Supporting Information for

Synergistic Effect of Foliar Exposure to TiO_2 Nanoparticles and Planting Density Modulate Metabolite Profile and Transcription to Alleviate Cadmium Induced Phytotoxicity to Wheat (*Triticum aestivum* L.)

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Summary

This supplementary contains additional texts, tables, and figures of data that support the conclusions of the study. This file contains 17 pages, 3 texts, 2 tables, and 11 figures.

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Text S1. Characterization of the nanoparticles.

Characterization of the TiO_2-NPs was conducted using a scanning electron microscope (SEM) (ZEISS Sigma 300, Germany), transmission electron microscope (TEM) (JEOL JEM 2100F, Japan) and an energy-dispersive X-ray (EDX) detector (Oxford X-MaxN 80T IE250, England). Both TEM, SEM, and EDX analyses confirmed the cubic shape of the TiO_2-NPs, with an average particle size of 30.52 nm. To assess the purity of the TiO_2-NPs, X-ray diffraction (XRD) analysis was conducted. Additionally, the fourier transform infrared spectroscopy spectrometer (Thermo Scientific Nicolet iS20, the United States) was utilized to analyze the structure and composition of the TiO_2-NPs. To determine the hydrodynamic size and zeta potential of the TiO_2-NPs in ultrapure water, stock solutions of 100 mg/L TiO_2-NPs were prepared. Prior to measurement, the solutions were bath-sonicated using a sonicator (KH5200DE, Kunshanhechuang Ultrasonic, Jiangsu, China) at 45 kHz for 30 min. The measurements indicated an average hydrodynamic size of 318 ± 15.4 nm, with a corresponding zeta potential of 5.90 ± 0.70 mV.

Text S2. Total antioxidant capacity assays.

Total phenolic: 5 mL of equal volume ratio of mixed extract (80% acetone: ethanol) was added to 0.1 g wheat leaves and then were extracted overnight (12 h). Then, 50 μL of leaves extracts was diluted with 450 μL distilled water, followed by adding 250 μL of folin-Ciocalteu (2 M) and 1.25 mL Na_2CO_3 (20 g/L). After centrifuged (2,000 rpm) for 10 min, absorbance of the supernatant was determined (735 nm) using a microplate reader (Varioskan LUX, Thermo Fisher Scientific, Vantaa, Finland), and a standard curve was determined using gallic acid.

Total antioxidant capacity: The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH = 3.6) and tripyridine triazine solution (10 mM) dissolved in 40 mM HCl and 10 mL $FeCl_3 \cdot H_2O$ (20 mM) (10:1; V:V) at 37 °C. The 50 μL test sample (the same with total phenolic) was then mixed with 1.5 mL FRAP reagent for 10 min (37 °C). The absorbance of the mixture was measured at 593 nm using a microplate reader (Varioskan LUX, Thermo Fisher Scientific, Vantaa, Finland). Activity of GSH, SOD, POD, CAT, and APX were determined using assay kits (Nanjing Jiancheng Bioengineering Institute).

Text S3. Cadmium subcellular distribution and speciation in wheat leaves.

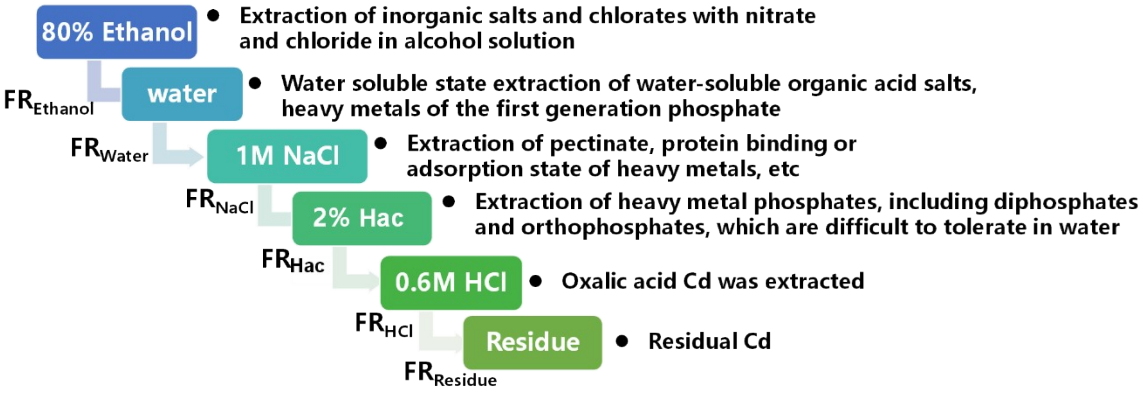
Cadmium subcellular distribution

Initially, the leaves were precisely weighed and homogenized in a chilled medium containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), and 1.0 mM dithioerythritol (DTE), which had been cooled to 4 °C prior to addition of the homogenate. The homogenate was then filtered through a nylon cloth (80 μm), with the residue trapped on the cloth designated as the cell wall fraction (F_{CW}). The filtrate was subsequently centrifuged at 1,500 g for 10 min at 4 °C using a refrigerated centrifuge. The pellet at the bottom of the tube represented the plastids (F_P). The supernatant from the first centrifugation was then centrifuged again at 5,000 g for 20 min. The resulting pellet was

designated as the nucleus fraction (F_N). The supernatant was further centrifuged at 15,000 g for 35 min to isolate the cell sediment, which was designated as the mitochondrial fraction (F_M). Finally, the last supernatant was designated as the soluble fraction (F_S). All the resulting cell components, both precipitates and supernatants, were dried at 70 °C until a constant weight was achieved. They were then digested with HNO_3 and H_2O_2. The Cd concentrations were subsequently determined using ICP-MS.

Cadmium speciation in wheat leaves

The detailed procedure is outlined below: The procedure involved overnight immersion of 1.00 g of wheat leaves in 10 mL of solution at 30 °C, followed by centrifugation at 5,000 g for 10 min. The pellet was re-suspended twice of the same extraction solution. And then fresh extractant was added for an additional 2-h extraction period. This 2-h extraction step was repeated three times, with the four resulting extraction solutions pooled. The Cd content of this pooled sample represented the Cd content of the specific component being analyzed.



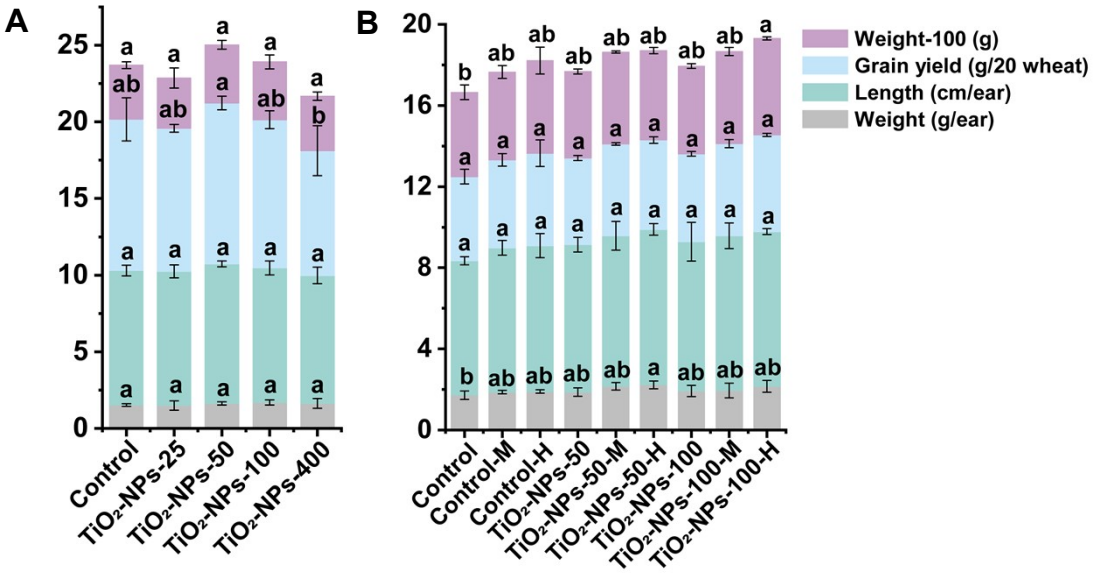


Figure S1. Basic growth index of wheat in pot (A) and field (B) experiment of wheat after foliar application of different concentration of TiO_2-NPs. Datas are means ± s.d. (n=3). Different letters above bars represent differences (***p* < 0.05**) determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

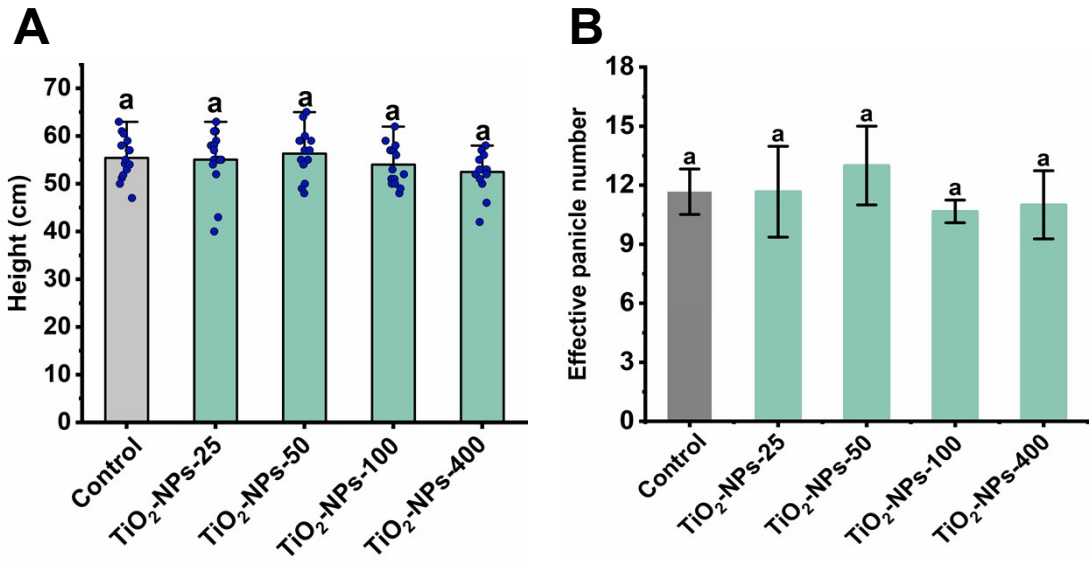


Figure S2. (A) Height and (B) effective panicle number of wheat after foliar application of different concentration of TiO_2-NPs in pot experiment. Datas are means ± s.d. (n=3). Different letters above bars represent differences (***p* < 0.05**) determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

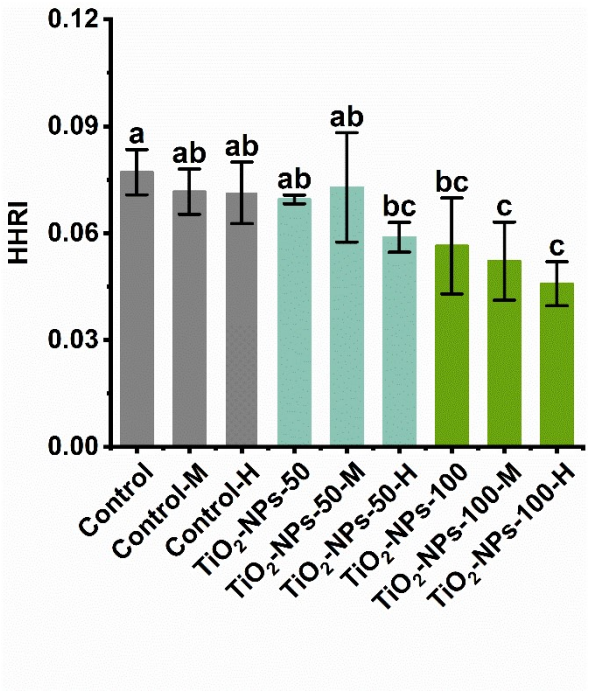


Figure S3. Human health risk index (HHRI) in field experiment of wheat after foliar application of different concentration of TiO_2-NPs. Datas are means ± s.d. (n=3). Different letters above bars represent differences (***p* < 0.05**) determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

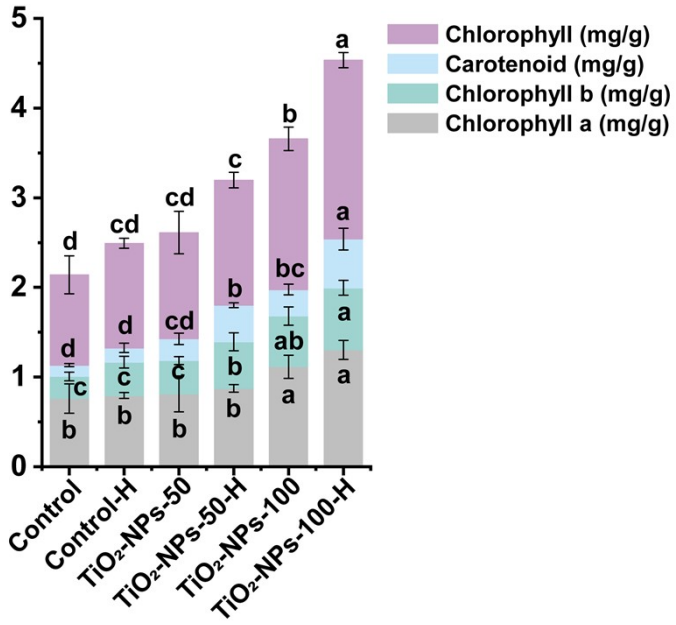


Figure S4. Chlorophyll content of wheat in field experiment after foliar application of different concentration of TiO_2-NPs. Datas are means ± s.d. (n=3). Different letters above bars represent differences (***p* < 0.05**) determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

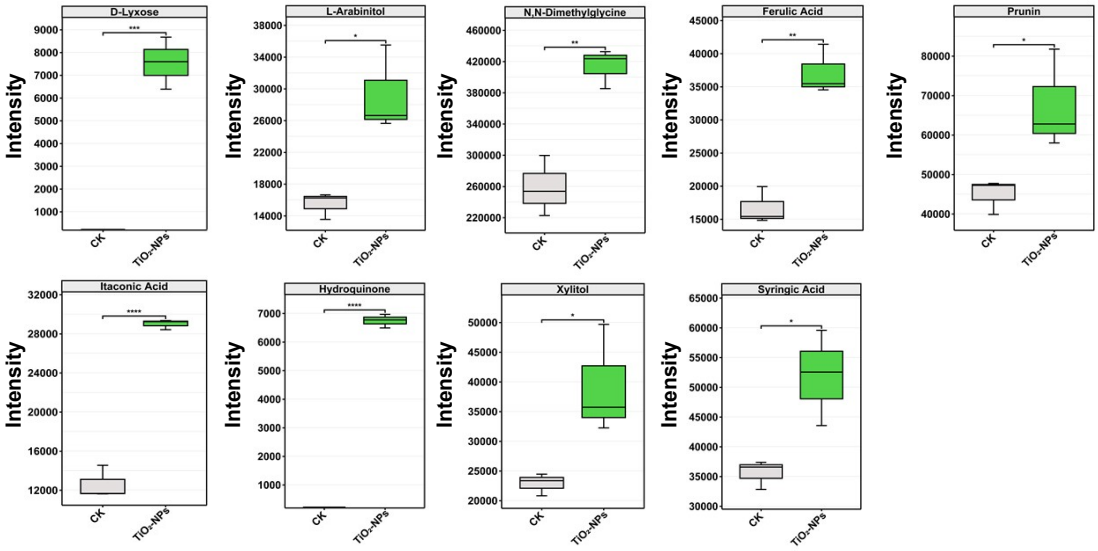


Figure S5. Antioxidant differential metabolites of wheat after foliar application of different concentration of TiO_2-NPs at 50 mg/L.

Note: ***p* < **0.05**, ****p* < **0.01,** *****p* < **0.001**.

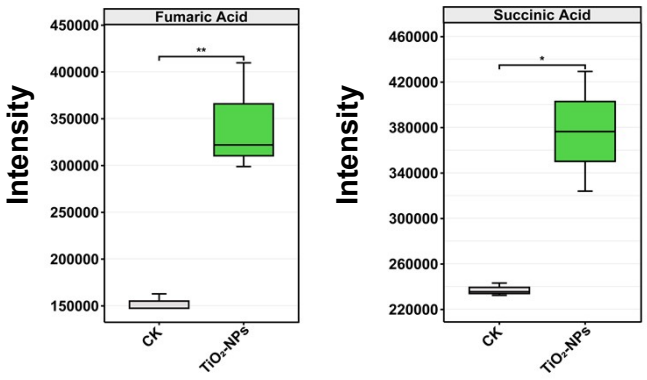


Figure S6. Differential metabolites associated with the tricarboxylic acid cycle of wheat after foliar application of different concentration of TiO_2-NPs at 50 mg/L.

Note: ***p* < 0.05**, ****p* < 0.01,** *****p* < 0.001.**

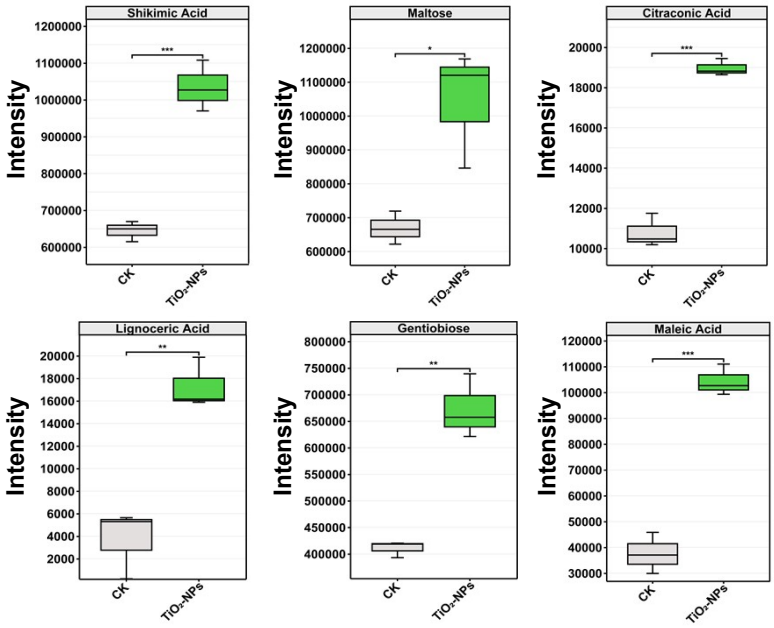


Figure S7. Differential metabolites associated with stress resistance of wheat after foliar application of different concentration of TiO_2-NPs at 50 mg/L.

Note: ***p* < 0.05**, ****p* < 0.01,** *****p* < 0.001.**

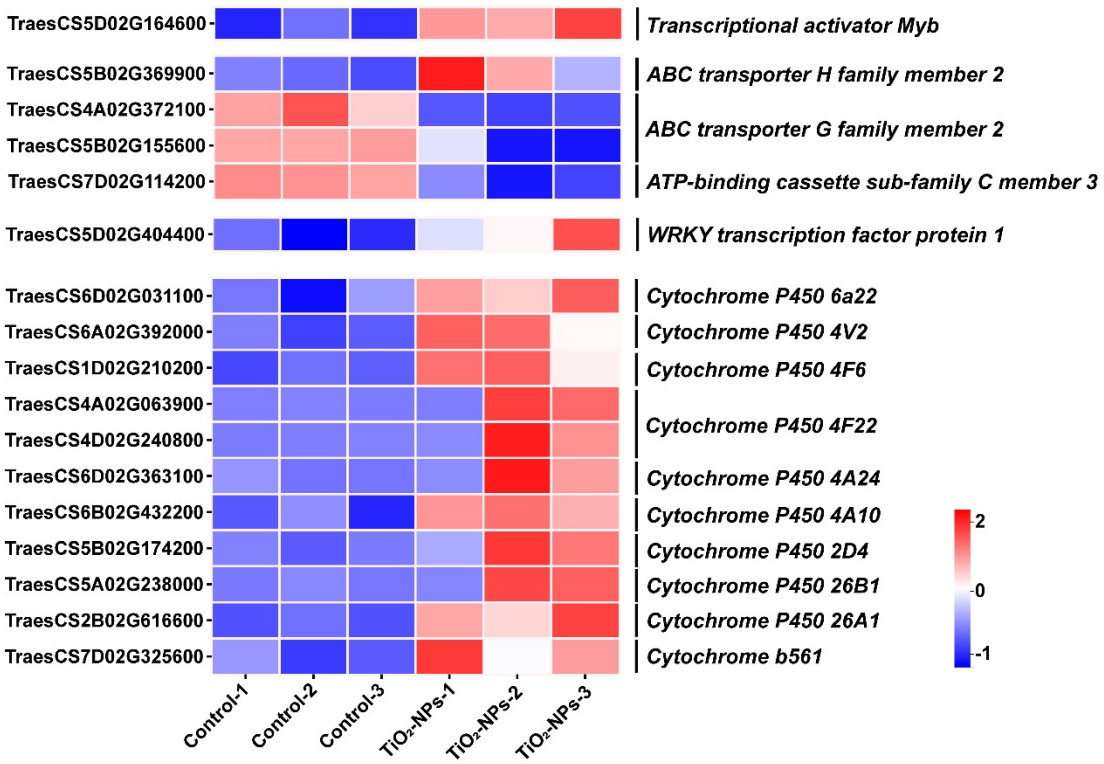


Figure S8. Expression of various stress-responsive transcription factors in wheat leaves after foliar application of different concentration of TiO_2-NPs at 50 mg/L.

Note: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

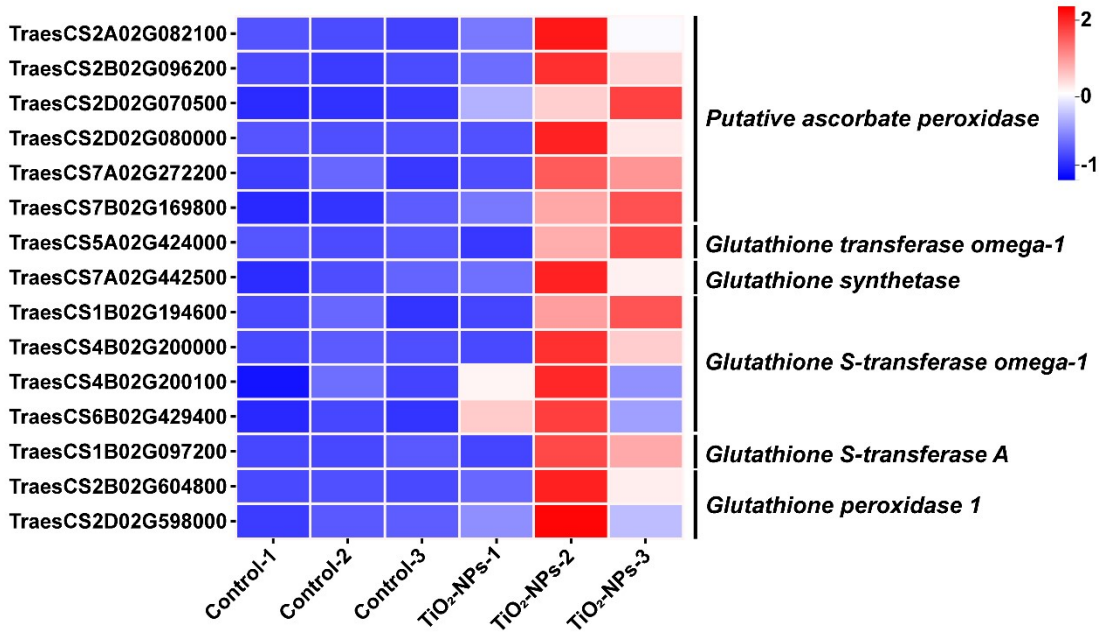


Figure S9. Expression of various glutathione transcription factors in wheat leaves after foliar application of different concentration of TiO_2-NPs at 50 mg/L.

Note: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

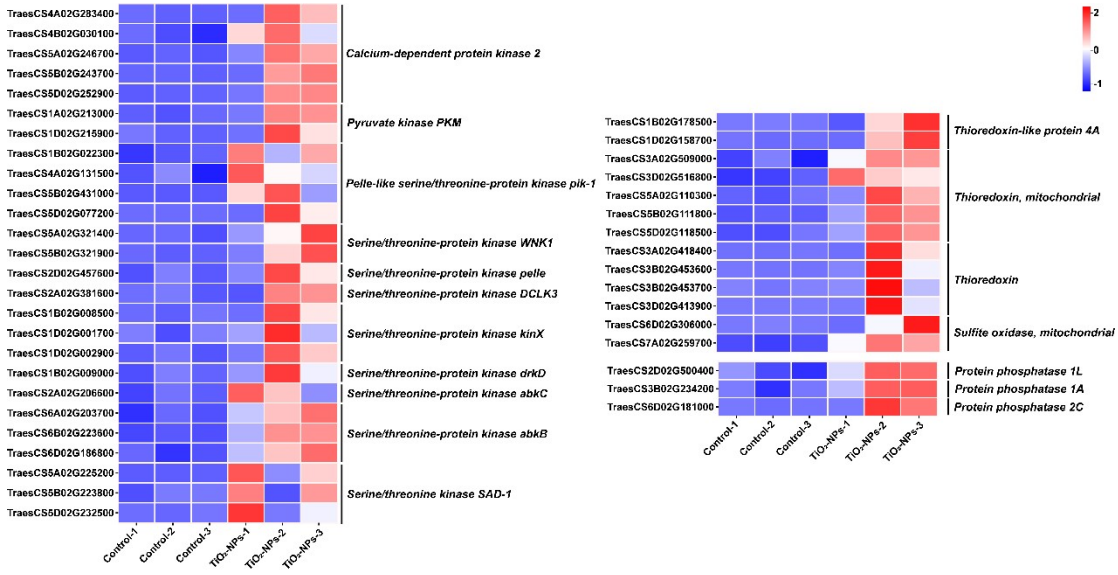


Figure S10. Expression of various serine and phosphatase transcription factors in wheat leaves after foliar application of different concentration of TiO_2-NPs at 50 mg/L.

Note: ***p* < 0.05**, ****p* < 0.01,** *****p* < 0.001.**

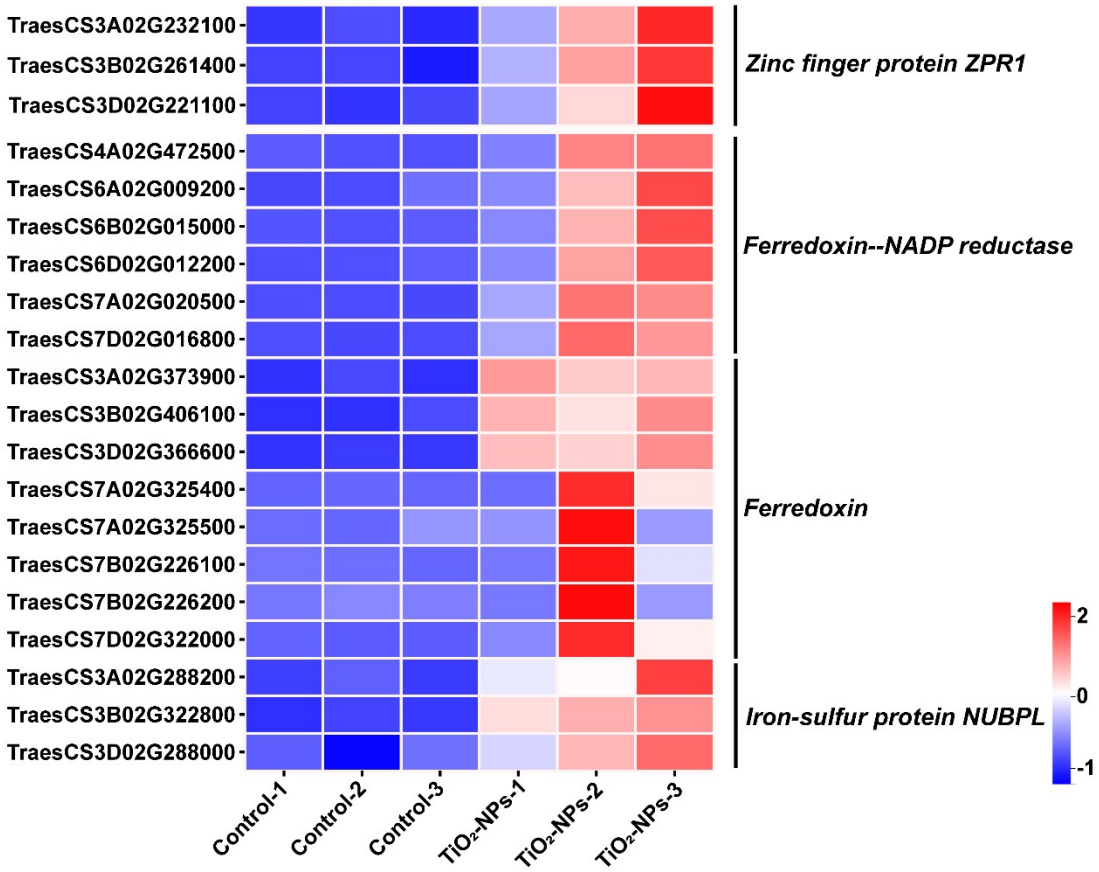


Figure S11. Expression of various zinc finger protein and iron related protein in wheat leaves after foliar application of different concentration of TiO_2-NPs at 50 mg/L.

Note: ***p* < 0.05**, ****p* < 0.01**, *****p* < 0.001.**

Table S1. Physicochemical properties of the experimental soil.

pH	Total nitrogen (%)	Organic matter (g/kg)	Cation exchange capacity (cmol/kg)	Olsen-phosphorus (mg/kg)	Olsen-potassium (mg/kg)	Amibient total Cd (mg/kg)	Available Cd (mg/kg)
8.13	0.21	38.12	18.3	14.86	8.58	1.08	0.61

Table S2. Statistics of sequencing data.

Treatments	Total reads (M)	Total bases (G)	Valid bases (%)	Q30(%)
Control-1	43.73	6.47	98.27	95.71
Control-2	46.98	6.88	97.03	95.26
Control-3	46.88	6.91	97.68	95.25
TiO_2-NPs-1	37.43	5.47	96.63	95.69
TiO_2-NPs-2	37.93	5.51	95.2	95.43
TiO_2-NPs-3	46.67	6.92	98.42	95.92