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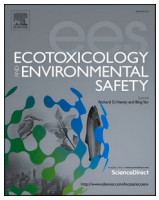

Molecular characterization and transcriptional response of *Lactuca sativa* seedlings to co-exposure to graphene nanoplatelets and titanium dioxide nanoparticles

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ABSTRACT

The widespread use of nanomaterials in agriculture may introduce multiple engineered nanoparticles (ENPs) into the environment, posing a combined risk to crops. However, the precise molecular mechanisms explaining how plant tissues respond to mixtures of individual ENPs remain unclear, despite indications that their combined toxicity differs from the summed toxicity of the individual ENPs. Here, we used a variety of methods including physicochemical, biochemical, and transcriptional analyses to examine the combined effects of graphene nanoplatelets (GNPs) and titanium dioxide nanoparticles (TiO_2 NPs) on hydroponically exposed lettuce (*Lactuca sativa*) seedlings. Results indicated that the presence of GNPs facilitated the accumulation of Ti as TiO_2 NPs in the seedling roots. Combined exposure to GNPs and TiO_2 NPs caused less severe oxidative damage in the roots compared to individual exposures. Yet, GNPs and TiO_2 NPs alone and in combination did not cause oxidative damage in the shoots. RNA sequencing data showed that the mixture of GNPs and TiO_2 NPs led to a higher number of differentially expressed genes (DEGs) in the seedlings compared to exposure to the individual ENPs. Moreover, the majority of the DEGs encoding superoxide dismutase displayed heightened expression levels in the seedlings exposed to the combination of GNPs and TiO_2 NPs. The level of gene ontology (GO) enrichment in the seedlings exposed to the mixture of GNPs and TiO_2 NPs was found to be greater than the level of GO enrichment observed after exposure to isolated GNPs or TiO_2 NPs. Furthermore, the signaling pathways, specifically the "MAPK signaling pathway-plant" and "phenylpropanoid biosynthesis," exhibited a close association with oxidative stress. This study has provided valuable insights into the molecular mechanisms underlying plant resistance against multiple ENPs.

1. Introduction

Nanotechnology is of great importance in agricultural practice (Lowry et al., 2024). Engineered nanoparticles (ENPs) are being used in various agricultural applications due to their unique physicochemical properties. These applications include the treatment of agricultural waste (Abdelsalam et al., 2023), development of novel pesticides (Mubeen et al., 2023), and enhancement of fertilizers (Maity et al., 2022). However, as nanotechnology becomes more integrated into the agricultural production, numerous studies have raised increasing concerns about the biosafety and environmental impact of ENPs (Murali et al., 2022). Research has indicated that ENPs can have detrimental effects on plants by inhibiting plant growth, destroying cellular structures, compromising cell wall integrity, damaging cell membranes and mitochondria, inducing oxidative stress, and causing genotoxicity (Gao et al., 2023). Therefore, the impact of ENPs on the agricultural environment is characterized by a mix of detrimental and beneficial outcomes.

Chemical contaminants are always present in the environment in the form of mixtures. The interactions between ENPs and other co-occurring contaminants, as well as their combined effects on agricultural plants, may differ significantly from the sum of the individual effects of the components present in the mixture (Zhang et al., 2022). It is known that ENPs can help plants resist heavy metal toxicity (Huang et al., 2024).

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However, when ENPs and heavy metals are co-exposed, they still exhibit toxicity to plants compared to plants that are not exposed to ENPs and heavy metals (Qiang et al., 2023). Once introduced into the surrounding ecosystem, ENPs have the potential to engage in interactions with other ENPs (Zhang et al., 2022). It is generally postulated that individual ENPs in a mixture may interfere with one another, resulting in the mixture being more or less toxic than the ENPs alone (Jośko et al., 2022; Rashid et al., 2023). However, there is limited research available on the combined toxicity effects of individual ENPs and the underlying molecular mechanisms in agricultural plants.

To date, graphene has attractive properties such as a large surface area, chemical stability, mechanical stability, tunable surface chemistry, and low toxicity, making it a promising material to be used as a base material for agricultural delivery systems (Bhattacharya et al., 2023). For instance, the potential application of graphene oxide, in combination with the pesticide fungicide pyraclostrobin, was examined as a nanocarrier for the advancement of nanopesticides designed specifically for agricultural use (Peng et al., 2022). This efficiency expands the applicability of graphene-based materials to sustainable agriculture (Antunes, 2023). Moreover, titanium dioxide nanoparticles (TiO_2 NPs) are one of the most commonly used ENPs in agriculture (Rodríguez-González et al., 2019). What is more, hybrid nanocomposites based on graphene and TiO_2 have become powerful nanomaterials in the field of wastewater treatment (Kusiak-Nejman and Morawski, 2019). Once these nanohybrid materials are unintentionally retained in the treated effluent, the practice of irrigating fields with this effluent may give rise to the contamination of the agricultural environment, subsequently causing detrimental impacts on the growth and quality of crops. Therefore, the co-existence of graphene and TiO_2 NPs in the agricultural environment emerges as a pressing concern that needs to be urgently investigated.

It is the purpose of this study to elucidate the molecular mechanism behind the toxicological effect of mixed ENPs on agricultural plants. The ENPs used in the study are specific types of nanoparticles, namely graphene nanoplatelets (GNPs) and TiO_2 NPs, as representative examples of graphene-family and metal oxide nanomaterials. Moreover, we used lettuce (*Lactuca sativa* L. var. *Ramose* Hort) as a model plant, as this plant was found to be the most sensitive crop among common food crops in terms of seed germination after exposure to ENPs (Gong et al., 2021). Additionally, lettuce is one of the most commonly grown hydroponic vegetables. Thereupon, a hydroponic system was used since hydroponic exposure allows to maintain the actual exposure concentration of ENPs in each different treatment (Mosa et al., 2018). We for the first time determined the individual and combined effects of GNPs and TiO_2 NPs on the seed germination and seedling growth of lettuce in a hydroponic system, and we subsequently elucidated the toxic mechanisms they induced at the molecular level in the different tissues of lettuce seedlings. Our research focused on oxidative stress response and transcriptome effects. We hypothesize that the interactions of GNPs with TiO_2 NPs significantly modulate the growth and physiological responses of the crop plant at the molecular level, thereby influencing critical biological processes that are essential for optimal plant development and productivity. To the best of our knowledge, this study represents the initial investigation into the RNA-seq analysis conducted on plants subjected to combinations of individual ENPs.

2. Materials and methods

2.1. Test materials

GNPs, which were 1–4 nm thick and had a particle size of 2 μm, and TiO_2 NPs with a primary size of 21 ± 5 nm (with a specific surface area of 50 ± 10 m^2/g and a purity of over 99.5 %), were purchased as powders from PlasmaChem GmbH in Berlin, Germany. The stock suspensions of ENPs were prepared by dispersing the powder in deionized (DI) water. Subsequently, these suspensions were underwent magnetic stirring for one hour to ensure proper dispersion of the ENPs before further utilization. The stock suspensions were then stored at 4 °C until ready for use. Lettuce seeds were obtained from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences.

2.2. Physicochemical analysis

GNPs and TiO_2 NPs, both individually and in combination, were characterized using a transmission electron microscope (TEM, JOEL 2100 f, JOEL Ltd., Tokyo, Japan) in DI water. The zeta potential (ζP) and hydrodynamic diameters (HD) of GNPs and TiO_2 NPs were determined at the beginning (0 h) and the end (7 d) in stationary suspensions of the individual and combined materials at a concentration of 10 mg/L, and were analyzed in DI water using a ZetaSizer instrument (Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK). To determine the translocation factor (TF) of TiO_2 NPs in lettuce, the total concentrations of Ti (μg/kg) in the roots and shoots of lettuce seedlings were measured using inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher iCAP-Q, Germany). The detection limit of Ti was 0.01 μg/kg. The evaluation of TF was based on the ratio of the total concentrations of Ti present in the shoots to those found in the roots (Wang et al., 2021).

2.3. Germination and growth of lettuce seeds

Neatly developed and plump lettuce seeds were selected, sterilized using sodium hypochlorite (0.5 % w/v) for 15 min, and rinsed with DI water (pH: 7.69, conductivity: 0.40 μS/cm, and total dissolved solids: 0.21 mg/L) for three times. The seeds were then incubated in DI water as the hydroponic growth media for lettuce seeds (Liu et al., 2021). Twenty-five seeds were randomly selected from the sterilized seeds using forceps, placed in glass Petri dishes, and assigned to a single treatment group with exposure concentrations of 0 (control), 10, and 100 mg/L of GNPs and TiO_2 NPs, respectively, and to a treatment group with a pairwise mixture at a single exposure concentration. The selection of higher concentrations of the test materials allows for the full extent of potential effects to be revealed, encompassing different response mechanisms in plants. The chosen concentrations are commonly employed in the phytotoxicity studies of ENPs (Leopold et al., 2022; Wu et al., 2021). In this study, 10 and 100 mg/L GNPs were denoted as G10 and G100, respectively. Similarly, 10 and 100 mg/L TiO_2 NPs were denoted as T10 and T100, respectively. In addition, the binary mixtures were named G10 + T10, G10 + T100, G100 + T10, and G100 + T100. Afterwards, all the experimental groups were sealed and incubated in an artificial climate chamber for 7 d in the dark at 18 ± 2 °C and 60 % humidity. A 7 d period serves as a suitable moderate observation window, enabling the initial effects of toxic substances on plants to be discerned without the confounding influence of prolonged exposure. This exposure duration for exposure was often established in accordance with existing research on the phytotoxicity of ENPs (Wu et al., 2021). The experiments were repeated twice at 7 d intervals. The seed germination potential and rate were recorded after 3 and 7 d, respectively. Germination percentage (%) = (number of seeds germinated / total number of seeds tested) × 100 %. Root elongation, shoot length, and fresh weight of lettuces were measured on day 7.

2.4. Antioxidant activity assays

Total protein (TP) contents, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) levels in the roots and shoots of lettuce seedlings were determined using commercial kits (Cat Nos. A045–2–2 for TP, A001–1–2 for SOD, and A003–1–2 for MDA assay kits) purchased from Nanjing Institute of Jiancheng Biological Engineering (Nanjing, China). For the antioxidant activity assay, the roots and shoots of lettuce seedlings were mechanically separated and ground using a mortar and pestle. Then, 3 mL of phosphate-buffered salt solution was added, and the roots and shoots were grinded on ice. After that, the grinded samples

were centrifuged at 1000 × g for 10 min at 4 °C. The collected supernatant was used to measure the TP contents, SOD activity, and MDA levels. The assay procedures for the biomarkers were in accordance with the manufacturer's instructions of the corresponding kits (Deng et al., 2013). Briefly, the three kits for TP, SOD, and MDA were analyzed by Coomassie Brilliant Blue, hydroxylamine, and barbiturate thiosulfate methods, respectively. Each group consisted of three replicates.

2.5. Gene expression assays

To determine the genes that were expressed differently in the roots and shoots of lettuce seedlings after being exposed to various treatments for a period of 7 d, the control and treatments (i.e., G100, T100, and G100 + T100) were dissected, and mRNA was extracted. Three qualified mRNA samples were used from both the control and each treatment to construct libraries. Transcriptome sequencing was subsequently conducted using the widely used Illumina platform (Meslier et al., 2022). The accession number assigned to the reference genome within the National Center for Biotechnology Information (NCBI) database is PRJNA173551. The ABI 7500 RT-qPCR system (Applied Biosystems, USA) was employed to validate the results obtained from transcriptome sequencing. The primer sequences for the selected ten differentially expressed genes (DEGs) are provided in Table S1. The reason for this selection is based on the principle of randomness (Chen et al., 2021). The 18SrRNA gene served as the internal control.

2.6. Statistical analysis

The data presented in this study are reported as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan's test with a significance level set at $p < 0.05$. The statistical analysis was conducted using IBM SPSS Statistics software, version 26.0, developed by IBM Corp. in Armonk, NY, USA.

3. Results and discussion

3.1. Physicochemical behavior

TEM images depicting the morphology of GNPs and TiO_2 NPs, both individually and in combination, are presented in Fig. 1A-C. The images demonstrate that both GNPs (Fig. 1A) and TiO_2 NPs (Fig. 1B) tend to agglomerate upon suspension in water. ENPs have a tendency to form agglomerates due to the attractive forces between them (Zare, 2016). Additionally, TiO_2 NPs were found to adhere to the surface of GNPs when both types of ENPs coexist (Fig. 1C).

It is important to understand the stability of ENP dispersions, as it may impact their subsequent toxicological effects (Zhang et al., 2022). To gain insight into their behavior (i.e., agglomeration and dispersion), the values of ζP and HD of GNPs and TiO_2 NPs alone and in combination were evaluated. As shown in Fig. 1D, the initial value of ζP of the mixture of GNPs and TiO_2 NPs (-10.3 mV) tended to be closer to the initial value of ζP of the component with the lower negative ζP value (-2.7 mV for TiO_2 NPs). Likewise, over 7 d, the value of ζP of the mixture of GNPs and TiO_2 NPs (-17.8 mV) tended to be closer to the value of ζP of the component with the lower negative ζP value (-12.0 mV for TiO_2 NPs). This also implies that the mixed system was not a stable suspension. In addition, the value of ζP of the mixture after 7 d showed a more negative charge compared to its initial value of ζP. This suggests that over time, the mixed particles may undergo sedimentation. Regarding the particle size distribution (Fig. 1E), the initial value of HD of TiO_2 NPs mixed with GNPs (1053 nm) was lower than the initial value of HD of both GNPs (2361 nm) and TiO_2 NPs (2142 nm). This means that the interaction of GNPs with TiO_2 NPs reduced the extent of agglomeration of individual ENPs. Similarly, a comparable trend was also observed in the value of HD after 7 d. Moreover, the value of HD of the binary mixture (854 nm) after 7 d was significantly lower than its initial value of HD ($p < 0.05$). This observation further corroborates the analysis of the ζP, indicating that sedimentation might occur within the mixed system, with smaller particles remaining suspended in water.

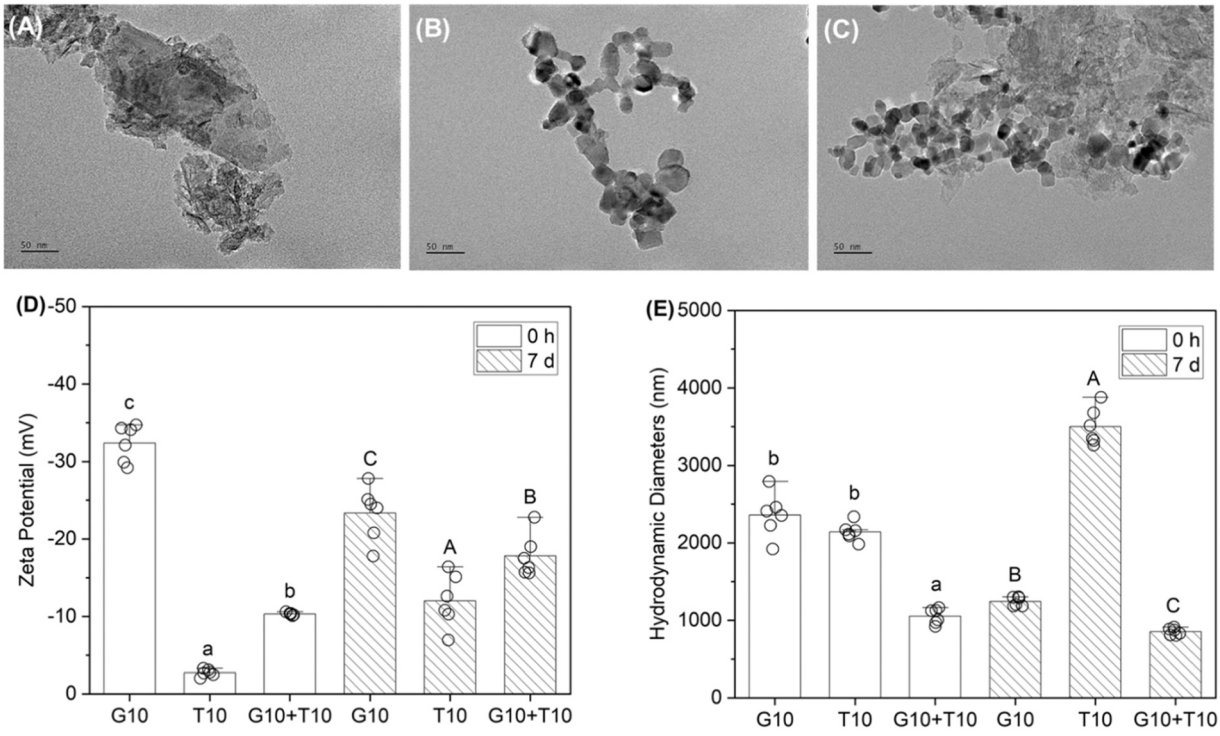

Fig. 1. TEM images of GNPs (A), TiO_2 NPs (B), and GNPs + TiO_2 NPs (C); Zeta potential (D) and hydrodynamic diameters (E) of single and binary mixtures of GNPs and TiO_2 NPs suspensions. G10: 10 mg/L GNPs, T10: 10 mg/L TiO_2 NPs, and T100: 100 mg/L TiO_2 NPs. The values reported are expressed as mean ± standard deviation ($n = 6$). Different letters represent statistically significant differences between the treatments ($p < 0.05$).

3.2. Seed germination and seedling growth effects

Effects of GNPs and TiO_2 NPs, individually and in combination, on the seed germination and seedling growth of lettuce are shown in Table S2 and Fig. S1. Overall, none of the ENP treatments significantly affected the relevant indicators of lettuce seed germination (Table S2), such as germination potential and germination percentage, compared to the control. Furthermore, the comparison revealed that no statistically significant variations were observed between the treatments in relation to the germination potential and germination percentage of lettuce seeds. After 7 d, the fresh weight of all the treatments was not significantly different from that of the control, except for the higher concentration of GNPs, as the fresh weight of the lettuce seedlings at this concentration was lower than that of the control. Similarly, the fresh weight of the higher concentration of GNPs was significantly 1.4 times lower than that of the higher concentration of GNPs in a binary mixture consisting of GNPs and TiO_2 NPs. In addition, no significant effect was observed for the treatments on the root length (Fig. S1A) and the shoot length (Fig. S1B) of lettuce seedlings compared to the control. The comparison also revealed no significant differences between the treatments in terms of root length and shoot length. Previous studies have also found that graphene-family nanomaterials (Castro et al., 2018) and TiO_2 NPs (Song et al., 2013) showed no toxic effects on the growth of lettuce.

3.3. Transport characteristics of Ti in lettuce

TF is an important indicator for assessing the uptake, distribution, and translocation of ENPs in plants (Wu et al., 2020). The TF values of total Ti from the roots to the shoots of lettuce seedlings under both single and mixed treatments are depicted in Fig. S2. The TF values decreased in the following order: T100 (1.72) > Control (1.51) > G100 (0.99) > G100 + T100 (0.37). The TF value of Ti in the control (1.51) was greater than 1, implying that Ti as metal preferred to migrate from the roots to the shoots. Moreover, the average concentrations of Ti in the roots and shoots in the control were 13.0 and 8.6 mg/kg, respectively. Furthermore, the TF value of Ti in the T100 treatment was greater than 1, implying that Ti in the T100 treatment also preferred to migrate from the roots to the shoots. However, in the T100 treatment, the average concentrations of Ti in the roots (10.7 mg/kg) and shoots (6.2 mg/kg) were lower than the average concentrations of Ti in the control roots and shoots. This implies that the lettuce seedlings did not show a translocation capacity for Ti present as TiO_2 NPs, whilst the presence of TiO_2 NPs limited the translocation of free Ti in the plants. Note that the TF value of Ti in the G100 treatment was lower than 1 and lower than the TF value of Ti in the control. This indicates that GNPs effectively restrained the migration of Ti in the plants through their inherent capacity to adsorb metals. Conversely, the TF value of Ti in the G100 + T100 treatment was lower than 1 and lower than the TF value of Ti in the G100 treatment. This implies that Ti also disliked migrating from the roots to the shoots. Meanwhile, in the G100 + T100 treatment, the average concentration of Ti in the shoots was 11.5 mg/kg, which was lower than the average concentration of Ti in the control shoots. However, interestingly, the average concentration of Ti in the roots was 31.6 mg/kg, which was higher than the average concentration of Ti in the control roots. Altogether, it can be concluded that the presence of GNPs prevented the migration of Ti from the roots to the shoots of lettuce seedlings but facilitated the accumulation of Ti present as TiO_2 NPs in the roots. This implies that the simultaneous exposure of plants to multiple ENPs can result in the potential for hyperaccumulation of ENPs in plant roots. Further advanced characterization techniques such as single-particle ICP-MS can be employed to discern the particle size distribution or particle concentration of ENPs within plant tissues, and the inclusion of micron-sized particles as a comparison can assist in more precisely defining the biological impacts of nanoparticles under combined pollution scenarios.

3.4. Antioxidant enzyme activity and lipid peroxidation

Plants that are exposed to ENPs often experience oxidative stress and subsequent oxidative damage (Li et al., 2024). This oxidative stress is commonly triggered by the buildup of reactive oxygen species (ROS) or a decline in antioxidant defense mechanisms (Klaunig et al., 1998). Oxidative stress can have significant effects on various biological processes within plants, including protein synthesis (Ayala and Machado, 2002). As shown in Fig. 2A, the TP content in the seedling roots exposed to each treatment group generally decreased by 22.6 – 80.9 % compared to the control group. For the single treatment group, the TP content in the roots exposed to GNPs decreased significantly by 31.4 – 75.9 % with increasing particle concentration ($p < 0.05$). The TP content in the roots exposed to TiO_2 NPs also exhibited a significant decrease by 72.6 – 80.9 % ($p < 0.05$). Moreover, T10 exerted a greater effect on the TP content in the roots than G10, whereas no significant difference was observed in the TP content in the roots between T100 and G100. Among all the mixed treatment groups, only the TP content in the roots exposed to G100 + T100 was significantly 3.2–4.1 times higher than the TP content in the roots exposed to the single treatment groups. As depicted in Fig. 2B, the TP contents in the shoots exposed to G10, G10 + T10, G10 + T100, and G100 + T10 decreased significantly by 16.7 – 23.6 % ($p < 0.05$).

The elimination of surplus ROS in living organisms depends on the antioxidant defense mechanisms (Sahu et al., 2022). One crucial element of the plant antioxidant enzyme system that counteracts oxidative stresses induced by unfavorable circumstances is SOD. SOD possesses the capability to facilitate the conversion of the superoxide anion into hydrogen peroxide through a process known as disproportionation (Feng et al., 2015). As illustrated in Fig. 2C, the SOD activity in the roots of seedlings exposed to the single treatment groups, except G10, increased significantly by 201 – 268 % ($p < 0.05$). This indicates that these treatments activated the antioxidant defense system to protect against the deleterious effects of ROS-induced oxidative stress. However, the SOD activity in the roots exposed to the mixed treatment groups did not exhibit a significant difference compared to the SOD activity in the control ($p > 0.05$). This means that SOD might have already scavenged ROS, thus allowing the subsequent activity to return to normal levels. As depicted in Fig. 2D, no significant differences were observed in the SOD activity in the shoots among the various treatment groups and the control ($p > 0.05$).

The elevation in ROS generation has the potential to induce lipid peroxidation in plants (Zhang et al., 2021). MDA is the most prevalent byproduct of lipid peroxidation in plant cells (Gavina et al., 2013). As shown in Fig. 2E, exposure to G100 or T100 alone significantly elevated the MDA contents to 70.2 or 77.1 nmol/mg prot in the roots relative to the control (12.6 nmol/mg prot) ($p < 0.05$), indicating that both treatment groups inflicted oxidative damage to the roots. However, the MDA contents in the roots exposed to the mixed treatment groups did not exhibit a significant difference compared to the MDA contents in the control ($p > 0.05$). This suggests that the combination of GNPs and TiO_2 NPs did not result in oxidative damage to the roots. This also means that SOD effectively eliminated ROS, and such defense mechanisms alleviated the oxidative damage effects induced by the binary mixtures. As depicted in Fig. 2F, there were no significant differences observed in the MDA contents in the shoots in between the various treatment groups and the control ($p > 0.05$). It can also be concluded that the analysis of MDA was consistent with the results of the SOD assessments.

In comparison, the single treatment groups caused oxidative damage to the roots but not to the shoots. This also suggests that the shoots exhibited less responses to oxidative stress than the roots. In addition, the response of the roots and shoots was similar in the case of the combined treatments of GNPs and TiO_2 NPs, as the mixed treatment groups did not cause oxidative damage in either the roots or shoots.

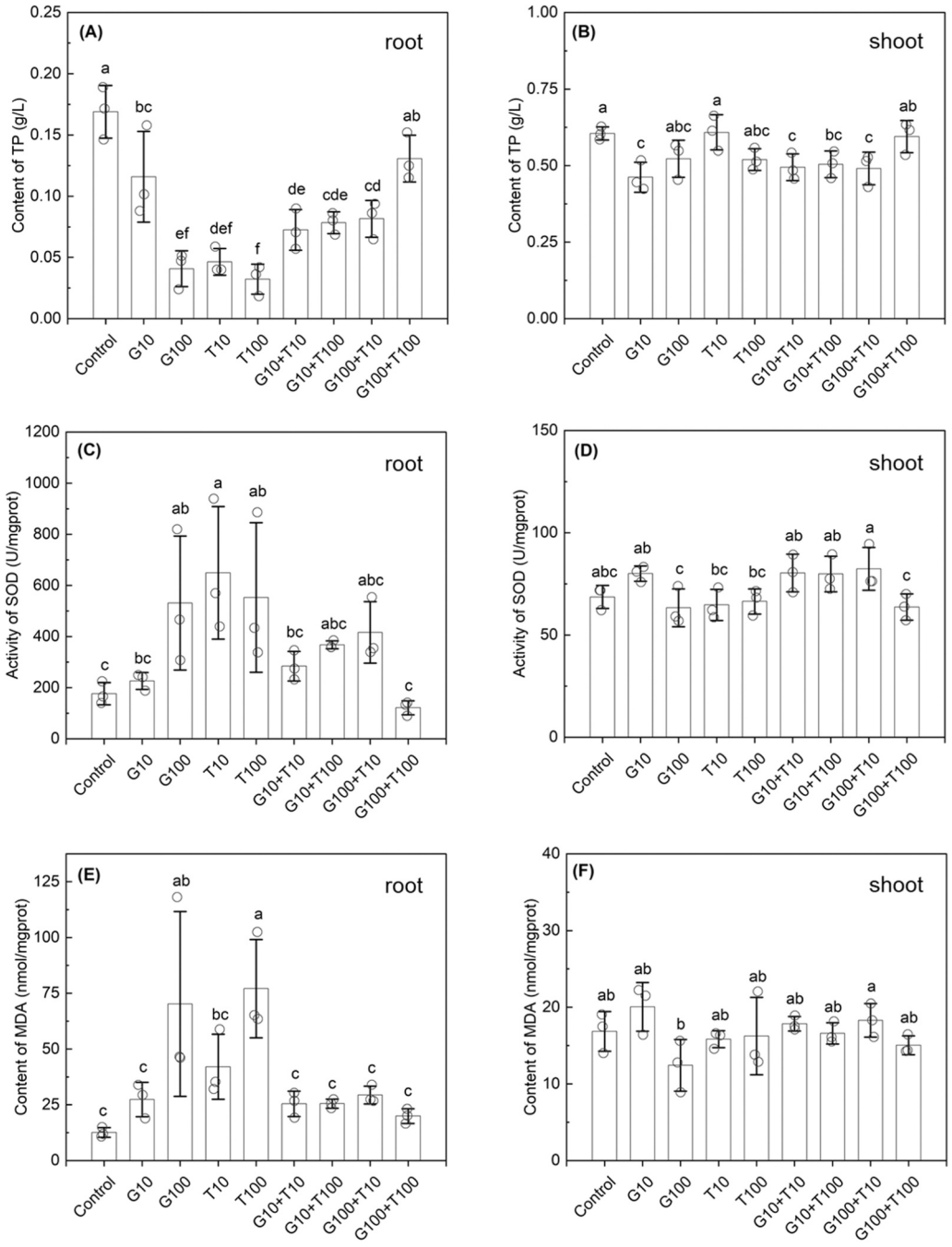

Fig. 2. Content of TP, activity of SOD, and content of MDA in the roots (A, C, and E) and shoots (B, D, and F) of lettuce seedlings (7 d) exposed to the single and binary mixtures of GNPs and TiO_2 NPs. G10: 10 mg/L GNPs, G100: 100 mg/L GNPs, T10: 10 mg/L TiO_2 NPs, and T100: 100 mg/L TiO_2 NPs. The values reported are expressed as mean ± standard deviation ($n = 3$). Different letters represent statistically significant differences between the treatments ($p < 0.05$).

3.5. Sequencing and analysis of transcriptome

3.5.1. Identification of DEGs

Venn diagrams (Fig. 3) were employed to ascertain the genes that exhibit exclusivity or co-expression in the roots and shoots of lettuce seedlings following exposure to four experimental conditions: control, G100, T100, and G100 + T100. Generally, the count of unique genes (UGs) consistently exceeded that of co-expressed genes, indicating the existence of discrete gene regulatory networks within each group during seedling growth.

As shown in Fig. 3A, there were 17,668 genes shared among the roots in the treatment groups studied. Compared to the control group, the number of UGs was lower in the plants exposed to G100 or T100 individually. However, the number of UGs was higher in plants exposed to both G100 and T100 compared to the control group. Moreover, the number of UGs in the suspension containing G100 + T100 exceeded the number of UGs in the suspension containing either G100 or T100. As shown in Fig. 3B, there were 19,043 genes shared among the seedling shoots after treatment with the various groups. The number of UGs in the group treated with G100 + T100 was higher than the number of UGs in the control group, and it fell in between the number of UGs in the groups treated with G100 and T100. In addition, a comparative analysis reveals that the number of co-expressed genes shared among the roots was lower than the number of co-expressed genes shared among the shoots. Instead, the number of UGs in the roots was greater than the number of UGs in the shoots.

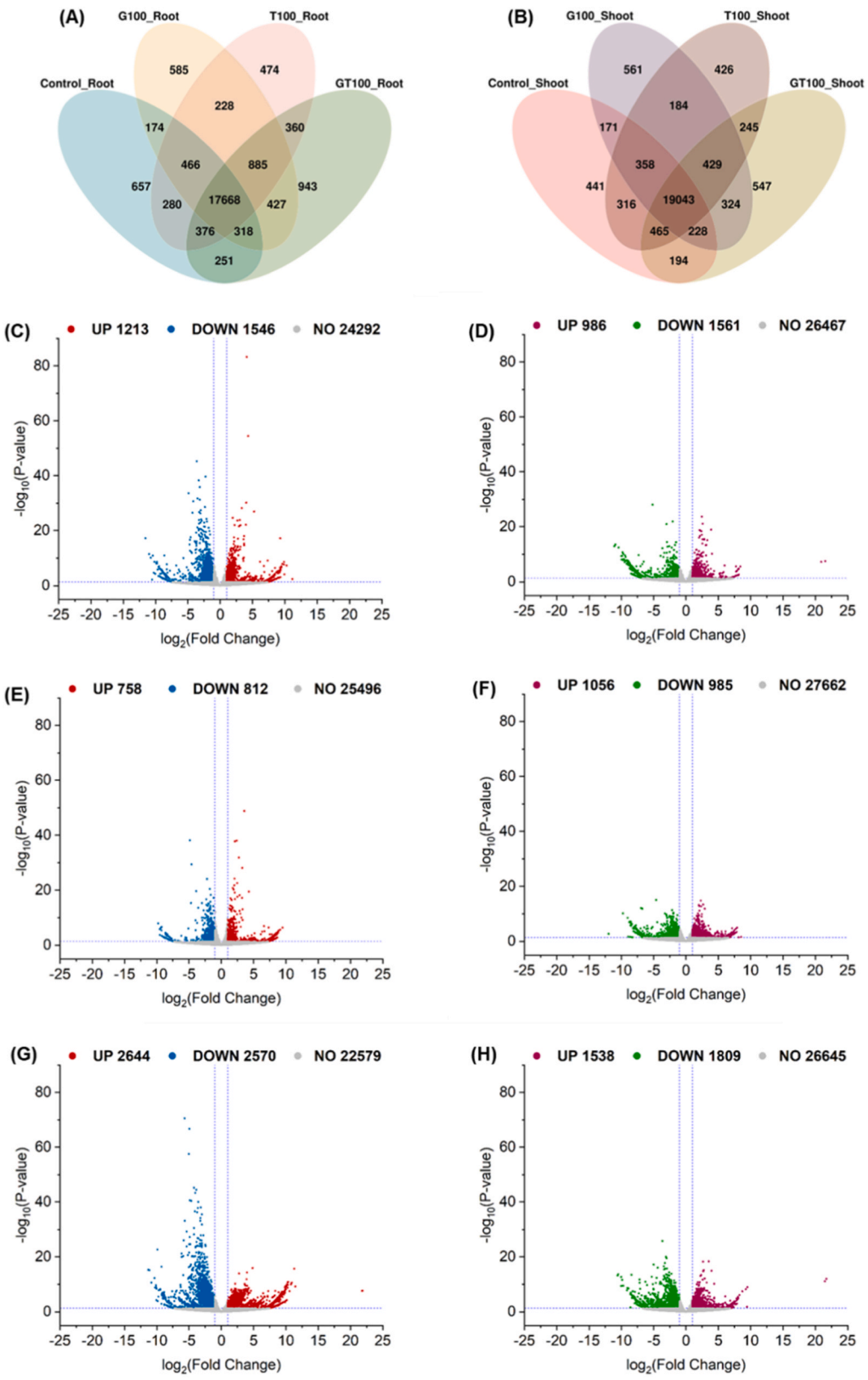

Fig. 3. Transcriptome analysis of the lettuce seedlings (7 d) exposed to 100 mg/L GNPs (G100), 100 mg/L TiO_2 NPs (T100), and 100 mg/L GNPs + 100 mg/L TiO_2 NPs (GT100). Venn diagrams show the overlap of gene expression in the roots (A) and the shoots (B) of the seedlings between different experimental groups. The threshold value was set to 1. Volcanic map of DEGs in the roots (C, E, and G) and shoots (D, F, and H) of the seedlings for different comparison groups (C and D: G100 vs control, E and F: T100 vs control, G and H: GT100 vs control), in which two vertical dotted lines in the figure distinguish DEGs that differed more than twofold, whereas DEGs above the horizontal dotted line had a p-value < 0.05. UP: up-regulated DEGs; DOWN: down-regulated DEGs; NO: non-differentially expressed genes.

The distribution of differentially expressed genes (DEGs) in the roots (Fig. 3C, E, and G) and shoots (Fig. 3D, F, and H) of lettuce seedlings after different treatments was analyzed. The results of this analysis are shown using a volcano map. In comparison to the control group, the number of up- and down-regulated DEGs induced in the roots decreased in the following order: G100 + T100 (Fig. 3G) > G100 (Fig. 3C) > T100 (Fig. 3E). This suggests that the roots exposed to the binary mixtures had the highest number of DEGs. Similarly, the number of up- and down-regulated DEGs in the shoots exposed to the binary mixture (Fig. 3H) was also higher than the number of up- and down-regulated DEGs in the shoots exposed to the single treatments (Fig. 3D and F).

In the present study, a correlation analysis was performed to assess the relationship between the outcomes obtained from the RNA-Seq and qRT-PCR for the DEGs (Fig. S3). The Pearson correlation coefficient, which was calculated to quantify the strength and direction of this relationship, was determined to be a value of 0.940. This high coefficient indicates a statistically significant correlation between the RNA-Seq and qRT-PCR results. Consequently, the transcriptome profile results were deemed reliable and accurate.

3.5.2. Functional divergence of genes involved in antioxidant system

To investigate the mRNA-level oxidative stress response, we examined the DEGs associated with antioxidant pathways in the roots (Fig. 4A and S4) and shoots (Fig. 4B and S5) of lettuce seedlings. These DEGs included homologs of SOD (Fig. 4), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GSH), laccase (LAC), lipoxygenase (LOX), and peroxidase (POD) (Fig. S4 and S5). Generally, the DEGs related to antioxidant pathways exhibited distinct expression patterns between the experimental groups and between the lettuce tissues. This suggests that gene expression plays a crucial role in maintaining cellular ROS homeostasis in the roots and shoots. We found that the expression of most of DEGs encoding APX, CAT, GSH, LOX, and POD in the roots and shoots was inhibited by a combination of G100 and T100. Moreover, the expression level of each gene in the roots and shoots exposed to the binary mixtures was generally lower than the expression levels in the case of exposure to the individual components of the mixtures. Thus, it can be seen that the mode of exposure of lettuce seedlings to GNPs and TiO_2 NPs, namely individual and binary exposure, affected gene expression. However, most of DEGs encoding LAC or SOD in the roots and shoots were found to be induced by a combination of G100 and T100. Meanwhile, there was a higher LAC or SOD gene expression in the roots and shoots exposed to the binary mixtures compared to the single components of the mixtures. It can therefore be concluded that these detoxifying enzyme genes could be involved in the initial resistance of lettuce seedlings to oxidative stress.

Notably, in response to GNPs + TiO_2 NPs stress, the expression of LAC was increased in the roots. LAC has been found widely in plants and can encode multicopper oxidases, which catalyze the oxidation of various substrates and reduce molecular oxygen to water (Niu et al., 2021). It was reported that antioxidant activity could be enhanced through LAC-mediated oligomerization of monomeric phenols (Botta et al., 2017). SOD is a crucial metalloenzyme family for the antioxidant defense system (Kula-Maximenko et al., 2022) and form the front line of defense against ROS-mediated injury (Kumar et al., 2022). As aforementioned, SOD-like genes in both the roots and shoots also showed a different expression pattern between individual and binary ENP exposure. Moreover, most of DEGs encoding SOD were highly expressed in the lettuce seedlings exposed to the combination of GNPs and TiO_2 NPs. This means that the activity of SOD was stimulated under the stress induced by exposure to GNPs + TiO_2 NPs. Furthermore, SOD might have scavenged superoxide anion ($O_2^{\cdot-}$), a highly reactive free radical, from the seedlings, reducing cellular damage from oxidative stress. This further validates the findings of biochemical tests, revealing changes in the SOD activity and the MDA contents. The combined exposure of GNPs with TiO_2 NPs did not trigger the formation of lipid peroxides in the seedlings (Fig. 2E), indicating that SOD effectively scavenged $O_2^{\cdot-}$ radicals. Consequently, the SOD activity was reinstated to control levels (Fig. 2C), leading to a balanced intracellular environment. Therefore, SOD constitutes an important antioxidant defense against oxidative stress induced by ENPs in lettuce seedlings.

3.5.3. GO enrichment analysis

The functional classification of DEGs in response to each treatment group was performed using gene ontology (GO) enrichment analysis. The GO terms were divided into three main categories, namely molecular function (MF), cellular component (CC), and biological process (BP). Fig. 5, S6, and S7 depict the top 30 most significantly enriched GO terms for the MF, CC, and BP. As shown in Fig. S6A, the DEGs enriched in the seedling roots exposed to GNPs were primarily associated with the MF of "sequence-specific DNA binding," the CC of "anchored component of membrane," and the BP of "protein ubiquitination." As shown in Fig. S7A, the DEGs enriched in the roots exposed to TiO_2 NPs were primarily associated with the "protein heterodimerization activity" in MF, "nucleosome" in CC, and "aromatic amino acid family biosynthetic process" in BP. Fig. 5A shows that the DEGs enriched in the roots exposed to GNPs + TiO_2 NPs were mainly associated with the "structural constituent of ribosome," "ribosome," and "translation" functions in terms of MF, CC, and BP, respectively. Note that the exposure groups involved the same MF (i.e., "sequence-specific DNA binding") and CC (i.e., "nucleosome," "chromatin," "protein-DNA complex," "DNA packaging complex," "chromosomal part," and "chromosome"). Furthermore, the degree of the same GO enrichments in the treatment group with GNPs + TiO_2 NPs was higher than that in both the GNPs and TiO_2 NPs.

As shown in Fig. S6B, the DEGs enriched in the seedling shoots exposed to GNPs were most involved in the MF of "sequence-specific DNA binding," CC of "proteasome core complex," and BP of "carbohydrate catabolic process." Fig. S7B shows that the DEGs enriched in the roots exposed to TiO_2 NPs were most involved in the functions of "calcium ion binding" in MF, "cell periphery" in CC, and "coenzyme metabolic process" in BP. Fig. 5B shows that the DEGs enriched in the roots exposed to GNPs + TiO_2 NPs were most involved in the "threonine-type endopeptidase activity" in MF, "proteasome core complex" in CC, and "catabolic process" in BP. The exposure groups had the same cost for MF

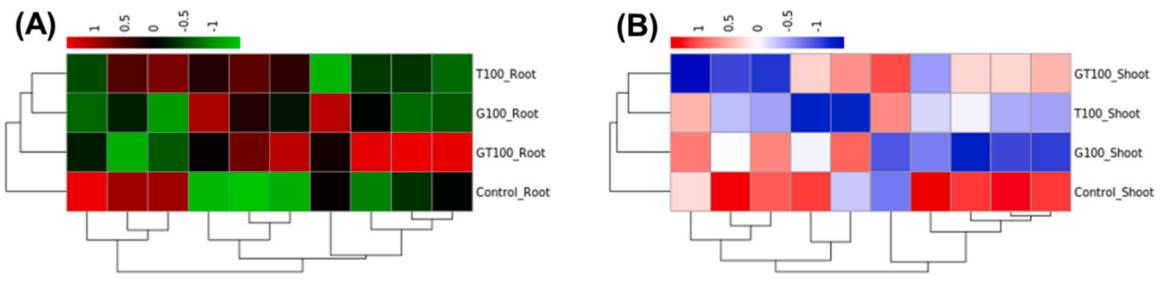

Fig. 4. Heat map showing the normalized expression of SOD-related DEGs in the roots (A) and the shoots (B) of lettuce seedlings (7 d) exposed to 100 mg/L GNPs (G100), 100 mg/L TiO_2 NPs (T100), and 100 mg/L GNPs + 100 mg/L TiO_2 NPs (GT100).

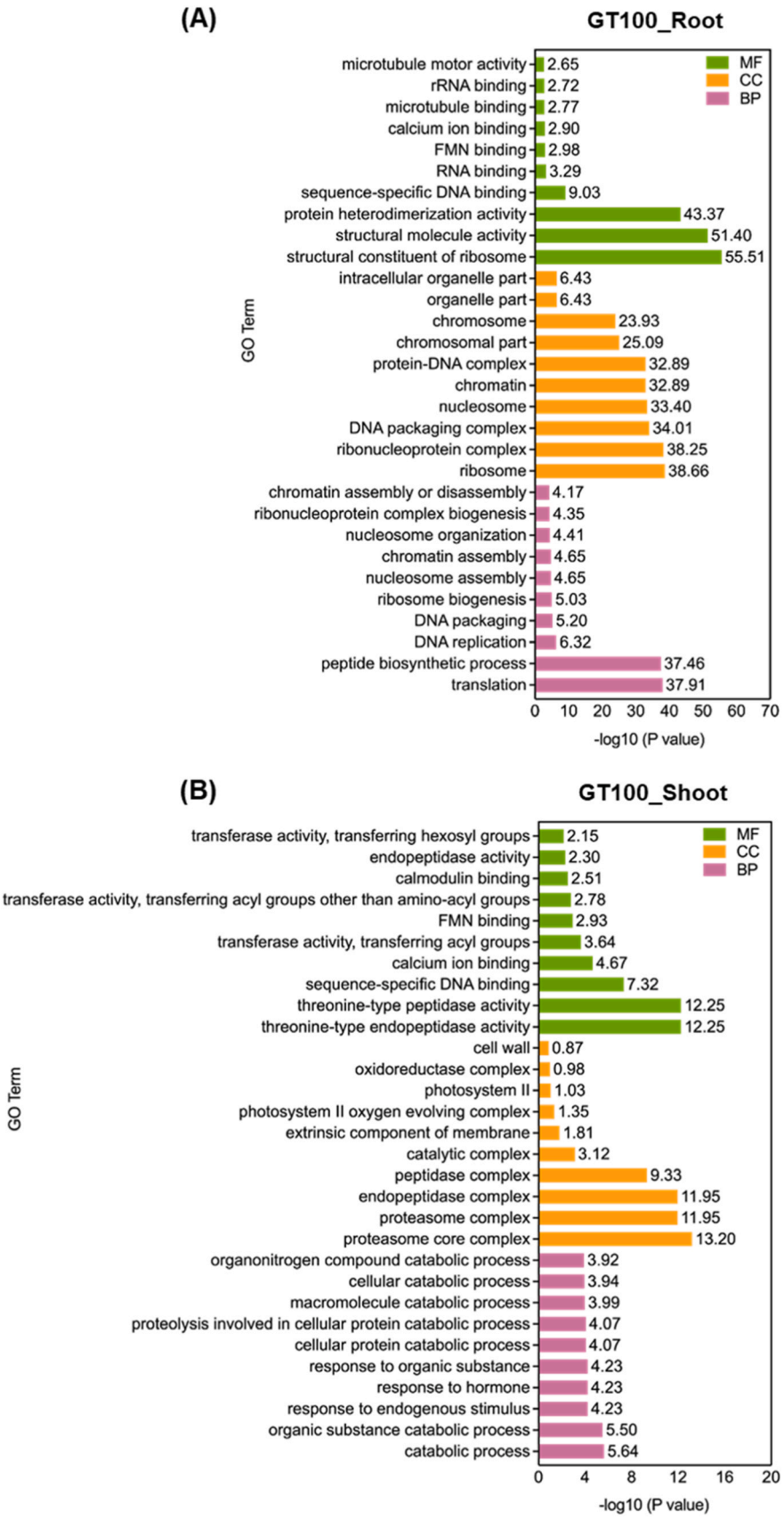

Fig. 5. Statistical classification of gene ontology annotation of DEGs in the roots (A) and the shoots (B) of lettuce seedlings (7 d) exposed to 100 mg/L GNPs + 100 mg/L TiO_2 NPs (GT100). MF: Molecular Function, CC: cellular component, and BP: biological process.

(i.e., "sequence-specific DNA binding"). Also, the degree of the same GO enrichments in GNPs + TiO_2 NPs was higher as compared to both the GNPs and TiO_2 NPs. Furthermore, we observed that the number of GO MF terms enriched in the roots exceeded those enriched in the shoots. Consequently, the functions diverged and were specifically expressed in the tissues of lettuce seedlings.

3.5.4. KEGG pathway analysis

To fully comprehend the intricate biological behavior of the transcriptome, the DEGs from various comparison groups were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. This analysis encompassed a range of biologically significant metabolic pathways. The role of DEGs was examined using the KEGG analysis, which revealed the top twenty pathways that were significantly enriched (Fig. 6, S8, and S9). The KEGG enrichment analysis demonstrated that these DEGs were involved in various signaling pathways. Specifically, the number of significantly ($p < 0.05$) regulated pathways in the roots of lettuce seedlings decreased in the following order: GNPs (Fig. S8A) > TiO_2 NPs (Fig. S9A) > GNPs + TiO_2 NPs (Fig. 6A). The three exposure groups share the same KEGG signaling pathways, specifically the "MAPK signaling pathway - plant" and "phenylpropanoid biosynthesis." Unlike the single groups of GNPs and TiO_2 NPs, GNPs + TiO_2 NPs showed significant regulation in several KEGG signaling pathways. These pathways include "DNA replication," "Ribosome biogenesis in eukaryotes," "Base excision repair," "Purine metabolism," "Arachidonic acid metabolism," "Mismatch repair," "ABC transporters," and "Biosynthesis of unsaturated fatty acids."

In the shoots of lettuce seedlings, the number of significantly ($p < 0.05$) regulated KEGG signaling pathways decreased in the following order: TiO_2 NPs (Fig. S8B) > GNPs + TiO_2 NPs (Fig. 6B) > GNPs (Fig. S9B). The three exposure groups share the same KEGG signaling pathways, namely "MAPK signaling pathway - plant," "Plant hormone signal transduction," "Phenylpropanoid biosynthesis," "alpha-Linolenic acid metabolism," and "Stilbenoid, diarylheptanoid, and gingerol biosynthesis." Unlike the single groups, the group treated with a combination of GNPs and TiO_2 NPs showed significant regulation in several KEGG signaling pathways. These pathways include "Alanine, aspartate and glutamate metabolism," "Phosphatidylinositol signaling system," "Fatty acid elongation," "Starch and sucrose metabolism," "Glycerophospholipid metabolism," and "Biosynthesis of various plant secondary metabolites."

In comparison, the enrichments of 8 KEGG signaling pathways were present in both the roots and shoots of lettuce seedlings after exposure to GNPs, while 7 KEGG signaling pathways were enriched in both the roots and shoots after exposure to TiO_2 NPs. In contrast to the single exposure, co-exposure to GNPs and TiO_2 NPs resulted in the identification of 3 KEGG signaling pathways that exhibited the same terms in both the roots and shoots. Thus, it can be concluded that the "MAPK signaling pathway - plant" and "phenylpropanoid biosynthesis" are the two most energy-intensive cellular processes in both the roots and shoots. These processes were found to be regulated and responsive to stress induced by the studied ENPs.

The MAPK signaling pathway is known to play a crucial role in regulating cellular functions, particularly in response to various stressors. Previous studies have shown that oxidative stress can activate the MAPK signaling pathway, leading to processes such as inflammation, apoptosis, and autophagy (Kim and Choi, 2015). One of the key indicators of oxidative stress is an increase in ROS, which are recognized as important regulators of the MAPK signaling pathway. In recent research, the MAPK signaling pathway in response to ENPs has been frequently observed in plants (Khan et al., 2023). In the present study, the KEGG enrichment analysis also identified the MAPK signaling pathway (Fig. 6, S8, and S9), indicating its involvement in the adaptation of lettuce seedlings to oxidative stress induced by ENPs.

As mentioned earlier, the "Phenylpropanoid biosynthesis" pathway was identified as one of the main stress response pathways in the lettuce seedlings. Diverse aromatic compounds such as benzenoids, coumarins, flavonoids, hydroxycinnamates, and lignin frequently contribute to the process of plant growth and development (Vanholme et al., 2010). The "Phenylpropanoid biosynthesis" pathway initiates with phenylalanine, serving as a precursor for the synthesis of the aromatic compounds (Vogt, 2010). The phenylpropanoid pathway is likely the most studied pathway of secondary metabolism in plants (Kundu et al., 2019). Furthermore, the "Phenylpropanoid biosynthesis" pathway is of significant importance in the cellular response to oxidative stress (Singh et al., 2023). Moreover, several derivatives or metabolites of phenylpropanoid in plant species are well known for their powerful antioxidant properties (Ekeuku et al., 2021). The increase in plant resistance is correlated with the multiple functions of phenylpropanoid compounds in plants, primarily consisting of their ability to scavenge ROS (Nhiem et al., 2011). Therefore, the phenylpropanoid metabolism pathway exhibited significant importance in the lettuce seedlings, as it contributed to the production of secondary products that played a crucial role in facilitating stress resistance responses.

In the present study, the combined exposure of GNPs and TiO_2 NPs did not have a significant phenotypic effect on seed germination and seedling growth of *L. sativa*. However, at the molecular level, this combination exerted a joint interaction on the root and shoot tissues of the lettuce seedlings. This molecular mechanism not only regulated the oxidative stress response in the plant cells but also resolved how the combined ENPs affected the expression pattern of key genes, signaling pathways, and the dynamic balance of the antioxidant defense system. To comprehensively explore this mechanism, future studies would need to integrate proteomics technology to meticulously analyze the changes in the expression products of key genes, thus revealing the fine regulatory network at the protein level. Meanwhile, comprehensive profiling using metabolomics technology would clearly illustrate the complete picture of metabolite changes in crops after combined exposure to ENPs, which is crucial for understanding how multiple ENPs interfere with and regulate plant metabolic pathways and how these metabolic changes are interconnected with oxidative stress responses. The aim of this study was to reveal the combined effect of GNPs and TiO_2 NPs on the crop plant at the molecular level, using ENPs with specific surface areas in the control experiment and in the mixture experiments. To further enhance the generalizability and specificity of the study, future studies would need to broaden the horizon and extend the research to different types and varieties of crops, as well as examine the performance of combinations of ENPs with different intrinsic properties (e.g., primary size, morphology, surface area, etc.) under various environmental conditions. Such a multidimensional study will not only help to verify the wide range and uniqueness of the combined effects of ENPs on crop plant growth but will also contribute to a more comprehensive understanding of the potential combined risk of multiple ENPs to plant ecosystems.

4. Conclusions

To sum up, we explored the molecular mechanisms associated with the physiological responses to oxidative stress in the roots and shoots of hydroponic lettuce seedlings exposed to GNPs and TiO_2 NPs, both individually and in combination. The level of oxidative damage observed in the roots of seedlings exposed to a combination of GNPs and TiO_2 NPs was less severe compared to the responses seen after exposure solely to GNPs or TiO_2 NPs. Using RNA sequencing technology, we found that the combination of GNPs and TiO_2 NPs resulted in a higher number of DEGs in the roots or shoots of the seedlings, compared to exposure to either GNPs or TiO_2 NPs alone. We then found that the DEGs related to antioxidant pathways exhibited functional divergence. Specifically, most of the DEGs encoding SOD exhibited elevated expression levels in the lettuce seedlings exposed to the combination of GNPs and TiO_2 NPs. Furthermore, the degree of enrichment of the same GO in the combined ENP mixtures was higher than the enrichment observed in the individual ENPs. Additionally, we identified that the signaling pathways, such as

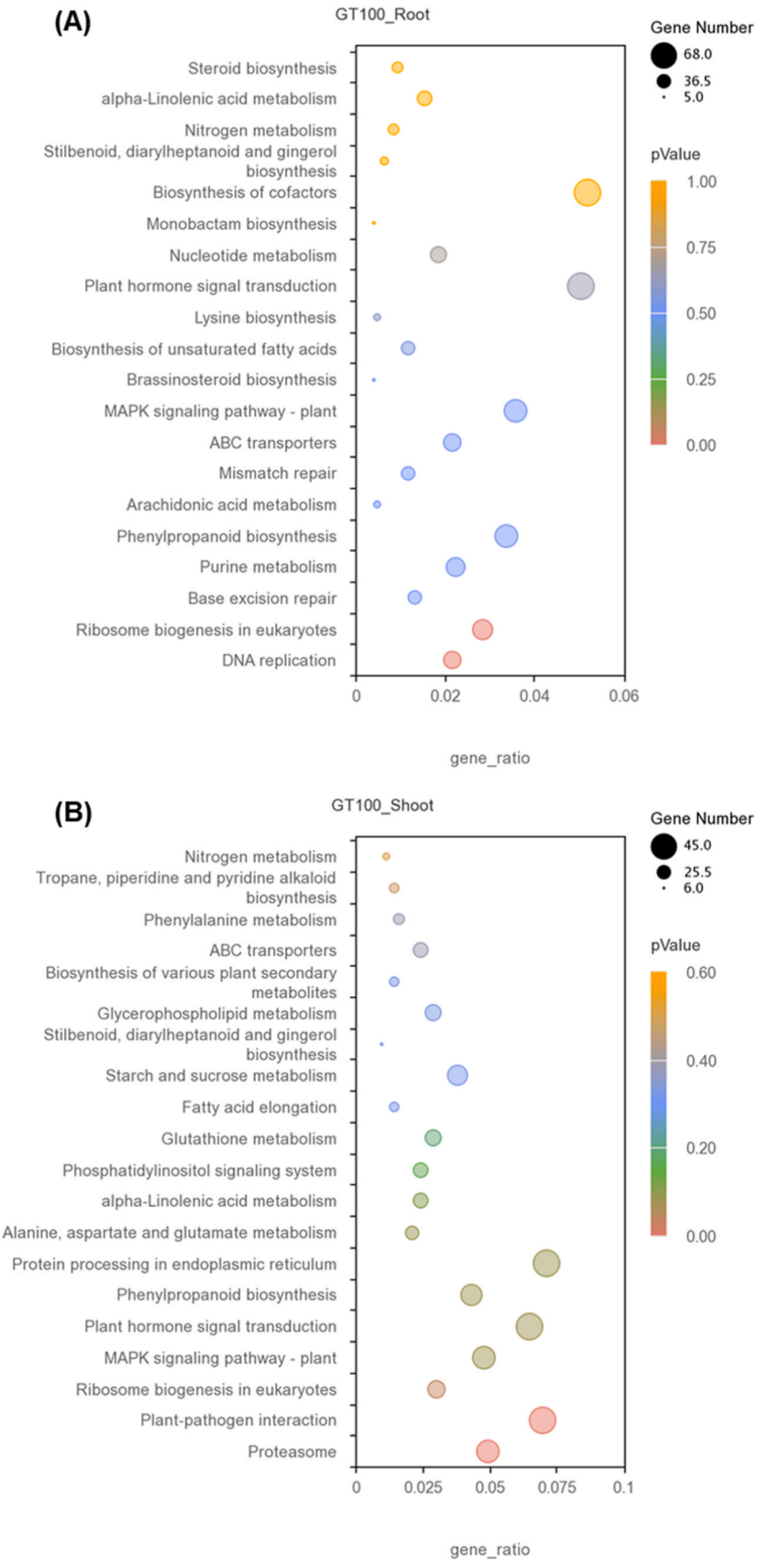

Fig. 6. KEGG pathway enrichment analysis of DEGs in the roots (A) and the shoots (B) of lettuce seedlings (7 d) exposed to 100 mg/L GNPs + 100 mg/L TiO_2 NPs (GT100).

the "MAPK signaling pathway-plant" and "phenylpropanoid biosynthesis" are closely associated with oxidative stress. These molecular-level findings are beneficial for evaluating and predicting the combined impacts of multiple ENPs on agricultural plants.

CRediT authorship contribution statement

Willie J.G.M. Peijnenburg: Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Xuancheng Yuan:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Zhuang Wang:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116761.

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