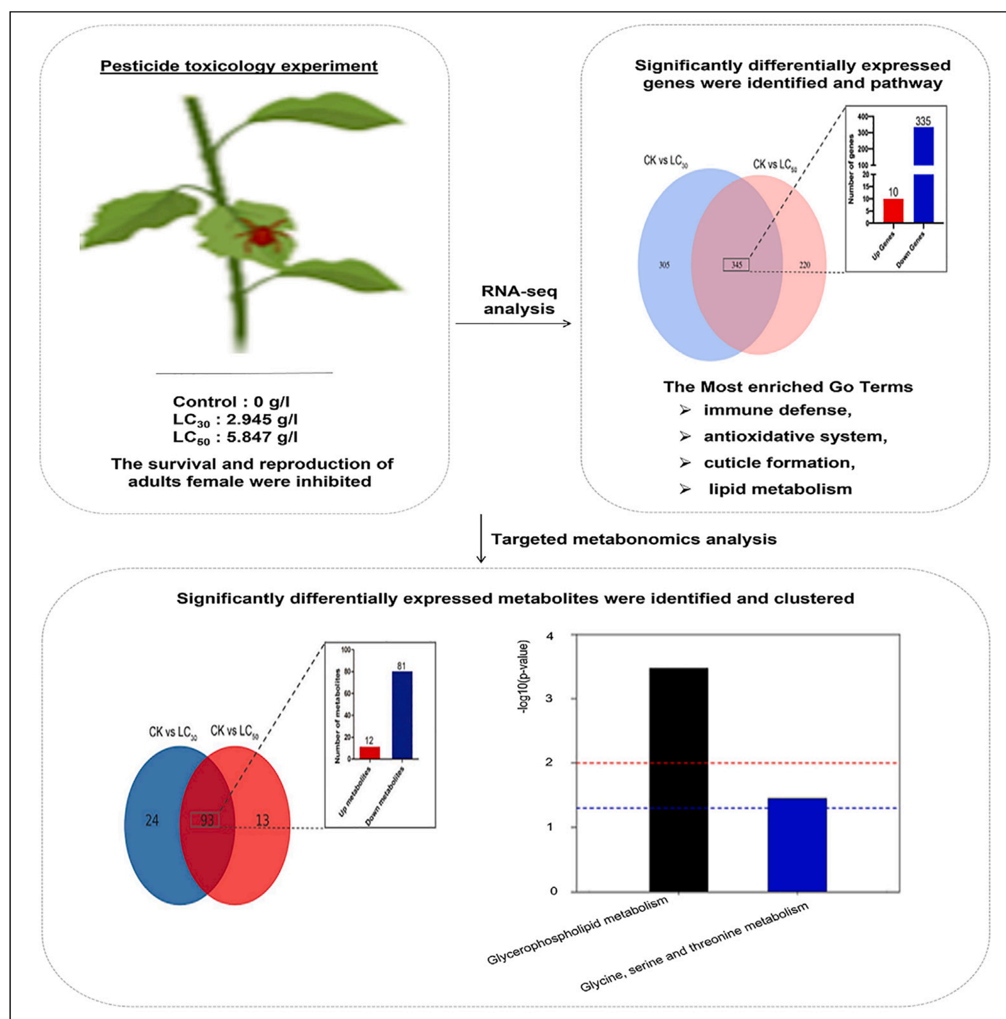

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Highlights

Spirobudiclofen has adverse effects on the evolution of *Panonychus citri*

Spirobudiclofen stress significantly aggravated transcriptomal changes

Sublethal concentrations of spirobudiclofen can affect lipid synthesis

Multiomics obtained mechanistic understanding foundation of spirobudiclofen toxicity

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Stress response and tolerance mechanisms of spirobudiclofen exposure based on multiomics in *Panonychus citri* (Acari: Tetranychidae)

Hongyan Wang,[1] Tianrong Xin,[1] Haifeng Wang,[1] Kexin Wen,[1] Yimeng Liu,[1] Jing Wang,[1] Zhiwen Zou,[1] Ling Zhong,[2] and Bin Xia[1,3,*]

SUMMARY

The toxicity of insecticides used in the field decreases gradually to sublethal concentrations over time. Therefore, it is necessary to study sublethal effects of pesticides for controlling population explosion. *Panonychus citri* is a global pest which control is based on insecticides. This study explores the stress responses of spirobudiclofen on the *P. citri*. Spirobudiclofen significantly inhibited survival and reproduction of *P. citri*, and the effects aggravated as concentration increased. The transcriptomes and metabolomes of spirobudiclofen-treated and control were compared to characterize spirobudiclofen molecular mechanism. Transcriptomics indicated stress induced by spirobudiclofen stimulated immune defense, antioxidative system, cuticle formation, and lipid metabolism, as deduced from RNA-seq analysis. Meanwhile, our study found that tolerance metabolism in *P. citri* was regulated by promoting the metabolism of glycerophospholipids, glycine, serine, and threonine. The results of this study can provide a basis for exploring the adaptation strategies of *P. citri* to spirobudiclofen stress.

INTRODUCTION

In agricultural production, pesticides have been widely used for many years to achieve timely and efficient pest control. Chemical pesticides are still important means of agricultural pest control for an extended period. However, excessive application of pesticides results in a series of environmental problems, such as the resistance of pests to pesticides,[1–3] extinction of natural enemies of pests, reduction in biodiversity, damage to other wild animals and plants,[4,5] and degradation of cultivated land and water quality.[6,7] Therefore, the scientific, reasonable, and safe use of pesticides is a significant challenge for agricultural pest control.

As a worldwide pest of citrus, *Panonychus citri* (Acari: Tetranychidae) has been focused on a considerable amount of research. *P. citri* is characterized by a short life cycle, high fecundity, fast reproductive rate, and rapid growth and development. These factors made *P. citri* adapt to pesticide selection pressure rapidly.[8] Hence, it has developed into one of the most severe pests in citrus orchards.[3] Spirobudiclofen, is a tetronic acid derivative acaricide that targets lipid biosynthesis.[9] Due to its direct toxicity to control *P. citri*, it is widely used in the field. The frequent use of insecticides in recent years has caused varying degrees of tolerance in the pest toward different kinds of insecticides.[1] Previous research has shown that the efficacy of pesticides depends on their direct application rate (lethal or sublethal or overdose).[10] Therefore, they may induce sublethal effects on some individuals with the difference in exposure dose between individuals and the passage of time.[11–13] Some examples of sublethal effects include changes in the biological and ecological behavior of pests, changes in fertility, the expansion of resistance, etc.[14,15]

In our study, the response of *P. citri* to spirobudiclofen is conducted by life tables, which are mainly manifested in the growth, reproduction, oviposition, and population change in the individuals. However, previous studies also focused on individual responses to pesticide stress analysis but did not evaluate the tolerance mechanism thoroughly. Omic technologies (such as transcriptomics and metabolomics) investigate biological systems at the molecular level relatively quickly. These methods can help determine the responses of organisms exposed to two concentrations of pesticides and further understand the

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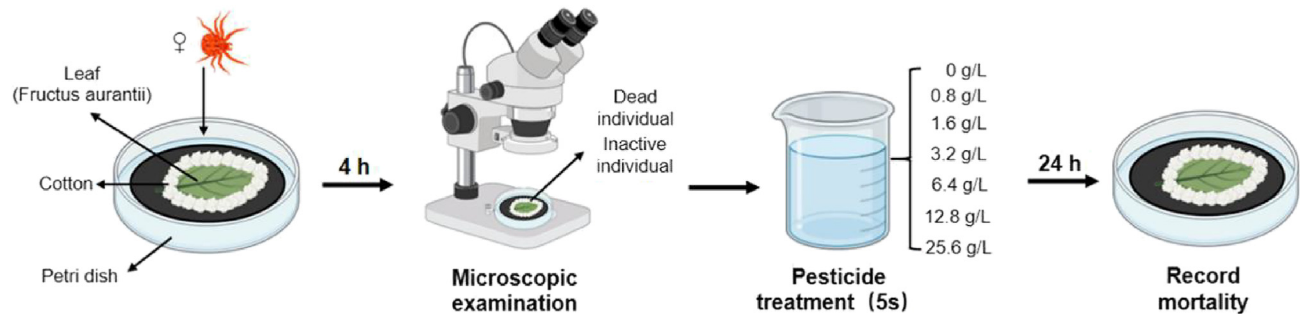

Figure 1. Schematic diagram of the bioassay method of spirobudiclofen treatment on adult females of *Panonychus citri*, in each replicate, 40 individuals are tested, four replicates are tested per concentration, and six concentrations are tested in each assay

potential biomarkers and toxicity mechanism of pesticides as an early prediction tool.[16,17] Meanwhile, transcriptomics and metabolomics investigate different aspects of the system and cell functional space and are highly complementary.[18,19] By analyzing these methods comprehensively, more molecular insights can be gained. At concentrations of environment-related contact, organisms are more sensitive at the molecular level than phenotypically.[17,20,21] The combined analysis of multi-omics technology is expected to reveal the responses of *P. citri* to spirobudiclofen efficiently and accurately. As a result, the results will provide a valuable foundation for applying the insecticide spirobudiclofen rationally.

RESULTS

Phenotypic responses after acute spirobudiclofen exposure

Toxicity of spirobudiclofen was assessed using leaf-dipping method (Figure 1). Concentrations resulting in 30% and 50% mortality were 2.945 g/L and 5.847 g/L (Table 1).

Stress response of *P. citri* to spirobudiclofen on growth and reproduction

In this study, we examined the effects of different concentrations of spirobudiclofen on the development time, adult longevity (the average time from adults until death), and the total life span of *P. citri* (Tables 2 and 3).

The adults female duration and oviposition period of *P. citri* treated with spirobudiclofen were significantly shorter than control. At the same time, the concentration effect was produced with the time increasing. However, All spirobudiclofen-treated groups were prolonged in egg duration. Compared to the control, spirobudiclofen-treated groups were significantly prolonged both pre-ovipositional period and total pre-ovipositional period. In addition, significant reductions in longevity and fecundity were found in all spirobudiclofen-treated groups.

The R_0、rm and λ of adults female were significantly reduced in spirobudiclofen-treated groups compared with control. Moreover, the mean generation time of *P. citri* was no significant difference between LC_{30} and LC_{50}, whereas they were decreased compared with the control.

Transcriptomic changes in *P. citri* after exposure to spirobudiclofen

Similar expression patterns were obtained in the qRT-PCR analysis of the eight selected DEGs. The transcriptomic data were validated by these results (Figure 2. and Table S2). The differences in the transcript profiles of the *P. citri* adult females between control and spirobudiclofen-treated groups were compared at the mRNA level by RNA-seq (Table S1).

Table 1. Toxicity of spirobudiclofen on adult females of *P. citri*

	Concentration g/L (95% CL)				
Acaricide	LC_{30}	LC_{50}	LC-P equation	χ^2	R
Spirobudiclofen	2.945 (2.257–3.610)	5.847 (4.905–6.896)	Y = - 1.031 + 1.547X	21.468	0.935

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Table 2. Stress response of *P. citri* to spirobudiclofen on the life history

Paremeter	Control	LC_{30}	LC_{50}
Egg duration (d)	$(4.80 \pm 0.20)^{b}$	$(5.47 \pm 0.13)^{a}$	$(5.58 \pm 0.06)^{a}$
Larva duration (d)	$(1.48 \pm 0.15)^{a}$	$(1.33 \pm 0.08)^{a}$	$(1.34 \pm 0.04)^{a}$
Nymph duration (d)	$(2.49 \pm 0.15)^{ab}$	$(2.44 \pm 0.11)^{b}$	$(2.88 \pm 0.06)^{a}$
Adults female duration (d)	$(12.93 \pm 0.23)^{a}$	$(7.93 \pm 0.18)^{b}$	$(6.62 \pm 0.06)^{c}$
Longevity (d)	$(23.31 \pm 0.13)^{a}$	$(19.32 \pm 0.38)^{b}$	$(18.65 \pm 0.09)^{b}$
APOP (d)	$(1.61 \pm 0.12)^{b}$	$(2.10 \pm 0.11)^{a}$	$(2.22 \pm 0.04)^{a}$
TPOP (d)	$(10.38 \pm 0.12)^{c}$	$(11.39 \pm 0.21)^{b}$	$(12.03 \pm 0.09)^{a}$
Oviposition period (d)	$(12.50 \pm 0.37)^{a}$	$(7.77 \pm 0.22)^{b}$	$(6.62 \pm 0.06)^{c}$
Fecundity (eggs/female/d)	$(5.35 \pm 0.25)^{a}$	$(3.46 \pm 0.15)^{b}$	$(3.12 \pm 0.03)^{b}$

Mean (+/− SE) of developmental time, stage mortality, longevity, and total preoviposition period (TPOP) of the *P. citri* treated with different concentrations of spirobudiclofen. Data followed by the same lower-case letter in the same row or the same capital letter in the same column were not significantly different based on a paired bootstrap test at the 5% significance level.

Transcriptome sequencing and DEG identification

Pairwise comparisons were performed in spirobudiclofen-treated groups to determine differentially expressed genes. Consequently, 646 (47 up-regulated and 599 down-regulated) DEGs were identified by transcriptome sequencing analysis between LC_{30} group and control. Moreover, 564 (81 up-regulated and 483 down-regulated) DEGs were identified between the control and LC_{50} group (Figure 3). Among these DEGs, 10 up-regulated (ABCG23, histone H3, etc.) and 335 down-regulated genes (such as chitinase, vitellogenin, elongation of very long chain fatty acids protein (ELOVL), fatty acid-binding protein, ceramide synthase, etc.) were commonly expressed in all spirobudiclofen- treated groups (Figure 3). Thus, *P. citri* seems to have evolved different adaptive mechanisms to adapt with spirobudiclofen stress.

Effects of GO and KEGG pathways on DEG signaling

Two comparisons (CK vs. LC_{30}, CK vs. LC_{50}) of DEGs were conducted with structured descriptions of their biological functions and systems. The most representative 20 GO terms are related to cellular component, molecular function, and biological process (Figure 4). The DEGs in the LC_{30} group were mainly involved in catalytic activity, chitin synthesis, blood circulation, sugar synthesis, and protein transport and significantly enriched in cell morphology, ganglioside catabolism, extracellular region, and ribosome (Figure 4). The DEGs in the LC_{50} group were mainly related to catalytic activity, low-density lipoprotein receptor, nuclear receptor, oxidase activity, the stratum corneum structural component, carotenoid synthesis, oxidative stress, sugar synthesis, oviposition process, peptide metabolism process and translation, extracellular region, and membrane component. These results showed that spirobudiclofen effectively stimulated GO terms and defense mechanisms such as oxidative stress, cuticle formation, and translation in *P. citri* to accelerate tolerance to spirobudiclofen.

KEGG pathway analysis further screened the DEGs expressed during spirobudiclofen stress. The DEGs in the LC_{30} group were significantly enriched in lysosomes, detoxifying enzymes, amino acids, and lipid metabolism. The DEGs in the LC_{50} group were significantly enriched in some amino acid and lipid metabolism

Table 3. Stress response of spirobudiclofen on the population parameters of *P. citri* offspring

Population parameters	Control	LC_{30}	LC_{50}
Net reproductive rate (R_0) (d^{-1})	$(56.80 \pm 2.42)^{a}$	$(21.29 \pm 0.69)^{b}$	$(8.91 \pm 0.05)^{c}$
Mean generation time (T) (d)	$(15.30 \pm 0.21)^{a}$	$(13.73 \pm 0.09)^{b}$	$(13.71 \pm 0.05)^{b}$
Intrinsic rate of increase (rm) (d^{-1})	$(0.26 \pm 0.04)^{a}$	$(0.22 \pm 0.02)^{b}$	$(0.16 \pm 0.01)^{c}$
Finite rate of increase (λ) (d^{-1})	$(1.30 \pm 0.05)^{a}$	$(1.25 \pm 0.03)^{b}$	$(1.17 \pm 0.01)^{c}$

The standard errors were calculated using the bootstrap programs with 100,000. Means in the same group with superscripts followed by different letters are significantly different between spirobudiclofen-treated and control using the paired bootstrap method at a 5% significance level.

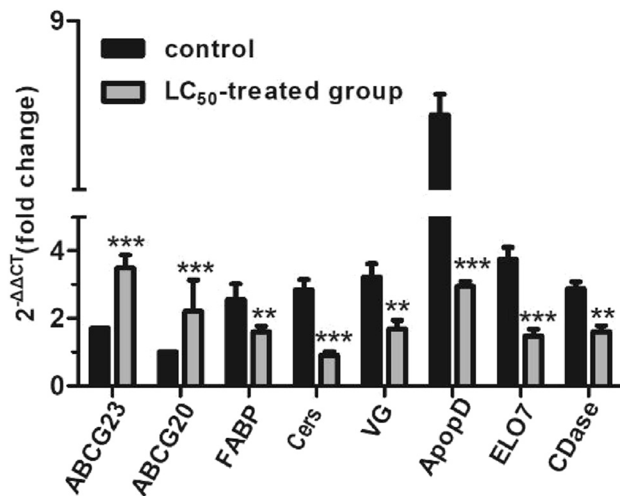

Figure 2. By using the $2^{-\Delta\Delta CT}$ method, gene expression (Mean $\pm$ SD) was quantified as a relative fold change
It was verified through RT-qPCR in the control and LC_{50}-treated groups of *Panonychus citri*. Asterisks represent the significant difference in DEG between the LC_{50}-treatment group and the control group (** p value <0.01 and *** p value <0.001, Student's t-test).

(Figure 4). Thus, KEGG results showed that *P. citri* reduced the toxicity of spirodiclofen by regulating the metabolism of amino acids and lipids and enhancing signal transduction.

Besides, the detailed functions of the shared DEGs associated with spirobudiclofen tolerance from the two comparison groups (CK vs. LC_{30}, CK vs. LC_{50}) were further classified by the KEGG pathways analysis. The mainly enriched DEGs involved in lipid metabolism were apolipoprotein D, elovl protein, etc (Figure 4). These results were consistent with the change of the stratum corneum in GO enrichment analysis.

Metabolomic changes in *P. citri* after exposure to acute spirobudiclofen

The differences in the lipid metabolite profiles between spirobudiclofen-treated groups and control were investigated by LC-MS-based targeted metabolomics.

Differential metabolite analysis

A pairwise comparison identified the differentially changed lipid metabolites in the spirobudiclofen-treated groups. Compared with the control, 117 and 106 lipid metabolites were significantly altered in the spirobudiclofen-treated groups (Figure 5). Among these metabolites, 12 up-regulated and 81 down-regulated lipid metabolites were expressed commonly among all groups (Figure 5). The metabolic disturbances observed were generally consistent with the transcriptome results in *P. citri* exposed to the

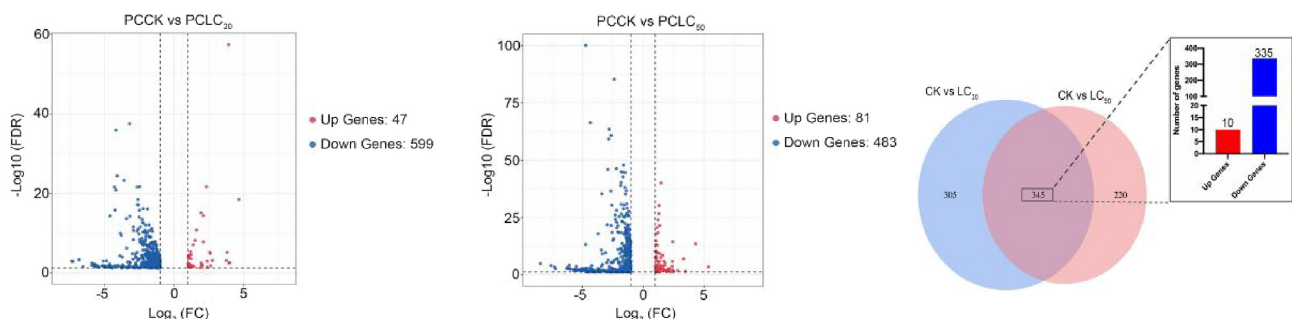

Figure 3. Overview of sugarcane transcriptome responses to *P. citri* after exposure to spirobudiclofen, Venn diagram showing transcripts upregulated or downregulated by *P. citri* at both sampling concentrations

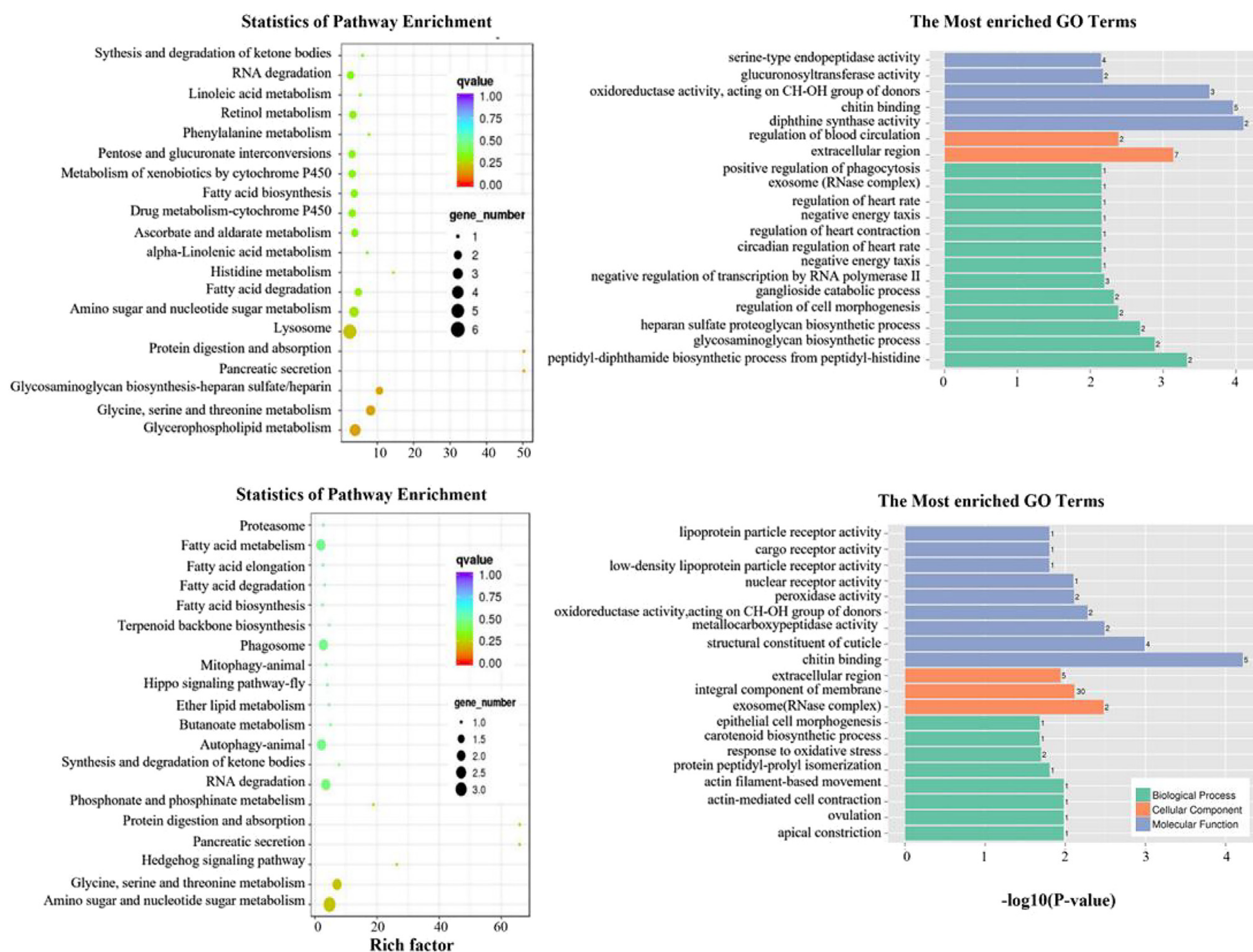

Figure 4. Bubble disgrams represent the top 20 pathways with significant DEG enrichment on KEGG
Histogram represents the enrichment of GO terms in significantly differential genes after spirobudiclofen exposure.

same levels of spirobudiclofen. The heatmap also revealed the presence of up-regulated (such as triacylglycerols and free fatty acids) and down-regulated lipid metabolites (such as phosphatidylcholine and phosphatidylethanolamine) in the spirobudiclofen-treated groups (Figure 5).

Multivariate statistical and pathway analysis

The PCA score curve showed significant difference between spirobudiclofen - treated groups and control (Figure 6). The OPLS-DA indicated proper separation between spirobudiclofen - treated groups and control (Figure 6). The enrichment analysis of KEGG pathways showed that spirobudiclofen - treated groups had obvious metabolism of glyceride, glycine, serine, threonine and other substances. These results showed that *P. citri* regulated glycerophospholipid and glycine, serine, and threonine metabolism to enhance the tolerance to spirobudiclofen.

DISCUSSION

The toxicity of pesticides on agricultural pest communities is a crucial global concern. Previous research has shown that pesticides have adverse effects on the development, growth, fecundity, and egg-hatching rate of insects.[22,23] Conversely, other study showed that the use of pesticides will have some positive effects (stimulatory effect) on the growth and reproduction of insects, which will allow the re-emergence of pests in the field.[24] For instance, sublethal concentrations of sulfoxaflor stimulate the fertility of *Sogatella furcifera*. Moreover, other research showed that APOP, TPOP and mean generation time of *Nilaparvata lugens* were significantly increased under the LC_{30} of triflumezopyrim.[25] Our study results demonstrated a

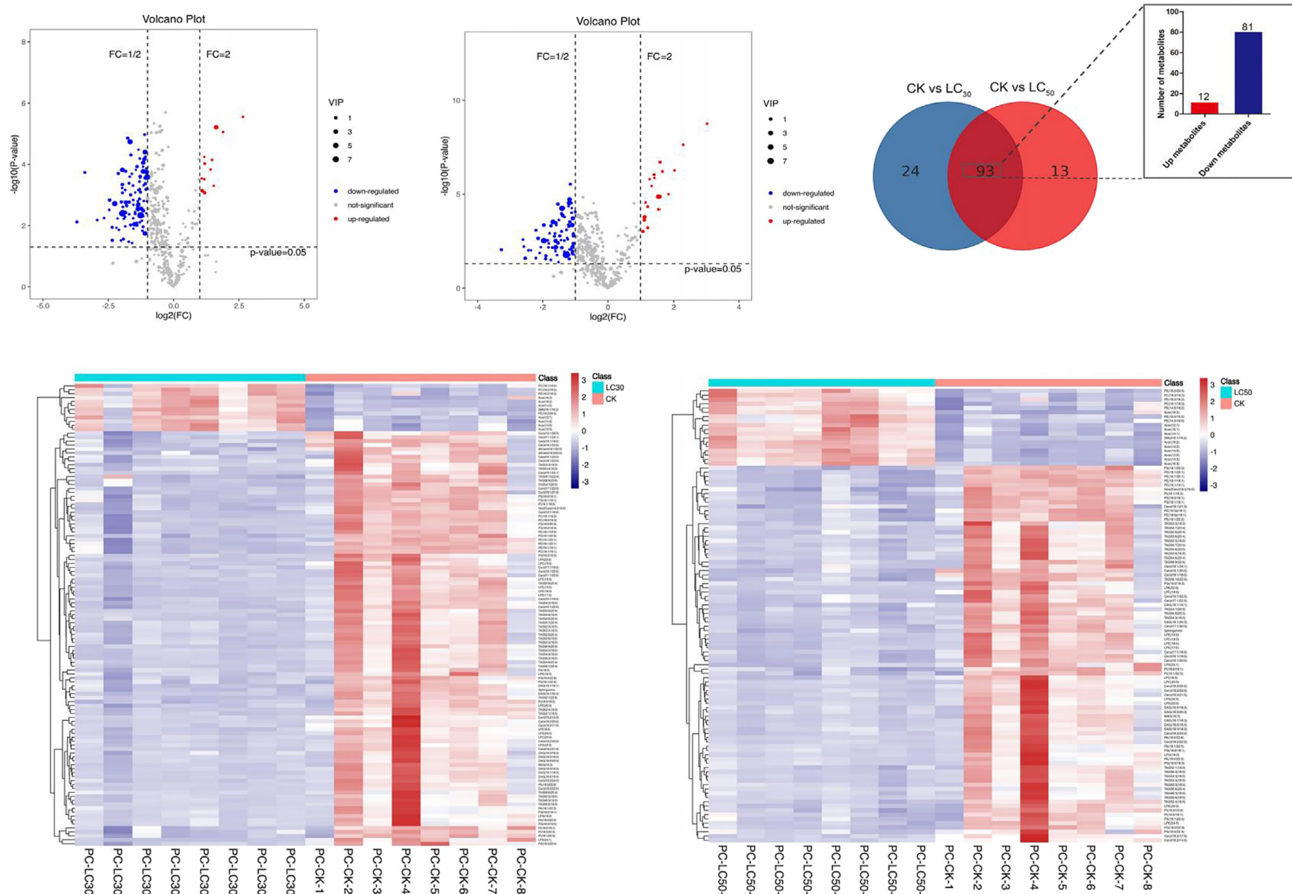

Figure 5. Overview of sugarcane metabolome responses to *P. citri* after exposure to two concentrations of spirobudiclofen

Venn diagram showing the number of lipid metabolites up-regulated or down-regulated by *P. citri* after exposure to both sampling concentrations. Heatmap of distinct lipid metabolites along the most affected pathways after spirobudiclofen exposure.

significant decrease in the population parameters (including r, λ, and R_0) and population expansion of *P. citri* following spirobudiclofen treatment. In addition, the growth period of the experimental group did not change significantly. However, the mean generation time, APOP, TPOP, egg-hatching ability, and other indicators all decreased significantly, which were different from the reported in previous reports. These changes illustrated that different species of pests respond differently to different insecticide stress.

The current study has widely reported the adverse effects of different pesticides on target pests (including *P. citri*). Recently, the treatment of *Plutella xylostella* with five pesticides (producing low lethal or sublethal concentrations) enhanced the tolerance of *P. xylostella*.[26] Similar induced resistance was also observed in body lice[27] and *Drosophila melanogaster*[28] after short-term exposure to sublethal concentrations of ivermectin. However, the molecular mechanism of the possible tolerance of *P. citri* to spirobudiclofen remains largely unknown. The present study constitutes the first analysis of the tolerance responses of *P. citri* to spirobudiclofen at the transcriptomic and metabolomic levels. Two concentrations of spirobudiclofen lead to substantial differential changes in the gene expression and metabolites of *P. citri*. Thus, the organism has developed several strategies to defend against spirobudiclofen stress. Concentration effects of spirobudiclofen were observed on the gene expression profiles of *P. citri* with the exposure concentrations increasing, which indicated toxic stress augmented. Similar effects also occurred on hexapods.[18,29,30]

Exposure concentrations have a strong influence on the toxicity of pesticide. The doses of pesticide absorbed and accumulated by organisms differ over a specific period. Hence, the amounts affect the

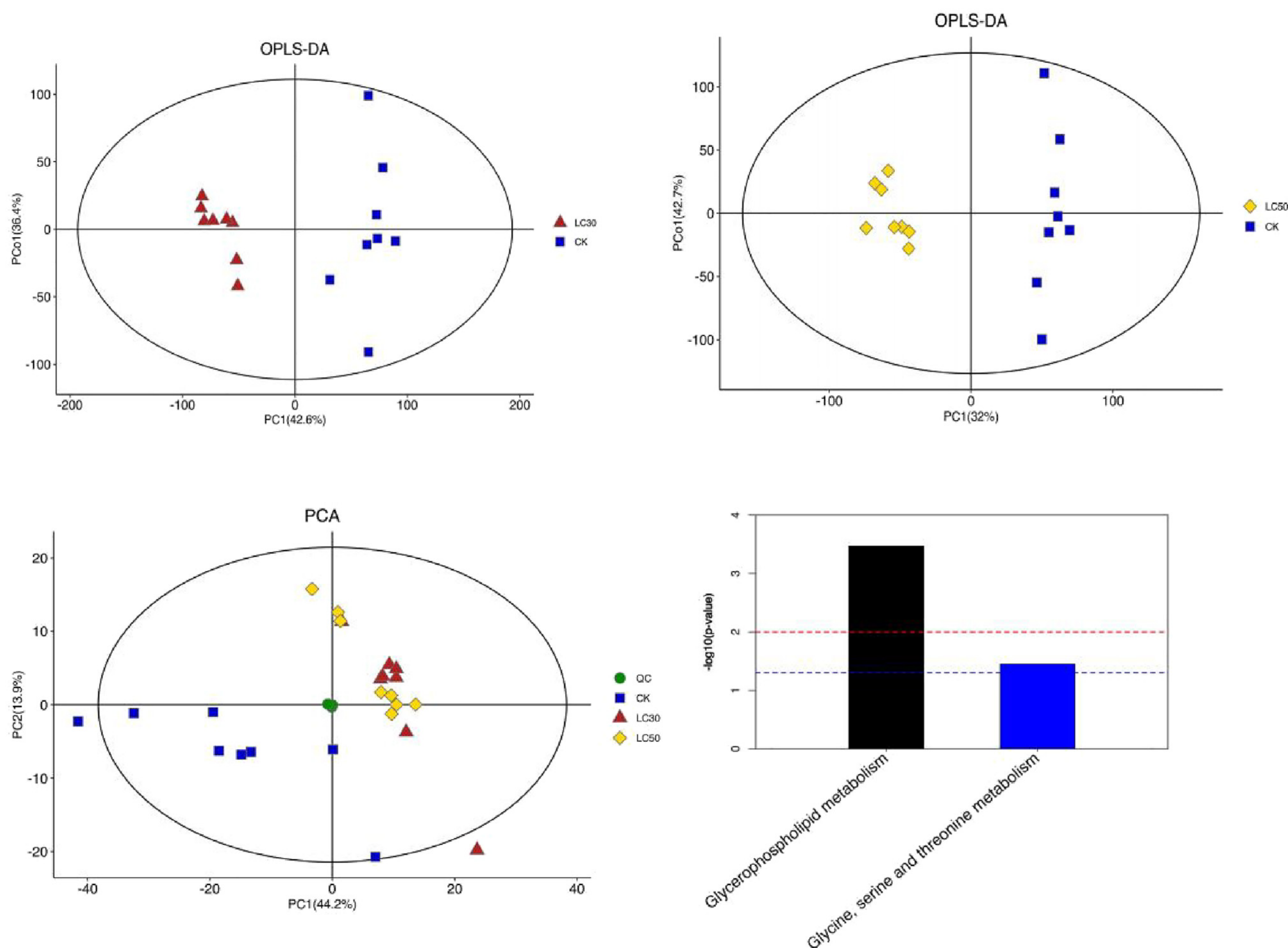

Figure 6. OPLS-DA score plots of *P. citri* samples (n = 8) from the control and spirobudiclofen-treated groups
Principal component analysis (PCA) of samples (n = 8) between control and spirobudiclofen-treated groups. Pathway annotation histogram illustrating the top 20 DEM pathways of significant enrichment.

exposure differently. They can help recombine the expression of genes to adapt to the pesticide stress.[31–33] The transcriptomic response of the two-treatment groups in the current study was distinct. However, many enriched top GO terms were shared among these groups. These common terms mainly included a structural constituent of the cuticle, chitin binding, lipoprotein particle (receptor) for cellular activity, and biological processes such as polysaccharide and amino acid synthesis (Figure 3). Our study identified significant changes in the expression of a series of genes (FABP, ELO7, APOPD, CerS, and CDase) associated with lipid synthesis and metabolism after spirobudiclofen exposure. Some studies suggest a link between reduced fat-related genes and resistance to pesticides in arthropods.[34,35] The cuticle provides close protection and strong support to the insect body to prevent infection by bacteria, parasitism, or predation by natural enemies.[36,37] At the same time, it resists the invasion of pesticides and other foreign substances, enabling the insect to adapt to the environment greatly. As a component of the stratum corneum of arthropods, lipid composition, and distribution characteristics are related to epidermis formation. Therefore, we speculate that changes in these genes are main factors for the tolerance of *P. citri* to spirobudiclofen.

Metabolites are the final downstream products of gene expression. Epigenetic regulation and post-translational modification can affect gene expression.[33] Targeted metabolomics data displayed significant changes in most lipid metabolites (glycerolipids, fatty acids, and fatty acyls) in *P. citri* after spirobudiclofen exposure (Figure 4). Significant differences were also observed in lipid metabolism-related genes after spirobudiclofen exposure (Figure 3, Tables S1 and S2). The down-regulation of specific genes may

compensate for the accumulation of lipid metabolites. Metabolites are the final products of gene transcription and regulate gene transcription.[38] Thus, exposure to spirodiclofen leads to abnormal lipid metabolism. In addition, some differential metabolites (such as sphingolipids and glycerophospholipids) were identified in our study. Previous study indicated that sphingolipids and glycerophospholipids will be involved in a series of biological functions, such as neurotransmission and immunity.[39] Glycerophospholipids are the main components of cell membranes, and their reduction can compromise membrane integrity.[38] Besides, a significant increase was observed in the content of amino acids like serine, threonine, and glycine. These amino acids are the basic units of proteins and are closely related to protein metabolism, these results also support the above transcriptomic findings (Figure 5).[40,41] Indeed, organisms respond to toxic stress by changing the levels of amino acids. Similar results have been reported in many previous studies. A recent study reported increased amino acid content in invertebrates due to pollutant stress.[18,40–43] Therefore, we speculated this factor could accelerate the development of tolerance to spirobudiclofen in *P. citri*.

Limitations of the study

Although the study results can provide resources for exploring the adaptation strategies of *P. citri* to spirobudiclofen, the functions of DEGs have not been analyzed. In our further study,we should focus on the functional analysis of the important DEGs or metabolites.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107111.

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AUTHOR CONTRIBUTIONS

H.W.: Writing: data analysis, completion of the experiments. T.X.: Experimental design, financial support. H.W., K.W., and Y.L.: Investigation. J.W.: mapping. Z.Z. and L.Z.: Funding acquisition. B.X.: Funding acquisition, experimental design.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Panonychus citri populations	the citrus orchard of Nanchang University in Nanchang City, Jiangxi Province (China)	N/A
Chemicals, peptides, and recombinant proteins		
spirobudiclofen 24% SC	Zhejiang Yulong Biotechnology Co., Ltd (Zhejiang, China)	IUPAC Chemical Name: Butyl 3 - (2,4-dichlorophenyl)- 2 - oxo- 1- oxaspiro [4,5] – dec - 3-en – 4 - yl carbonate
Critical commercial assays		
RNAiso Plus	Takakura Matsumoto, Japan	CAT# D9108A
PrimeScriptTM RT Master Mix Reagent Kit	Takara Bio Inc., Kusatsu, Japan	CAT# RR047A
Deposited data		
Raw and analyzed data	NCBI database	PRJNA976604
Oligonucleotides		
Primer sequences for the genes used for qRT-PCR, see Table S2	Table S2	N/A
Software and algorithms		
Oligo	http://www.downza.cn/soft/278916.html	v0.7.0
SPSS	https://spssau.com/indexs.html	v0.24.0
Graphpad prism	San Diego, CA, United States	v0.8.0

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfifilled by the lead contact, xiabin9@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Metatranscriptome data has been deposited at NCBI and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- All relevant data supporting the fifindings of this study are available from the lead contact upon request.
- The published article and supplemental information include all data generated and analyzed during this study. This paper does not report original code.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

P.citri population was collected from citrus orchards of Nanchang in Jiangxi province. Leaves fed with mites were sampled randomly from citrus trees and collected mites were reared in an artificial climate incubator at 28 ± 1°C temperature，60% ±10% (RH) relative humidity with a 16: 8 h (L:D) photoperiod.

METHOD DETAILS

Exposure experiment and sampling

The ecotoxicological effects of chemical compounds are currently evaluated by toxicity tests that are performed on organisms.[44] The sublethal concentrations of spirobudiclofen on *P.citri* were determined by leaf impregnation with mites.[13,45] Based on the pre-test results, the insecticide was diluted to 7 concentrations with triton X-100 (such as Figure 1). The exposed device is shown in Figure 1. Adult individuals(n=60) were picked up and transferred to the leaf disc. After 4 hours, the dead and inactive individuals were picked out under the microscope. (The number of remaining individuals was 45 in each group, 3 biological repetitions in each group) which were immersed in the configured pesticide solution of different concentrations for 5s. Taking them out, the excess solution was sucked with absorbent paper and placed in the artificial incubator for 24h. In addition, triton X-100 was treated as a positive control, and the mortality in the control group is less than 10% as the effective test. After 24h treatment, the number of deaths of *P. citri* was counted. The obtained data were processed on Graphpad and SPSS to obtain the virulence regression linear equation, the value of median lethal concentration LC_{50} and sublethal concentration LC_{30}.

Similarly, the adults female of *P.citri* were treated with the determined sublethal concentration of spirobudiclofen (n=500 per group). After 24 hours, Samples were collected for transcriptomics and metabolomics.

Effect of sublethal concentrations on biological parameters of offspring from treated *P.citri* females

Adults female（n=90）of the same age were placed on the discs after treatment of leaf discs with two sublethal concentrations and triton X-100 by the leaf dip method. After 24 h, surviving females were transferred separately to untreated leaf discs (2 cm in diameter). Subsequently, after 24h of egg-laying, eggs were saved. Fifty eggs were used for the next experiment at each sublethal concentration. All stored eggs (n=150) were checked daily, and developmental times and survival rates were recorded. Newly emerged females were coupled with a male for mating. The population parameters were calculated for each individual, such as pre-adult duration and survival rate of each stage, adult lifespan, and daily fecundity.

Transcriptomic analysis by RNA-sequencing

The female adults *P.citri* in the control group, sublethal concentration (LC_{30} and LC_{50}) of spirobudiclofen-treatment groups were used for transcriptome sequencing (There were approximately individuals(n=500) prepared in three biological replicates per condition.). Transcriptome sequencing was performed by Biomarker Technologies Co., Ltd. (Beijing, China). According to the instructions, the total RNA was isolated with Trizol reagent (Takakura Matsumoto, Japan). Then, the sequencing was performed using Illumina HisSeq platform. After sequencing, clean reads were obtained by filtering raw reads, examining sequencing error rate and GC content distribution. Then, the mapping between high-quality clean readings and reference genomes using Hisat2 v2.0.5 software (Yu and Liu, 2020), and prediction of some new genes (assembly and functional annotation) were conducted.

Read-count data were analyzed using the DEseq2 package, and the corrected p value (Benjamini and Hochberg False discovery rate) < 0.05, $|Log2FoldChange| > 1$ was used to define differentially expressed genes (DEGs).[13,46] The DEGs were used for functional enrichment analysis, such as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology (GO) terms analysis. The RNA-sequencing procedures were conducted by Novogene. RNA-sequencing raw data are deposited in the NCBI databases.

Quantitative real-time polymerase chain reaction (qRT-PCR) validation

To verify the RNA-Seq results, 8 DEGs were randomly selected to perform quantitative real-time PCR (qRT-PCR). The extracted RNAs (1000 ng) (n = 3 replicates) were reverse-transcribed into cDNA. In brief, according to the manufacturer's instructions, the isolation of total RNA from *P. crtri* and the synthesis of first strand cDNA was done using the Trizol reagent (Takara, Kusatsu,Japan) and the PrimeScript™ RT Master Mix Reagent Kit (Takara, Kusatsu, Japan), respectively. qRT-PCR reactions were performed using the Applied Biosystems StepOnePlus Real-Time PCR System (Foster City, CA, USA). The PCR program setup was divided into two stages: 95°C for 30 s; 40 cycles of 95°C for 5 s and then 60°C for 30 s. The relative expression analysis of target genes was calculated by the $2^{-\Delta\Delta CT}$ method. The qRT-PCR primers (Table S2) were designed using oligo7.0 software, and GADPH gene was considered as the reference gene.

Metabolomics analysis by UHPLC-MS

P.citri from the control, LC_{30} and LC_{50} exposure groups were determined by LC−MS-based targeted metabolomics analysis. The procedures of it were based on Qtrap 5500 liquid chromatography triple quadrupole mass spectrometer (AB Sciex, USA), more than 1000 kinds of lipids were analyzed by the schedule MRM model.

Statistical and pathway analyses of the metabolomics data were performed for the control group, LC_{30} and LC_{50} groups using MRMPROBS software. The data were corrected for the batch effect using the built-in module for MRMPROBS. Then, the student's test and fold change analysis were used to complete to screen for metabolites differing in concentrations between spirobudiclofen groups and the control group. Unsupervised principal component analysis (PCA) and supervised partial least squares-discriminant analysis (OPLS-DA) was performed to get a global overview of the metabolic changes. Metabolites with variable importance in the projection (VIP) greater than 1 were regarded as significant and responsible for group separation.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

To assess the treatment effects between the control and each sublethal concentration of spirobudiclofen groups, statistical significances were determined by ANOVA using the SPSS 24.0, Multiple comparisons were conducted using Duncan's test, and $p<0.05$ was considered as significant. The figures were generated by Graphpad.

Gene expression levels were normalized by Fragments Per Kilo-base of transcript sequence per Million base pairs (FPKM). The DEGs were identified using the criterion of $p < 0.05$ and at least 2-fold expression difference. Hierarchical cluster heatmap of FPKM of DEGs was plotted to globally present the change trend of DEGs. GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases were used for the enrichment analysis of function and pathway of DEGs basing the hypergeometric distribution in cluster Profiler R package(Tang et al., 2018b; Zhu et al., 2018), and the threshold of significant enrichment was $p < 0.05$.

PCA was conducted to compare the difference of metabolites level between control and each sublethal concentrations of spirobudiclofen groups, and degree of variability among replicated samples within every group. Partial least squares discrimination analysis (OPLS-DA) was conducted at metaX to maximize the separation between different groups and to identify the responsible metabolites causing the separation. Variable importance in projection (VIP) value of first principal component which represent the contribution of metabolite to classification was calculated. The p-value was calculated using univariate analysis (t-test). The metabolites with VIP > 1 and $p<0.05$ and fold change ≥ 2 or ≤ 0.5 were identified as differential changed metabolites (DCMs). Heatmap of normalized expression values of DCMs was plotted using Pheatmap package in R language(Li et al., 2017). To present change trend of DCMs, KEGG databases were used for the enrichment analysis of pathway of DCMs(He et al., 2020).