Supporting information

Nano-chitosan boosts sesame plant anti-herbivore defenses and seed nutritional metabolites

Zhenggao Xiao [a], Haihua Ji [a], Le Yue [a], Feiran Chen[a], Xiu-Ping Yan [b], Zhenyu Wang [a, c, *], Sergio Rasmann [d]

[a] Institute of Environmental Processes and Pollution Control, School of Environment and Ecology, Jiangnan University, Wuxi 214122, China

[b] Institute of Analytical Food Safety, School of Food Science and Technology, Jiangnan University, Wuxi 214122, China

[c] Jiangsu Collaborative Innovation Center of Technology and Material of Water Treatment, Suzhou University of Science and Technology, Suzhou 215009, China

[d] Institute of Biology, University of Neuchatel, Neuchatel 2000, Switzerland

* Corresponding author:
E-mail address: wang0628@jiangnan.edu.cn (Z. Wang)

Number of pages: 20
Number of texts: 5
Number of tables: 3
Number of figures: 9

Supplementary method

Text S1. Measurement of leaf oxidative stress and antioxidant enzyme activity

Leaf H_2O_2 content was determined as described by previous study,[1] with slight modifications. Briefly, 100 mg of fresh leaf was homogenized in an ice bath with 1.5 mL 0.1% (w/v) trichloroacetic acid, and then centrifuged at 12000 rpm at 4 °C for 15 min and the supernatant collected. Afterward, 0.5 mL of the supernatant was added to 0.5 mL of potassium phosphate buffer (10 mM, pH 7), and the absorbance of the supernatant was measured on a microplate reader (Varioskan Lux, Thermo, USA) at 390 nm.

Leaf superoxide anion ($O_2^{\bullet-}$) content was analyzed according to the previously reported method with a slight modification.[2] Briefly, fresh leaf (100 mg) was ground in 1.5 mL cold potassium phosphate buffer (pH 7.8) and the mixtures were centrifugation at 12000 rpm at 4 °C for 10 min. The supernatant (1 mL) was added to phosphate buffer (0.9 mL) and hydroxylamine hydrochloride (0.1 mL) and the mixtures reacted for 30 min at 25 °C, then 17 mmol L^{-1} sulfanilic acid (1 mL) and 7 mmol L^{-1} α-naphthylamine (1 mL) were added the reaction mixture and the solution was continues to react at 25 °C for 20min. The absorbance of the supernatant was measured on a microplate reader (Varioskan Lux, Thermo, USA) at 530 nm.

Leaf lipid peroxidation was estimated in terms of malondialdehyde (MDA) concentrations as described by previous studies,[3, 4] with a slight modification. Fresh leaves (100 mg) were homogenized in 1.5 mL of chilled 80% ethanol, and centrifuged at 12000 rpm at 4 °C for 20 min. The extract (1 mL) was then mixed with 4 mL aqueous solution of 20% trichloroacetic acid and the aqueous solution of 0.67% 2-thiobarbituric acid, incubated at 90 °C for 30 min. The reaction stopped by putting the tubes in an ice bath and then centrifuged at 12000 rpm for 30 min. The absorbance of the supernatant was recorded on a microplate reader (Varioskan Lux, Thermo, USA) at 440, 532 and 600 nm.

The activity of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in sesame leaves were determined according to a previous study with minor modification.[5] Briefly, Fresh leaves (100 mg) were ground in 50 mM pre-cooled PBS

(pH 7.8) containing 1% polyvinylpyrrolidone. The homogeneous was centrifuged (12000 rpm, 4 °C, 20 min), and the supernatants were enzyme extracts. SOD activity was determined by photochemical NBT method in 3 mL reaction mixture. The reaction mixture, including 50 mM PBS (pH 7.8), 75 μM NBT, 10 μM EDTA-Na_2, 13.05 mM methionine, 2 μM riboflavin, and 100 μL enzyme extracts, was shaken and exposed to light for 20 min. Two hundred μL of the reaction mixture was transferred to a multifunctional microplate and measured on a microplate reader (Varioskan Lux, Thermo, USA) at 560 nm. One unit of SOD is defined as being present in the extract, which can inhibit by 50% of NBT photoreduction. POD activity was measured with guaiacol as the substrate. The absorbance of 200 μL reaction mixture (20 mM potassium phosphate buffer (pH 6.0), 0.56% guaiacol, 0.01% H_2O_2, and 6.67 μL enzyme extracts) was recorded for 3 min at 470 nm. The increase of 0.01 of OD value per min was defined as one unit of POD. For CAT activity, the absorbance of 200 μL of the reaction mixture (15 mM phosphate buffer (pH 7.0), 0.05% H_2O_2, and 6.67 μL enzyme extracts) was recorded for 3 min at 240 nm. The reduction of 0.01 of OD value per min was used as one unit of CAT.

Text S2. Measurement of calcium ion fluxes by Non-invasive Micro-test Technique system (NMT)

The net fluxes of Ca^{2+} in leaf mesophyll cells was measured using Non-invasive Micro-test Technique system (NMT) (NMT100S-SIM-XY, Xuyue, Beijing, China) as previous studies described.[6, 7] Briefly, potassium chloride (0.1 M) was used for silver chloride, and then pre-pulled and moisturized microelectrodes (4-5 μm, XY-DJ-01) were filled with the electrolyte solution (Ca^{2+}: 100 mM $CaCl_{2)}$ to a length of about 1.0 cm followed by filling of selective liquid ion-exchange cocktails (LIXs, Ca^{2+}: XY-SJ-Ca, 40-50 μm, Younger, USA). The microelectrodes were connected to the NMT system with a silver chloride wire and then calibrated in solutions with 0.1 and 0.05 mM $CaCl_2$ for Ca^{2+} flux (Nernstian slope 28 ± 5 mV per decade). All measurements were recorded at least 75 times. Each treatment was measured using six biological replicates. Ca^{2+} fluxes were measured away from the cell surface about 1-2 μm, and

the data were analyzed using imFluxes V2.2 software.

Text S3. Determination of leaf phytohormones by HPLC-MS/MS

The content of phytohormones (JA, SA, and ABA) in sesame leaves were determined according to a previous method,[7] with a slight modification. Briefly, 100 mg sesame leaves were ground in liquid nitrogen and transferred into 1 mL of cold extraction solution (ethyl acetate containing 10 $\mu g \cdot mL^{-1}$ BHT-butylhydroxytoluene). The mixture was vortexed for 15 min, sonicated in an ice bath for 15 min, centrifuged at 12000 rpm for 10 min at 4 °C, and then transferred the supernatant to a new centrifuge tube. The solution was gently blown dry with nitrogen, reconstituted with 200 μL of 70% methanol, vortexed for 5 min, sonicated in an ice bath for 5 min, and centrifuged at 12000 rpm at 4°C for 10 min. Finally, pipette 100 μL of the supernatant into the input sample for LC-MS/MS using an UHPLC system (Vanquish, Thermo Fisher Scientific, Germany). A standard solution of JA, SA, and ABA (Sigma-Aldrich, Switzerland) was measured every ten samples for quality control. The mobile phase A (H_2O, 0.01% formic acid) and B (acetonitrile, 0.01% formic acid formic acid in acetonitrile) were used for the elution gradient: 0 min, 5 % B; 1.5 min, 5 % B; 9 min, 70 % B; 10 min, 70 % B; 10.1 min, 5 % B; 15 min, 5 % B. The flow rate and injection volume were 0.35 mL min^{-1} and 5 μL, respectively. The concentration of phytohormones was conducted by using a calibration equation obtained by linear regression from five calibration points for each analysis. The R^2 value for the JA, SA, and ABA standard was 0.9992, 0.9984, and 0.9991, respectively.

Text S4. Analysis of sesame leaf metabolites by HPLC-MS/MS

The extraction and identification of sesame leaves metabolites were according to a previous study with a slight modification.[8] Briefly, sesame fresh leaves (100 mg) were powdered in liquid nitrogen and transferred to 2 mL tubes, and then added into 1.5 mL of 80% methanol/water with 0.1% formic acid and 0.2 mg/L 2-chloro-L-phenylalanine as internal standard. Subsequently, the mixture was vortexed for 1 min and ultrasonicated for 30 min in ice, and then centrifuged at 4°C, 12000 rpm for 15

min. After that, the supernatant was freeze-dried and re-dissolved in 200 μL solvent of methanol, acetonitrile, and water (4:4:2, v/v/v) followed by centrifuging at 4 ℃, 12000 rpm for 15 min. Eventually the supernatant was detected analyzed by LC-MS/MS (Thermo Fisher, Germering, Germany). The quality control (QC) samples were prepared with a mixture of the same amount of all samples. The mobile phase A (H_2O, 0.1% formic) and B (acetonitrile, 0.1% formic acid) were used for the elution gradient: 0 min, 5 % B; 1.5 min, 5% B; 10 min, 100 % B; 11min 100%B; 11.5 min, 5 % B; 14 min, 5 % B. The injection volume and the flow rate were 5 μL and 0.35 mL min^{-1}, respectively. The parameters of ESI were: sheath gas pressure, 35 arbitrary units; aux gas flow, 15 arbitrary units; sweep gas flow, 0 arbitrary units; capillary temperature, 320 °C; aux gas heater temperature, 350 °C. Spray voltage was 3.5 kV for ESI (pos) and -3.0 kV for ESI (neg).

Text S5 Measurements of the direct entomotoxic effect of potential anti-herbivore compounds suspension

To assess the direct entomotoxic effects of potential antiherbivore compounds, third-instar *S. litura* caterpillars were individually reared on the potential antiherbivore compound solution (5 mg/kg, 10 mg/kg, 50 mg/kg, 100 mg/kg, and 200 mg/kg), or on the artificial diet added with isovolumetric deionized water, as control. Larval weight was recorded before placing the larvae on the diet (Time = 0 h), then in turn at 12 h, 24 h, 48 h, and 72 h after the onset of the experiment.

137 **Table S1. Primer sets used in this study**

Gene	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	References
PAL	GCCTGTTAGAAAACCAGTCCCTA	GCTGGAAAGCGGGAGACC	[9]
C4H	AGTGGGTCACTCATTCTGGTT	GCTTCTTCTGGATCTCCGG	[9]
COMT	TGTGATCCACTGCTACCCGA	TGTCCTGTGCTTTCCCTTAGTC	[9]
4CL	TCTCCCCTTGCATTCTTACTG	TCTTCTTGAAACCAACTCCACTT	[9]
CCR	TCGGGGATGAGAAAGAGACC	CGATGGAATCATAGTCAAGCAA	[9]
HCT	AGGATTATGCTCTGCCCAAA	GTTGCAATCGCAGCTCAGT	[9]
C3H	CGGCCTCCTTTGGGATATGA	GGTCACTACCAACCACTCGG	Gene ID: 105162700
CCoAOMT	CCCCACGATGCCAAGATACT	GTAGCCGATCACACCACCAA	[9]
CAD	TACCGGTATTCCACCCGTTG	GTTATGCTTTTCCTCCCGAGC	Gene ID: 105169503
DIR	CCGGATGATGTTCACTCGCT	GACCCCACCGATGATGTTGT	Gene ID: 105170376
CYP8IQI	CGCGGAAGACGGATGAGT	GATTCCTGCAACGAAAGCAAA	[10]
CYP92B14	TCGATTACTCAGACGTGACATGG	GGCTTTCCTGATAGGGAGTGTAG	[10]
DXS	GCGATCGATTACATACTCTTTGC	GTGGGAATATACGGAGGGGC	Gene ID: 105165070
MCS	GAAGCTCACTCTGATGGTGATGT	GGGCTGACCTTTGGTCTTTG	Gene ID: 105164054

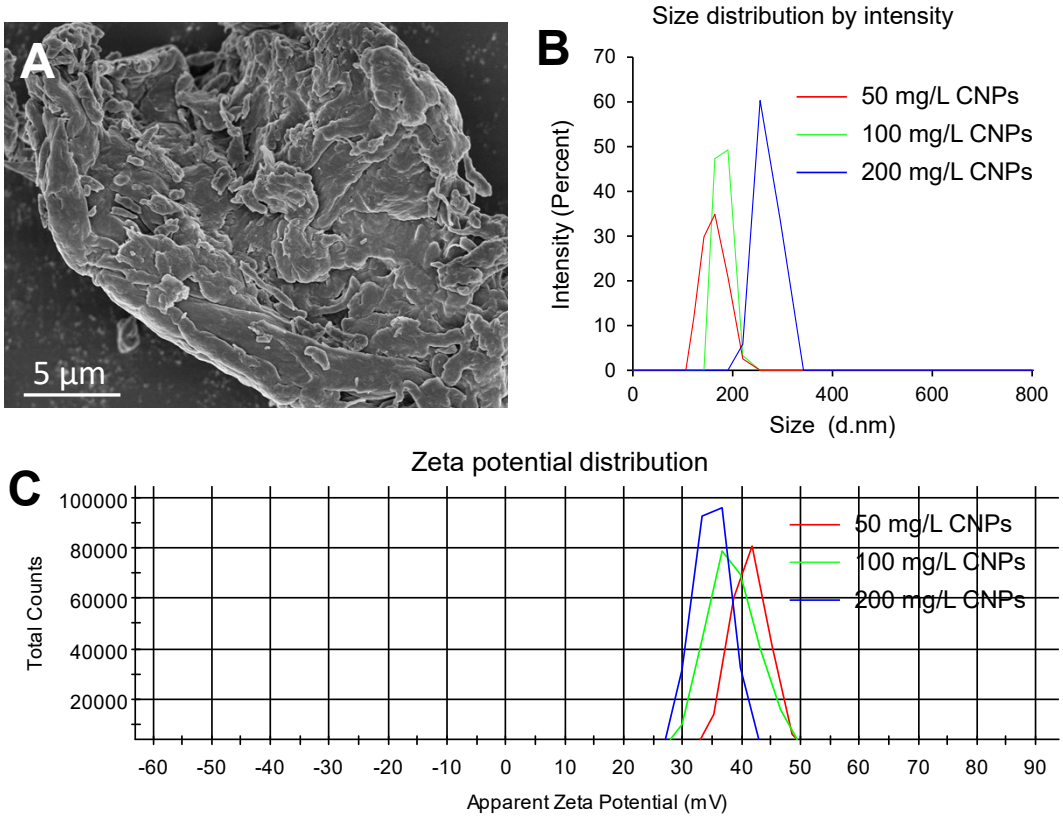
HDR	GCGCAAGTATGGGGTCGAAA	CCACCAACTCACCATGCATCA	Gene ID: 105169604
GPPS	GACCGAATAGCCGTCGTCAT	GGATTGGCGGTCAGAGGAAA	Gene ID: 105175753
CDPK11	GGAATTATGTGCTGGCGGC	CATCAGAAAGATACTGTCCAGGT	Gene ID: 105159534
CDPK29	GTTCTTTCCTTATCCAGGCCGA	GTTGCAATCGCAGCTCAGT	Gene ID: 105165972
Actin	GTTGGTCTCTTTGAGGAC	CAGCTGGATGTCTTTTGG	[11]

Table S2. Average hydrodynamic particle diameters (nm) and zeta potentials (ζ) of CNPs at different dose (50, 100, 200 mg/L). Data was shown as means ± SD (n = 5).

Materials	Dose (mg/L)	Particle mean size (nm)	Zeta potential (mV)
Chitosan nanoparticles (CNPs)	50	155.3 ± 11.0	41.3 ± 3.1
	100	178.9 ± 14.8	38.4 ± 4.2
	200	266.6 ± 22.0	35.0 ± 4.8

Table S3. Two-way ANOVA results table showing the interactive effects of chitosan and herbivore treatment (*S. litura*) on sesame plant growth, oxidative stress, antioxidant enzyme, Ca^{2+} and phytohormones. The test results are shown with the test statistic F-value and significance levels ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ and $^{NS}p > 0.05$)

Plant traits	Data transformation	Chitosan	Herbivore	Chitosan × Herbivore
Shoot biomass	Non	7.4 ***	14.3 ***	0.3 NS
Root biomass	Log	1.6 NS	2.5 NS	0.7 NS
Leaf Chlorophyll	Non	30.2 ***	30.0 ***	1.9 NS
Leaf H_2O_2	Log	2.7 NS	258.1 ***	6.0 **
Leaf $O_2 \cdot^-$	Non	16.1 ***	0.5 NS	3.5 *
Leaf MDA	Non	1.6 NS	18.3 ***	4.6 **
Leaf SOD	Non	122.3 ***	272.0 ***	2.8 NS
Leaf POD	Log	40.0 ***	85.2 ***	3.7 *
Leaf CAT	Log	33.5 ***	494.1 ***	4.5 **
Leaf Ca^{2+}	Log	136.5 ***	112.3 ***	9.8 ***
Leaf JA	Log	34.7 ***	619.2 ***	0.4 NS
Leaf SA	Non	15.6 ***	108.3 ***	3.9 *
Leaf ABA	Non	7.7 **	225.2 ***	6.6 **



146

147 **Fig. S1** SEM analysis of bulk chitosan (A). Particle size distribution (B) by intensity
148 and Zeta potential distribution (C) of CNPs at the dose of 50, 100, 200 mg/L,
149 respectively.

150

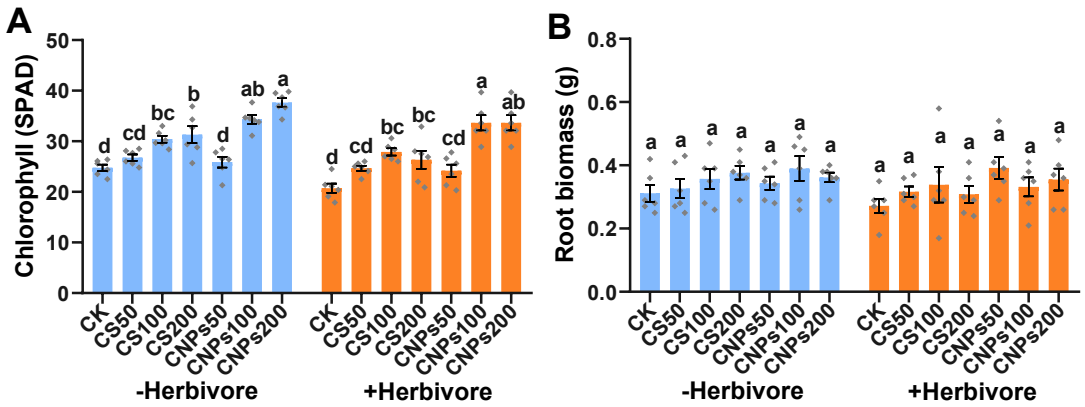
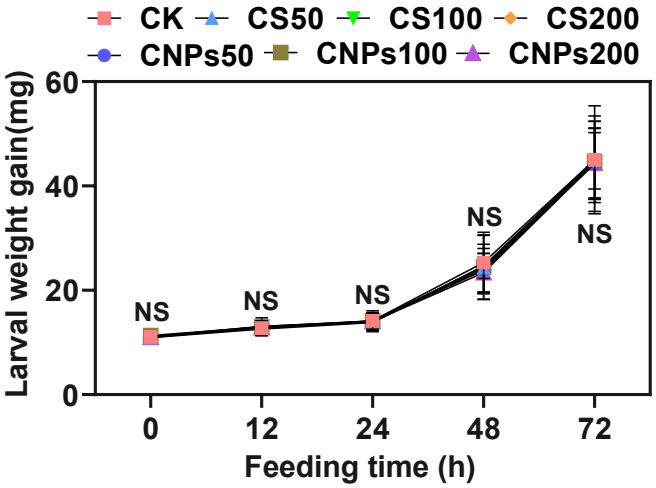


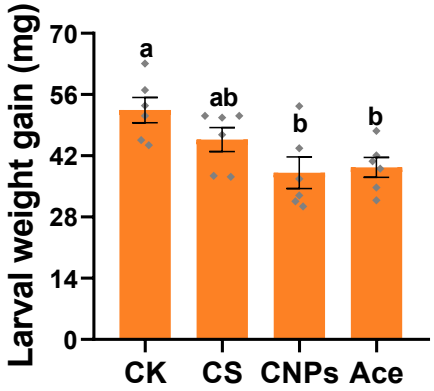
Fig. S2 Effect of CNPs on sesame plant growth functional traits. (A) Sesame leaf chlorophyll and (B) root biomass in the absence (-Herbivore, blue bars) and presence (+Herbivore, orange bars) of *S. litura* caterpillars. Data are means ± SE (n = 6). Significant differences among the treatments are marked with different lowercase letters in the absence and presence of *S. litura*, respectively (Tukey's HSD test, $p < 0.05$).



159

160 **Fig. S3** Toxicity bioassay. Dot plots show the growth of *S. litura* caterpillars feeding
161 on artificial diet added with different concentration (CK = control, 50, 100, and 200
162 mg/kg) of bulk chitosan (CS) and chitosan nanoparticles (CNPs) during a 72 h
163 feeding period. Data are means ± SE (n = 10) (Tukey's HSD test, $p < 0.05$, NS, Non-
164 significance).

165



166

167 **Fig. S4** *S. litura* larval growth on sesame leaves exposed to CNPs. Chitosan
168 treatments include CK, control plants; CS, bulk chitosan; CNPs, chitosan
169 nanoparticles. Data are means ± SE (n = 6). Significant differences between the
170 treatments are marked with different lowercase letters (Tukey's HSD test, $p < 0.05$).

171

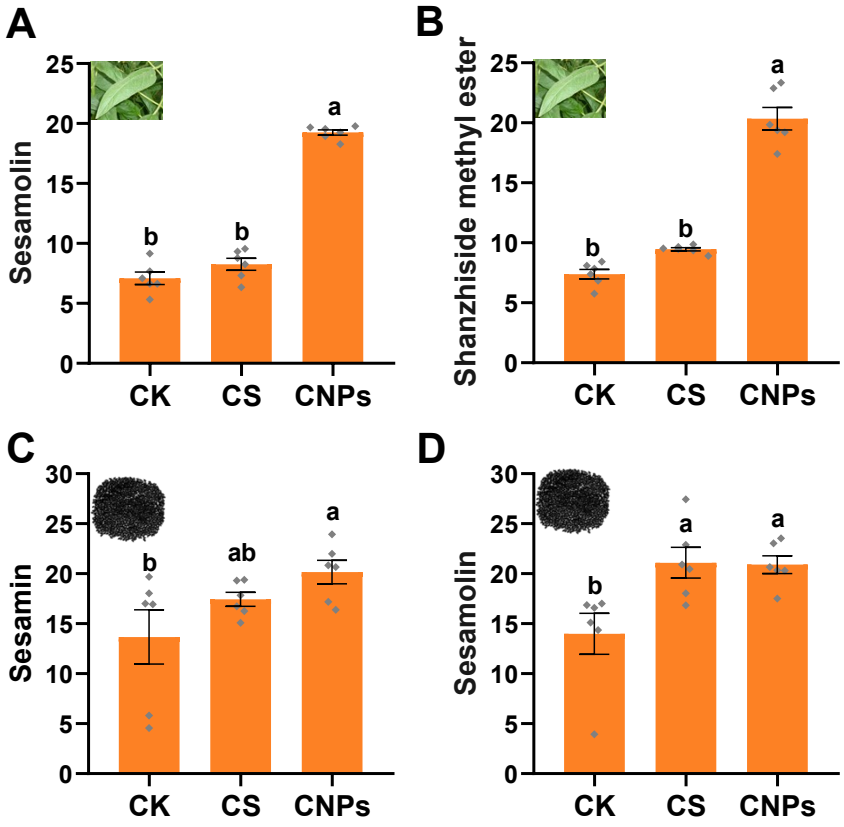


Fig. S5 The relative abundance of metabolites in sesame plants exposed to CNPs in response to herbivory. (A) Sesamolin and (B) shanzhiside methyl ester in leaves; (C) sesamin and (D) sesamolin in seeds. Chitosan treatments include CK, control plants; CS, bulk chitosan; CNPs, chitosan nanoparticles. Data are means ± SE (n = 6). Significant differences among the treatments are marked with different lowercase letters (Tukey's HSD test, $p < 0.05$).

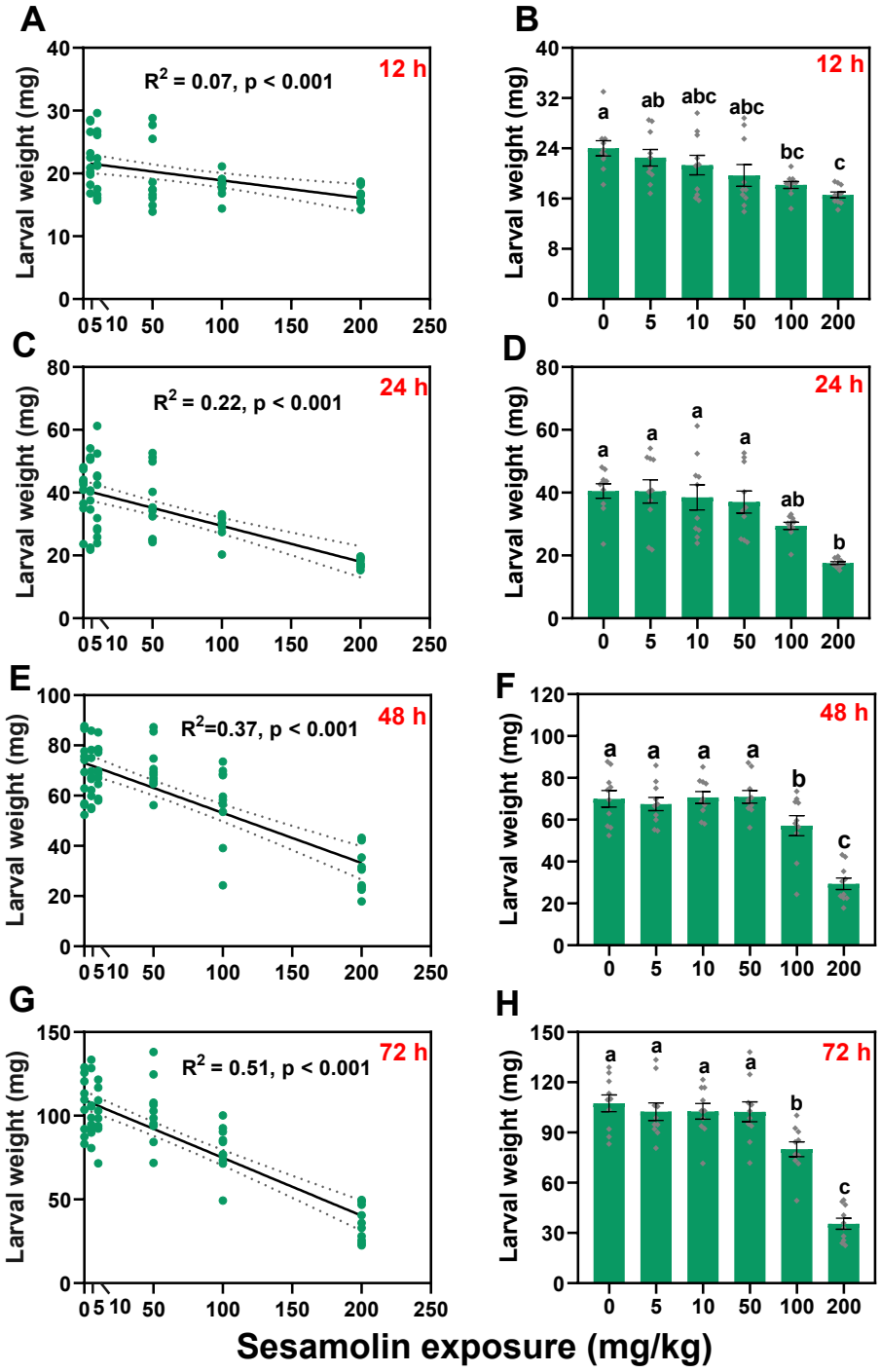


Fig. S6 Toxicity bioassay. Sesamolin-mediated toxicity effects on *S. litura* larval weight and the *in vitro* feeding experiment with artificial diet within 72 h feeding period exposed to sesamolin with various doses (0, 5, 10, 50, 100 and 200 mg/kg). Data are means ± SE (n = 10). Significant differences among the treatments are marked with different lowercase letters (Tukey's HSD test, $p < 0.05$).

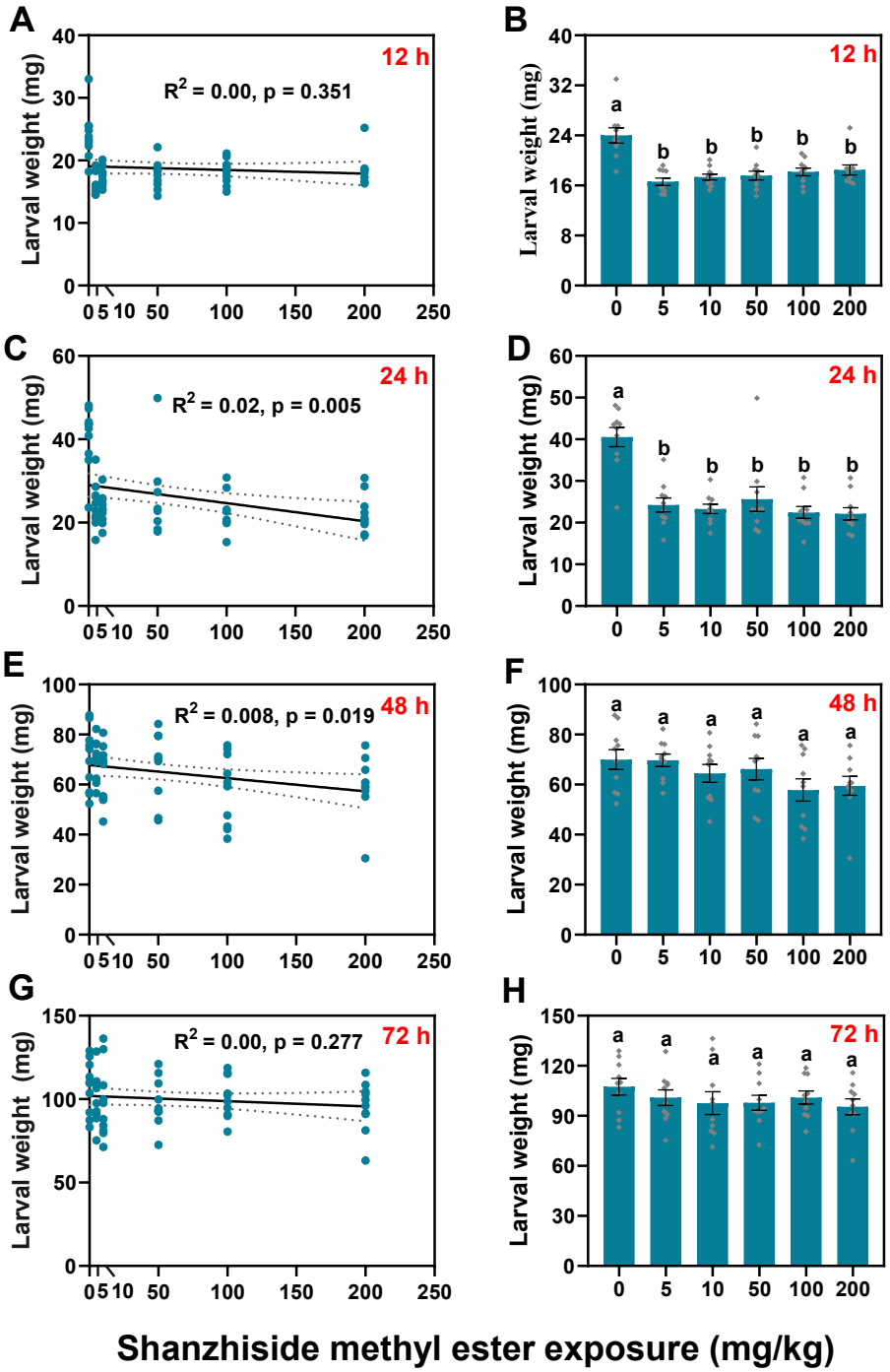


Fig. S7 Toxicity bioassay. Shanzhiside methyl ester-mediated toxicity effects on *S. litura* larval weight and the *in vitro* feeding experiment with artificial diet within 72 h feeding period exposed to shanzhiside methyl ester with various doses (0, 5, 10, 50, 100 and 200 mg/kg). Data are means ± SE (n = 10). Significant differences among the treatments are marked with different lowercase letters (Tukey's HSD test, $p < 0.05$).

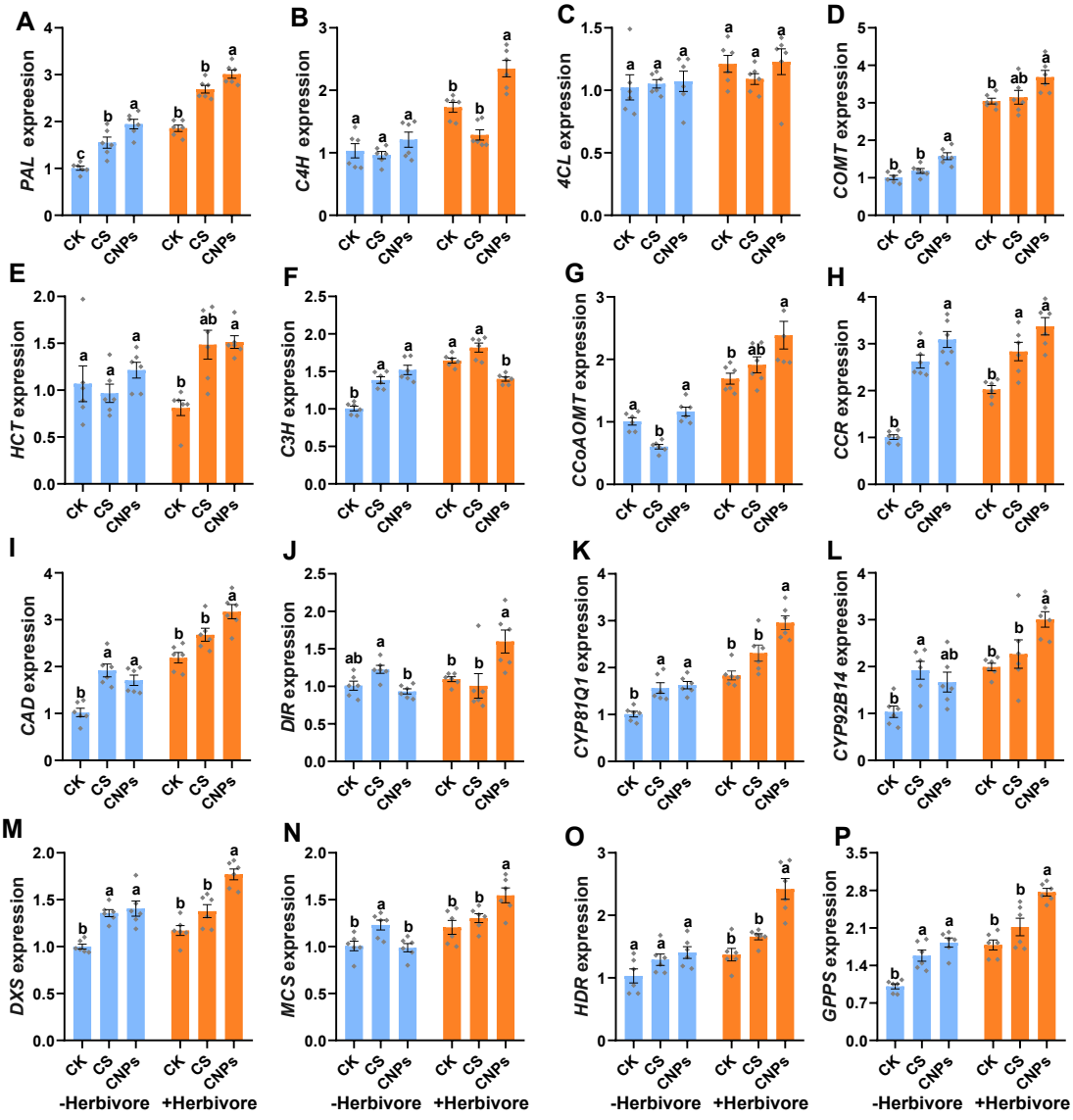


Fig. S8 Effect of CNPs on the biosynthesis gene expression of defense compounds. Shown is the relative expression of genes: (A-L) sesamolin and (M-P) shanzhiside methyl ester in sesame leaves in sesame leaves in the absence (-Herbivore, blue bars) and presence (+Herbivore, orange bars) of *S. litura* caterpillars. Data are means ± SE (n = 6). Chitosan treatments include CK, control plants; CS, bulk chitosan; CNPs, chitosan nanoparticles. Significant differences among the treatments are marked with different lowercase letters in the absence and presence of *S. litura*, respectively (Tukey's HSD test, $p < 0.05$).

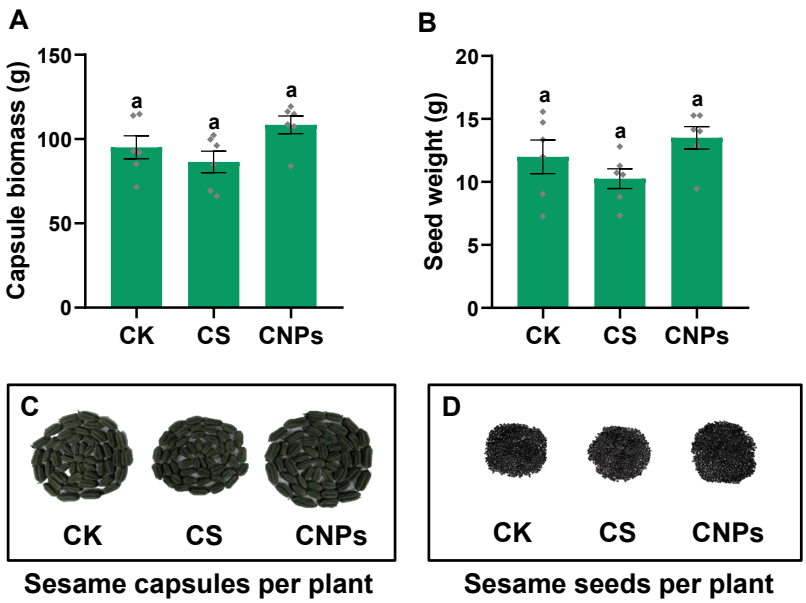


Fig. S9 Effect of CS and CNPs on the sesame yield under field condition. (A) Capsule biomass, (B) seed weight, photos of (C) sesame capsules and (D) seeds per plant. Chitosan treatments include CK, control plants; CS, bulk chitosan; CNPs, chitosan nanoparticles. Data are means ± SE (n = 6). Significant differences among the treatments are marked with different lowercase letters (Tukey's HSD test, $p < 0.05$).

References

1. M. S. Sheteiwy, H. Shao, W. Qi, P. Daly, A. Sharma, H. Shaghaleh, Y. A. Hamoud, M. A. El-Esawi, R. Pan, Q. Wan and H. Lu, Seed priming and foliar application with jasmonic acid enhance salinity stress tolerance of soybean (*Glycine ma*x L.) seedlings, *J. Sci. Food. Agric.*, 2021, **101**, 2027-2041.
2. J. Liu, L. Gai and H. Zong, Foliage application of chitosan alleviates the adverse effects of cadmium stress in wheat seedlings (*Triticum aestivum* L.), *Plant. Physiol. Biochem.*, 2021, **164**, 115-121.
3. Sofy., A. A. Hmed, A. E. M. Alnaggar, R. A. Dawoud, R. F. M. Elshaarawy and M. R. Sofy, Mitigating effects of bean yellow mosaic virus infection in faba bean using new carboxymethyl chitosan-titania nanobiocomposites, *Int. J. Biol. Macromol.*, 2020, **163**, 1261-1275.
4. A. Akcin, The effects of fulvic acid on physiological and anatomical characteristics of bread wheat (*Triticum aestivum* L.) cv. flamura 85 exposed to chromium stress, *Soil. Sediment. Contam.*, 2021, **30**, 590-609.
5. X. Luo, X. Cao, C. Wang, L. Yue, X. Chen, H. Yang, X. Le, X. Zhao, F. Wu, Z. Wang and B. Xing, Nitrogen-doped carbon dots alleviate the damage from tomato bacterial wilt syndrome: systemic acquired resistance activation and reactive oxygen species scavenging, *Environ. Sci. Nano.*, 2021, **8**, 3806-3819.
6. Y. Li, Z. Tang, Z. Pan, R. Wang, X. Wang, P. Zhao, M. Liu, Y. Zhu, C. Liu, W. Wang, Q. Liang, J. Gao, Y. Yu, Z. Li, B. Lei and J. Sun, Calcium-mobilizing properties of salvia miltiorrhiza-derived carbon dots confer enhanced environmental adaptability in plants, *ACS Nano*, 2022, **16**, 4357-4370.
7. Z. Wang., J. Tang, L. Zhu, Y. Feng, L. Yue, C. Wang, Z. Xiao and F. Chen, Nanomaterial-induced modulation of hormonal pathways enhances plant cell growth, *Environ. Sci. Nano.*, 2022, **9**, 1578-1590.
8. Z. Wang, X. Le, X. Cao, C. Wang, F. Chen, J. Wang, Y. Feng, L. Yue and B. Xing, Triiron tetrairon phosphate ($Fe_7(PO_4)_6$) nanomaterials enhanced flavonoidaccumulation in tomato fruits, *Nanomaterials,* 2022, **12**, 1341.
9. L. Wei, H. Zhang, Y. Duan, C. Li, S. Chang, H. Miao and R. Hovav, Transcriptome

comparison of resistant and susceptible sesame (*Sesamum indicum* L.) varieties inoculated with *Fusarium oxysporum* f.sp.sesami, *Plant Breeding*, 2016, **135**, 627-635.

10. J. Murata, E. Ono, S. Yoroizuka, H. Toyonaga, A. Shiraishi, S. Mori, M. Tera, T. Azuma, A. J. Nagano, M. Nakayasu, M. Mizutani, T. Wakasugi, M. P. Yamamoto and M. Horikawa, Oxidative rearrangement of (+)-sesamin by CYP92B14 co-generates twin dietary lignans in sesame, *Nat. Commun.*, 2017, **8**, 2155.

11. R. Zhou, Komivi Dossa , Donghua Li , Jingyin Yu , Jun You, Xin Wei and X. Zhang, Genome-wide association studies of 39 seed yield-related traits in sesame (*Sesamum indicum* L.), *Int. J. Mol. Sci*., 2018, **19**, 2794.