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Activation of antioxidant and detoxification gene expression in cucumber plants exposed to a $Cu(OH)_2$ nanopesticide†

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Cu-containing nanopesticides are increasingly being used as fungicides in modern agriculture. However, their fate, transport and toxicity in crop plants have been less studied. Here, we exposed 3 week-old cucumber plants cultivated in artificial media to different concentrations of a $Cu(OH)_2$ nanopesticide (0, 2.5 and 25 mg) for 7 d. The physiological and molecular responses were investigated. In order to elucidate the contribution of copper ions to the response, we also exposed the plants to $CuSO_4$. Results showed that the $Cu(OH)_2$ nanopesticide did not reduce the photosynthetic pigment production. In contrast, 10 mg Cu ions induced a significant decrease in photosynthetic pigment levels (around 25%) and leaf chlorosis symptoms. Foliar exposure to 25 mg $Cu(OH)_2$ nanopesticide induced significant changes in mRNA levels of antioxidant and detoxification-related genes; 6 genes (*SOD, GPX4, GPX, MDAR, POD, WRKY6*) were up-regulated up to 9-fold, and one (*cAPX*) was down-regulated by 32%. The $Cu(OH)_2$ nanopesticide at both dose levels (2.5 and 25 mg per plant) decreased the transcript production of a stress-related gene (*DNAJ*) by 40% and 80%, respectively. The up-regulation of the transcript levels of *SOD, GPX4, GPX, MDAR, POD,* and *WRKY6* and down-regulation of *DNAJ* was also observed in $CuSO_4$ treated plants (with increases of up to 7-fold), indicating that most of the responses are due to released copper ions. We postulate that the increased mRNA levels of antioxidants and detoxification enzymes reflect plant adaptation to over-generated reactive oxygen species (ROS) triggered by copper ions. The activated genes could serve as potential biomarkers of nanopesticide exposure and may be applicable to other plant/Cu nanopesticide interactions.

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† Electronic supplementary information (ESI) available: Physicochemical properties of the $Cu(OH)_2$ nanopesticide (Table S1); primers used for real-time RT-PCR assays (Table S3); essential (Table S3) and non-essential element concentrations (Table S4) in cucumber leaves treated with $CuSO_4$; effects of the $Cu(OH)_2$ nanopesticide (Fig. S1) and $CuSO_4$ (Fig. S3) on the biomass and root length of cucumber seedlings; image of cucumber leaves showing toxic symptoms due to Cu^{2+} induced damage (Fig. S2); Cu bioaccumulation in cucumber plants after foliar application of $Cu(OH)_2$ (Fig. S4) and $CuSO_4$ (Fig. S5) for 1 week; effects of the $Cu(OH)_2$ nanopesticide (Fig. S6) and $CuSO_4$ (Fig. S7) on photosynthetic pigment content; PCA analysis of gene expression in cucumber leaves exposed to the $Cu(OH)_2$ nanopesticide (Fig. S8); gene expression in cucumber leaves after foliar application of $CuSO_4$ (Fig. S9); effects of the $Cu(OH)_2$ nanopesticide and $CuSO_4$ on the total phenolic content in cucumber leaves (Fig. S10); dissolution kinetics of the $Cu(OH)_2$ nanopesticide (Fig. S11); sample preparation for SEM (Fig. S12). See DOI: 10.1039/c7en00358g

Introduction

The application of nanotechnology in modern agriculture for use in plant protection and nutrition has become increasingly popular in the past decade.[1,2] Among the nanoagrochemicals used in agriculture, nanopesticides have received greater attention than nanosensors or nanofertilizers.[3,4] Recently, many inorganic nanoparticles (NPs) (*e.g.*, Si,[5] TiO2,[6] Ag,[7] Cu,[8] CuO,[9] and Al[10]) have been found to have the capacity to suppress bacterial and fungal pathogens and are being increasingly considered in plant protection products. As these nanopesticides emerge in the market, understanding their bioaccumulation and toxicity in crop plants is of great importance for human and ecological health.[11]

Although several previous studies have been published related to nanopesticides applied to soils, the prescribed method for introducing copper-based nanopesticides is foliar application to protect fruits and leaves. For foliar sprayed pesticides, the leaf interface characteristics (trichomes, cuticular folds and wax crystals) are key factors impacting the adhesion and retention of pesticides on leaf surfaces.[12] For example, lotus leaves have a superhydrophobic surface; water

droplets with pesticide will not be retained on similar leaf surfaces.[13] Some pesticide formulators add a commercial surfactant to the mix to address this issue. Cucumber leaves have a thin film and trichomes which can entrap droplets, making it easier to deposit pesticides on their surfaces. Therefore, cucumber (*Cucumis sativus*) may accumulate more nanopesticide and be more vulnerable to contaminants compared to plants which have a large contact angle between the leaf surface and water droplets, *e.g.* maize.

Investigating the toxicity of nanoparticles in plants at a molecular level provides a more comprehensive and deeper understanding of plant response.[14–18] Using gas chromatography-mass spectrometry (GC-MS), Atha *et al.* observed DNA damage in radish (*Raphanus sativus*), perennial ryegrass (*Lolium perenne*), and annual ryegrass (*Lolium rigidum)* after exposure to 0–1000 mg L^{-1} of CuO NPs.[14] Using microarrays, Kaveh *et al.*[15] observed that some genes in *Arabidopsis thaliana* were up- or down-regulated in response to 0–20 mg L^{-1} of Ag NPs and Ag^{+}. More recently, Pagano *et al.* applied reverse transcription quantitative polymerase chain reaction (RT-qPCR) to investigate the transcriptional response of tomato (*Solanum lycopersicum*) and zucchini (*Cucurbita pepo*) to various NPs (CuO, CeO_2, and La_2O_3) (0–500 mg L^{-1}) and observed that some gene expressions were activated after exposure to the NPs, and an NP-specific response was noted.[16]

Copper is a redox-active metal; redox cycling between Cu(I) and Cu(II) results in production of reactive oxygen species (ROS) through Fenton and Harber–Weiss reactions.[19,20] Enzymatic antioxidants such as superoxide dismutase (*SOD*), catalase (*CAT*), and peroxidase (*POD*), and non-enzymatic antioxidants such as ascorbic acid, carotenoids, tocopherols and phenolics compose the plant antioxidant defense system, which can protect the plant by quenching excess ROS.[21] Our recent metabolomics study demonstrated that foliar application of a $Cu(OH)_2$ nanopesticide on lettuce leaves induced oxidative stress and triggered antioxidant and detoxification defenses. A number of low molecular weight antioxidants, such as *cis*-caffeic acid, chlorogenic acid, 3,4-dihydroxycinnamic acid and dehydroascorbic acid, were consumed to defend against oxidative stress.[17] The response of the enzymatic antioxidant defense system to oxidative stress induced by the $Cu(OH)_2$ nanopesticide has not been studied.

In this study, 3 week-old cucumber seedlings planted in artificial growth media were exposed *via* foliar application to different concentrations of a $Cu(OH)_2$ nanopesticide or $CuSO_4$ for 1 week. Dosing was normalized as mass (mg) of Cu applied per plant during the 1 week foliar exposure, for ease of comparison. To assess whether the $Cu(OH)_2$ nanopesticide or $CuSO_4$ elicited a noticeable toxicity response in the cucumber plants, the expression of 18 genes (*RBOH*, *MAPK1*, *MAPK3*, *WRKY30*, *WRKY6*, *HSP70*, *DNAJ*, *GST*, *POD*, *CAT*, *CAPX*, *MDAR*, *GPX4*, *GPX2*, *GPX*, *SOD*) related to regulatory and oxidative stress defense was determined using RTq-PCR. In addition, the total phenolics and carotenoids were analyzed. Physiological parameters (biomass, photosynthetic pigments, macro and micro-nutrient content) were also determined. By understanding the antioxidant enzyme gene expression and antioxidant levels, we aimed to determine at a molecular level the plant's strategy for increasing tolerance to stress induced by $Cu(OH)_2$ nanopesticides.

Materials and methods

$Cu(OH)_2$ nanopesticide

The $Cu(OH)_2$ nanopesticide used in this study was in the form of a commercial biocide (Kocide 3000, Dupont). The primary particle size is ~50 to >1000 nm.[22,23] The hydrodynamic diameter of Kocide 3000 in NanoPure water (pH 7) is 1532 ± 580 nm and the zeta potential is −47.6 ± 43 mV, measured *via* Dynamic Light Scattering (Malvern Zetasizer Nano ZS-90). The micronized particles in Kocide 3000 are made up of $Cu(OH)_2$ nanosheets, bound together by an organic composite that disassociates in water.[22] The copper content in Kocide 3000 is 26.5 ± 0.9%; the other elements detected by SEM-EDS are C, O, Na, Al, Si, S, and Zn.[22] The physicochemical properties of $Cu(OH)_2$ nanopesticide are presented in Table S1.† The dissolution kinetics of $Cu(OH)_2$ in different solutions indicates that up to around 30% of the initial Cu is released in days to weeks (Fig. S11†).[22]

Plant growth and experimental design

Cucumis sativus seeds were purchased from Seed Savers Exchange (Iowa, USA). One cucumber seed was sown 1 cm deep in each 0.5 L Poly-Tainer container (7.5 × 7.5 cm^2) containing sand (Quikrete Washed Plaster Sand), Sunshine Advanced Growing Mix#4 (SunGro Horticulture), vermiculite (Therm-O-Rock), coco coir (Canna), perlite (Therm-O-Rock), and 1 tablespoon of 4-4-4 fertilizer per gallon at a ratio of 1 : 3 : 1 : 2 : 2 by volume. For this mechanistic investigation, artificial growth media were used to exclude the extraneous variables introduced by soil exposure. A total of eighteen pots of cucumber seedlings were grown in a greenhouse for 3 weeks before foliar application of the $Cu(OH)_2$ nanopesticide. The temperature in the greenhouse was maintained at 28 °C by day and 20 °C by night. $Cu(OH)_2$ nanopesticide suspensions were prepared at 100 and 1000 mg L^{-1} in NanoPure water and sonicated (Branson 8800, Danbury, CT, USA) in a temperature control bath for 30 min until full dispersion prior to application. A hand-held spray bottle was used for spraying. Each spray was ~0.8 mL. During spraying, the spray bottle was set at 20 cm above the plants to ensure that the aerosols went directly onto the leaf surfaces. The cucumber plants were sprayed a total of 3 times per day for 7 days (7 days were selected because preliminary experiments showed early response started within 7 days); the amount sprayed each time was around 4 ml per pot (0.8 ml times 5). The total amount of nanopesticide suspension applied was 500 ml for 7 days at various concentrations, resulting in a total application of 0, 2.5 and 25 mg of Cu as $Cu(OH)_2$ nanopesticide per plant.

In order to elucidate the contribution of copper ions to the physiological or metabolic changes, another set of 3 week-old cucumber plants were exposed to different

concentrations of $CuSO_4$ solution (0, 10, 100 and 500 mg L^{-1}) for 1 week, corresponding to 0, 0.21, 2.1 and 10 mg Cu as $CuSO_4$ per plant.

Physiological analysis and Cu and element content

After 7 d of exposure to the $Cu(OH)_2$ nanopesticide or $CuSO_4$ at various levels, cucumber plants were harvested and thoroughly washed with deionized water to remove residual nanoparticles and soil particles. The shoot and root biomass and root length were measured. Then the shoot was divided into stems and leaves. After oven drying for 3 days at 70 ° C, dried tissues were digested with a mixture of 4 ml of H_2O_2 and 1 ml of plasma pure HNO_3 (v/v: 4 : 1) using a microwave oven system (Multiwave Eco, Anton Paar) at 180 °C for 1 hour. The standard reference materials NIST 1547 and 1570a were also digested and analyzed as samples. The recoveries for all elements were between 90 and 99%. Cu and 6 other micronutrients (Na, Mn, Fe, Ni, Zn, Mo), 4 macronutrients (Mg, P, K, Ca), and 13 non-essential elements (Al, Ti, V, Cr, Co, As, Se, Ag, Cd, Sb, Ba, Tl, Pb) were analyzed using inductively coupled plasma mass spectrometry (ICP-MS 7900, Agilent, USA). The standard solution was diluted from an ICP-MS environmental calibration standard (Agilent, USA), which contains 1000 mg L^{-1} each of Fe, K, Ca, Na, and Mg, and 10 mg L^{-1} each of Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Se, Tl, V, Zn, Th, and U in 10% HNO_3.

Photosynthetic pigments

Chlorophyll a and b and the total carotenoid content were determined based on Sesták *et al.* (1971) with some modifications.[24] Specifically, 0.01 g of cucumber leaves were immersed in 5 ml of 80% methanol for 12 h to extract the pigments. The mixture was centrifuged for 10 minutes at 3000 rpm. Absorbance at 666 and 653 nm was used for chlorophyll a and b, and at 470 nm for carotenoids. Results were expressed as mg of total chlorophyll or carotenoids per gram of fresh weight or per plant.

Total RNA extraction and antioxidant enzyme gene expression analysis

Cucumber leaves were frozen in liquid nitrogen and then ground using mortar and pestle. The resulting powders were stored at −80 °C. Total RNA was extracted from 0.05–0.1 g of freeze dried leaf powder using a Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO) following the supplier's recommendation. Three independent biological replicates per treatment were used. Traces of genomic DNA were removed using an On-Column DNase I Digest Set (Sigma-Aldrich). The quality and quantity of RNA were assessed using a Thermo Scientific Nanodrop Lite Spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse transcription (RT) reaction was performed using a High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Waltham, Massachusetts). For each RT reaction, the total RNA input was up to 1 μg. RT-qPCR was performed following an SYBR green I-based qPCR method; the basal PCR system used was GoTaq Hot Start DNA Polymerase (Promega).[25] The PCR reaction parameters were optimized as follows: the final concentration of SYBR green I was 0.5×; the final concentration of DMSO was 1%; the final concentration of $MgCl_2$ was 4 mM; the final concentration of dNTP was 0.4 mM and the final concentration of each primer was 0.5 mM. Triplicate 20 μl PCR reactions were set up and amplification was carried out using a CFX96 Real-Time System (Bio-Rad) using the following temperature profiles: 2 min at 95 °C, 40 cycles of 20 s at 95 °C, 20 s at 59 °C, 15 s at 72 °C plus plate reading, followed with a standard melting curve program. The qPCR data were processed and analyzed using the CFX Manager software (version 3.0). In order to control for differences in amounts of the starting material, the expression level of genes of interest was normalized by the expression level of actin (ACT1). The design of the primers for *Cucumis sativus* was based on the genome database Phytozome 11.0.5 (http://genome.jgi.doe.gov/cucumber/cucumber.home.html). The sequences of the primers are listed in Table S2.†

Determination of total phenolics

The total antioxidant capacity was determined following Singleton and Rossi's procedure (1965).[26] Ground cucumber leaf samples (0.01 g) were mixed with 5 mL of 80% methanol, and the mixture was placed in an end-over-end shaker on a Dayton-6Z412A Parallel Shaft (USA) roller mixer with a speed of 70 rpm at room temperature for 12 h to ensure full extraction. After centrifugation at 2000 g for 10 min, the supernatant was used to determine the total content of phenolic compounds.[26] Specifically, 50 μL of the methanolic extract was mixed with 450 μL of DI water and 250 μL of 2 M Folin–Ciocalteu reagent. The mixture was added to 1.25 mL of 20 g L^{-1} Na_2CO_3, incubated at 25 °C for 20 min and then centrifuged at 2000 g for 10 min. The supernatant absorbance was measured at 735 nm using a UV-vis spectrometer (Shimadzu UV-1800, Japan). The standard curve was prepared using gallic acid (GA) with a regression R^2 = 0.998. The absorbance was converted to phenolic content in terms of milligram of GA equivalent (GAE) per gram of dried weight (DW).

Scanning electron microscopy analyses

A Nova NanoSEM 650 scanning electron microscope (SEM) made by FEI, Hillsboro, OR was used to image $Cu(OH)_2$ particles on the surface of the nanopesticide-treated cucumber leaves. The SEM was operated in low vacuum mode, which allowed for direct analysis of fresh leaves without the need for chemical fixation. The SEM's beam voltage was set at 7 kV, and a chamber pressure of 0.68 Torr was used. Data were collected using a low vacuum detector (LVD) at a working distance of ~5 mm. The sample preparation process is shown in Fig. S12.† Specifically, a paper punch was used to carefully cut out sphere-shaped leaf fractions. The paper punch allowed us to cut around the perimeter of the circular leaf fractions without disturbing the leaf surface around the core,

where the SEM data were collected. The spherical leaf fractions were deposited onto aluminum SEM stubs using a thin layer of fast drying colloidal silver paint (Ted Pella, Redding, CA) and viewed under the microscope without sputtering.

Statistical analysis

The data are presented as the mean ± standard deviation of 6 replicates. Comparisons between groups with respect to biomass, photosynthetic pigments, mineral content and gene expression levels were carried out using one-way ANOVA followed by Tukey–Kramer post hoc tests, performed using SPSS.

Results and discussion

Biomass accumulation and root elongation

After foliar exposure to the $Cu(OH)_2$ nanopesticide for 7 d, no visible toxicity symptoms were observed in exposed cucumber plants. In addition, the root length and root and leaf biomass did not change significantly compared to the control (Fig. S1†).

In the $CuSO_4$ treatment, dosing with 0.21 and 2.1 mg Cu per plant did not induce visible toxicity symptoms; however, leaf chlorosis was observed on day 4 after spraying at a 10 mg Cu dose (yellow spots in Fig. S2†). Foliar chlorosis is a common initial toxicity symptom of excess of Cu in plants.[27] Similar to the $Cu(OH)_2$ nanopesticide, Cu^{2+} did not induce significant biomass reduction during the 7 d exposure (Fig. S3†), although obvious leaf chlorosis occurred at a higher $CuSO_4$ dose. It is likely that significant changes in growth rate and biomass would only be observed with longer exposure. It is noteworthy that dosing with 0.21 mg of $CuSO_4$ increased the root biomass ($p < 0.05$), indicating that a low dose of Cu promotes root growth.

Cu bioaccumulation in upper plant tissues

ICP-MS data showed that the Cu bioaccumulation in leaves after 7 d exposure was 18, 514 and 3855 mg Cu per kg dry weight (DW), from foliar spray of 0, 2.5 and 25 mg Cu as $Cu(OH)_2$ nanopesticide, respectively (Fig. S4†). Cu bioaccumulation in leaves treated with 0, 0.21, 2.1 and 10 mg Cu as $CuSO_4$ was 13, 70, 400 and 1391 mg Cu per kg DW (Fig. S5†). The Cu content was determined after harvested leaves were washed with deionized water, reflecting strongly bound or absorbed Cu as either nanopesticide or Cu^{2+} on or in the leaves. The Cu content in leaves treated with 25 mg Cu as $Cu(OH)_2$ nanopesticide was 2.8 times higher than for the 10 mg Cu^{2+} treatment, reflecting a similar Cu deposition rate regardless of whether Cu was introduced as a nanopesticide or in the ionic form. SEM imaging (Fig. 1) showed that cucumber leaf surfaces have many cuticles (Fig. 1A and D), which can easily entrap nanoparticles and other chemicals. After 24 hours of foliar application of nanopesticide, numerous small particles were attached to the leaf surface (Fig. 1E). The diameter of stomata in 3 week-old cucumber leaves is around 14 μm (Fig. 1C), which is large enough for nano-scale, even micro-scale, particles to enter through the guard cells.

In stems, the Cu content was 12, 78 and 771 mg Cu per kg DW for the 0, 2.5 and 25 mg $Cu(OH)_2$ nanopesticide treatments, respectively (Fig. S4†). The Cu detected in the stems may be translocated from leaf tissues or through direct diffusion from the stem epidermis to the xylem, because the stems were also exposed to $Cu(OH)_2$ during spraying. Cu bioaccumulation in stems treated with 0, 0.21, 2.1 and 10 mg Cu as $CuSO_4$ was 6.6, 12, 67 and 108 mg Cu per kg DW (Fig. S5†), respectively.

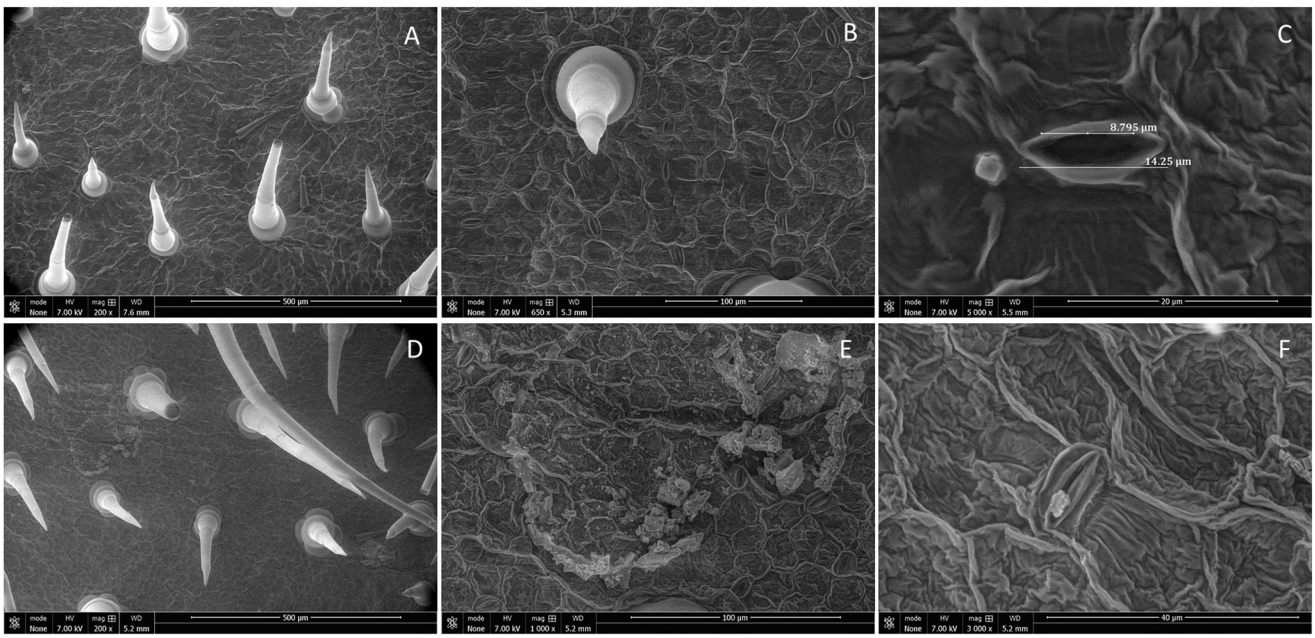


Fig. 1 SEM micrographs of the surface of cucumber leaves reveal the adaxial cuticle morphology: control without exposure to nanopesticide (A–C); 24 h after exposure to $Cu(OH)_2$ nanopesticide (D–F).

Translocation to roots

Cu in roots did not increase with foliar application of the $Cu(OH)_2$ nanopesticide, and in fact, decreased slightly with increasing $Cu(OH)_2$ dose (Fig. S4†). The Cu concentration in roots was 218, 180 and 162 mg Cu per kg DW for the control, and 2.5 mg and 25 mg Cu with nanopesticide treatments. Cu in leaves exposed to 25 mg $Cu(OH)_2$ nanopesticide markedly decreased (25.6%, $p = 0.051$) compared to the control. This indicates that neither $Cu(OH)_2$ particles nor dissolved Cu ions were significantly translocated from leaves and stems to roots. Similarly, we did not observe higher levels of Cu in cucumber roots after foliar application of $CuSO_4$ (Fig. S5†), which suggests that Cu ions were not translocated from aerial parts to the roots. These results indicate that Cu has very poor basipetal mobility in cucumber plants. This is in contrast to studies that demonstrated the translocation of Cu from CuO NPs from upper parts to roots *via* the phloem in maize[28] or eggplants[29] and suggests species specificity for this process.

It is also possible that cucumber plants have a strategy for sequestering Cu in leaves and stems, chelating Cu^{2+} with amino acids or organic acids.[30] However, this mechanism cannot entirely explain the decrease in root Cu concentrations induced by the $Cu(OH)_2$ nanopesticide. We hypothesize that the gene regulation of copper transporters was altered. *COPT1* is a high-affinity transporter specific for the Cu ion and it plays an important role in Cu acquisition by roots.[31] Yruela[32] reported that the expression of the *COPT1* gene is negatively regulated by increasing Cu. Therefore, it is possible that the copper transporter protein was down-regulated in the roots due to the excess Cu in the shoots. However, in the $CuSO_4$ treatment, the Cu concentrations remain unchanged. This suggests that the *COPT1* gene expression was not altered in this treatment. Further studies are needed to identify the underlying mechanism.

Photosynthetic pigment changes

Photosynthetic pigments, which are responsible for capturing solar energy, are also sensitive indicators of stress.[19] Results showed that the $Cu(OH)_2$ nanopesticide at both levels did not induce a decrease in photosynthetic pigment levels (Fig. S6†). After exposure to 25 mg $Cu(OH)_2$ nanopesticide, chlorophyll a and b production was increased by 51% ($p = 0.059$) and 38% ($p = 0.058$), respectively, compared to the control. In contrast, for the $CuSO_4$ treatment, chlorophyll a and b and carotenoid concentrations did not change significantly at lower (0.21 and 2.1 mg Cu) doses, but they significantly decreased (26.2%, 25.4% and 24%, respectively) ($p < 0.01$) at the 10 mg dose (Fig. S7†). This is consistent with the observed foliar chlorosis symptoms after the 10 mg Cu as $CuSO_4$ treatment (Fig. S2†). Chlorosis is due to the loss of chlorophyll. Protochlorophyllide reductase, an enzyme involved in the reduction of protochlorophyll to chlorophyll, is well known to be inhibited by copper.[33] The inhibition of the reductive steps in the biosynthetic pathways of photosynthetic pigments is due to the high redox potential of many heavy metals.[34,35] These results demonstrate that the impact of ionic copper on the photosynthetic pigments is different from that of the $Cu(OH)_2$ nanopesticide, which at higher doses enhanced the biosynthesis of the photosynthetic pigments *via* an unknown mechanism, while a high dose of ionic Cu resulted in chlorosis. The difference likely reflects the difference in Cu^{2+} release rates or also a nano-specific response or process.

Effect of $Cu(OH)_2$ nanopesticide and Cu^{2+} on macro and micro nutrients

Essential nutrients for normal plant growth are composed of macro (N, P, K, Ca, Mg, S) and micronutrients (Fe, Mn, Zn, Cu, Mo, and Ni). There are also non-essential elements with unknown biological and physiological functions (*e.g.* Cd, Sb, Cr, Pb, As, Co, Ag, Se). As shown in Tables 1 and 2, exposure to 25 mg Cu from the $Cu(OH)_2$ nanopesticide significantly increased the concentration of a number of elements including Na, K, Fe, Al, Co, Ni, V, and Ag. A previous study found that the commercial $Cu(OH)_2$ nanopesticide contains Na and Al.[22] Additional ICP-MS analysis showed that in addition to Na and Al, Ni and a number of non-essential elements such as Ti, V, Co, Se, Ag and Pb were also present in the $Cu(OH)_2$ nanopesticide, which explains the increased concentration of these elements in cucumber leaves.

Fe and Mo, which are not present in the nanopesticide, also changed significantly. Fe increased by 61% ($p < 0.01$) in leaves exposed to 25 mg nanopesticide, compared to the control. The mechanism for the increased Fe in leaves in response to the $Cu(OH)_2$ nanopesticide is unknown. In addition, the concentration of Mo in stems and roots decreased by 17% and 24%, respectively ($p < 0.05$), indicating that foliar application of the nanopesticide decreased the Mo uptake *via* the roots. Mo is needed to convert nitrate to ammonia within the plant, and is an essential component of two enzymes involved in nitrogen metabolism.[36] Less Mo may cause reduced availability of molybdoenzymes, such as nitrate reductase and nitrogen-fixing nitrogenase, impacting nitrogen metabolism with unknown impacts on the plants.[36]

As shown in Table S3,† foliar application of $CuSO_4$ significantly increased the concentration of a number of macro nutrients in leaves, including Mg, P, K, Ca, and Fe, in a dose-dependent manner. Interestingly, the concentration of Mg, P, K, and Fe in roots significantly decreased at the 10 mg $CuSO_4$ dose ($p < 0.01$). This indicates that plants attempted to translocate more of these ions from roots to leaves. The up-regulation of metal ions in leaves may reflect osmotic adjustment to maintain normal metabolism, and could be regarded as a protective mechanism in response to excessive Cu.[37] As mentioned before, the $Cu(OH)_2$ nanopesticide did not induce the alteration of macro nutrients in leaves, possibly due to the slower release of Cu^{2+}, as supported by previous dissolution experiments.[22] In addition, exposure to the $Cu(OH)_2$

Table 1 Effect of $Cu(OH)_2$ nanopesticide at different doses (0, 2.5 and 25 mg as Cu) on cucumber plant essential nutrients (mg per kg DW)

	Na	Mg	P	K	Ca	Mn	Fe	Mo
Leaf								
Control	1589 ± 276	130 189 ± 1147	9396 ± 384	46 070 ± 3030	34 287 ± 3396	70 ± 8	96 ± 4.5	0.43 ± 0.126
2.5 mg	1719 ± 542	12 583 ± 665	8907 ± 593	46 909 ± 2124	33 660 ± 1679	72 ± 11	108 ± 11.4	0.29 ± 0.041
25 mg	2542 ± 382**	12 043 ± 2300	9446 ± 1078	47 076 ± 6902	32 437 ± 7282	72 ± 15	155 ± 23**	0.36 ± 0.076
Stem								
Control	8249 ± 1315	6226 ± 698	13 843 ± 654	118 125 ± 3455	14 054 ± 753	28 ± 3	49 ± 19.1	0.21 ± 0.028
2.5 mg	9120 ± 1256	6030 ± 667	13 663 ± 503	130 259 ± 7582**	14 364 ± 746	32 ± 5	54 ± 10.6	0.17 ± 0.055*
25 mg	9520 ± 2147	5825 ± 224	14 452 ± 972	130 507 ± 7567**	14 794 ± 339	30 ± 4	51 ± 8.4	0.17 ± 0.018
Root								
Control	14 954 ± 2493	5617 ± 966	10 445 ± 1108	51 720 ± 7120	11 879 ± 487	82 ± 22	533 ± 92.7	0.44 ± 0.029
2.5 mg	16 962 ± 2654	6213 ± 1450	9874 ± 436	53 927 ± 3057	11 185 ± 572	67 ± 15	830 ± 1004	0.39 ± 0.041
25 mg	14 011 ± 3362	5274 ± 969	9672 ± 1032	48 812 ± 6508	10 795 ± 374	82 ± 50	443 ± 204	0.33 ± 0.061*

All data are mean ± SD (n = 6). *$P < 0.05$, **$P < 0.01$, as compared to the control. DW means dry weight.

Table 2 Effect of $Cu(OH)_2$ nanopesticide at different doses (0, 2.5 and 25 mg as Cu) on cucumber plant essential nutrients (mg per kg DW)

	Al	Co	Ni	Ti	V	Ag	Pb
Leaf							
Control	18 ± 5.5	0.060 ± 0.009	0.541 ± 0.13	218 ± 48	0.034 ± 0.009	0.004 ± 0.003	0.083 ± 0.018
2.5 mg	31 ± 5.8*	0.065 ± 0.014	0.794 ± 0.6	347 ± 159	0.041 ± 0.011	0.007 ± 0.002	0.16 ± 0.161
25 mg	153 ± 510**	0.13 ± 0.024**	1.08 ± 0.36	699 ± 143**	0.079 ± 0.018*	0.033 ± 0.011**	0.18 ± 0.042
Stem							
Control	19 ± 22	0.11 ± 0.009	0.219 ± 0.05	2350 ± 4342	0.552 ± 1.062	0.012 ± 0.003	0.071 ± 0.022
2.5 mg	31 ± 14	0.672 ± 0.617**	0.213 ± 0.06	336 ± 158	0.062 ± 0.043	0.008 ± 0.001	0.074 ± 0.016
25 mg	40 ± 17*	0.373 ± 0.145**	0.291 ± 0.11	304 ± 88	0.031 ± 0.013	0.012 ± 0.004	0.075 ± 0.027
Root							
Control	669 ± 181	1.08 ± 0.149	2.65 ± 0.54	4454 ± 1321	1.16 ± 0.168	0.011 ± 0.077	1.28 ± 0.212
2.5 mg	803 ± 561	1.32 ± 0.331	2.61 ± 0.66	5287 ± 3658	1.51 ± 1.321	0.045 ± 0.021	1.17 ± 0.292
25 mg	491 ± 182	1.08 ± 0.13	1.87 ± 0.51	3105 ± 1227	1.24 ± 0.938	0.034 ± 0.015*	1.00 ± 0.171

All data are mean ± SD (n = 6). *$P < 0.05$, **$P < 0.01$, as compared to the control. DW means dry weight.

nanopesticide induced a decrease in Mo in cucumber roots and stems, while ionic Cu did not impact Mo uptake in roots. In addition, Na was significantly decreased ($p < 0.05$) in all the tissues (root, stem and leaf) when plants were exposed to 10 mg $CuSO_4$ (Table S3†). It is interesting to note that the K^+/Na^+ ratio in leaves increased with Cu^{2+} dose in a dose-dependent fashion ($p < 0.01$) (data not shown). Wang *et al.*[38] proposed that maintaining a high cytosolic K^+/Na^+ ratio (increasing K^+ and preventing Na^+ from accumulating in the leaves) is critical for plant growth and salt tolerance. This can explain the above-mentioned decrease in Na in all tissues. Plants decrease Na uptake to increase the K^+/Na^+ ratio as an active protection mechanism. Taken together, these results demonstrate that the $Cu(OH)_2$ nanopesticide and ionic Cu differ in their impacts on elemental nutrient uptake in cucumber plants.

Changes in gene expression in response to $Cu(OH)_2$ nanopesticide

Antioxidant enzymes, which play a major role in quenching ROS, are an important component of a plant's defense system. Therefore, high expression of antioxidant genes is hypothesized to be a protective response to ROS generated by copper ions released from the $Cu(OH)_2$ nanopesticide. We examined the expression of 16 stress-responsive genes (*RBOH*, *MAPK1*, *MAP3K3*, *WRKY30*, *WRKY6*, *HSP70*, *DNAJ*, *GST*, *POD*, *CAT*, *CAPX*, *MDAR*, *GPX4*, *GPX2*, *GPX*, *SOD*) using RT-qPCR. Since a previous study found that soil-based exposure to Cu NPs inhibited the uptake of Fe in cucumber leaves,[39] we also analyzed two Fe uptake related genes (*FRO4* and *IRT1*). Principal component analysis (PCA) was applied to the gene expression datasets to help identify general similarities and differences between the control and $Cu(OH)_2$ treated groups. The PCA score plot (Fig. S8†) shows that the control and nanopesticide treated plants were clearly separated along principal component 1 (PC1), especially at the high dose (25 mg), indicating that the gene expression profile changed. A heat map (Fig. 3) also indicates a trend in up-regulation upon exposure to 25 mg Cu from the nanopesticide compared to the control. The one-way ANOVA analysis indicated that 8 out of the 18 genes studied were significantly up- or down-regulated in cucumber leaves, mostly in the 25 mg nanopesticide treatment (Fig. 3).

Antioxidant genes (*SOD*, *CAT*, *POD*, *GPX*, *cAPX*, *MDAR*). We found that dosing with 25 mg $Cu(OH)_2$ resulted in an increased expression level of *SOD*, *GPX4*, *GPX*, *MDAR*, and *POD* genes by up to 9-fold (Fig. 3). It is reported that *SOD*, *CAT*,

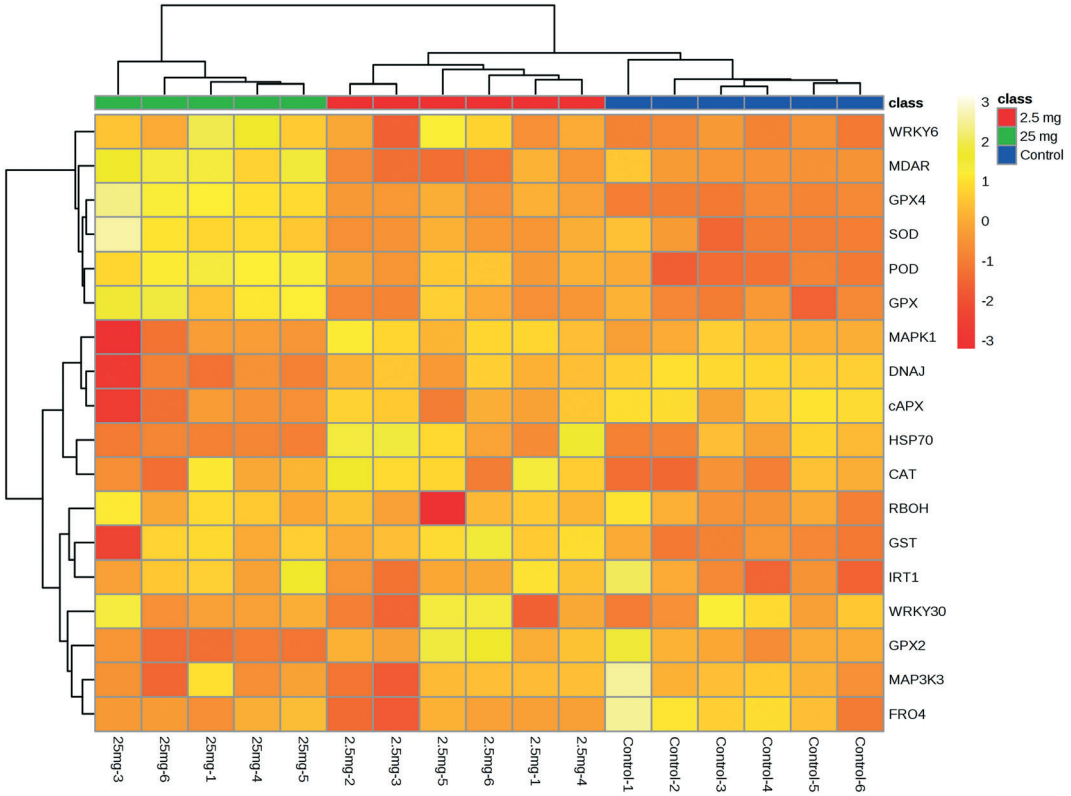


Fig. 2 Heatmap illustrating the expression level of 18 transcripts in all samples exposed to the $Cu(OH)_2$ nanopesticide at different doses (0, 2.5 and 25 mg as Cu). The data presented are log 10 transformed read counts per transcript. MetaboAnalyst 3.0.

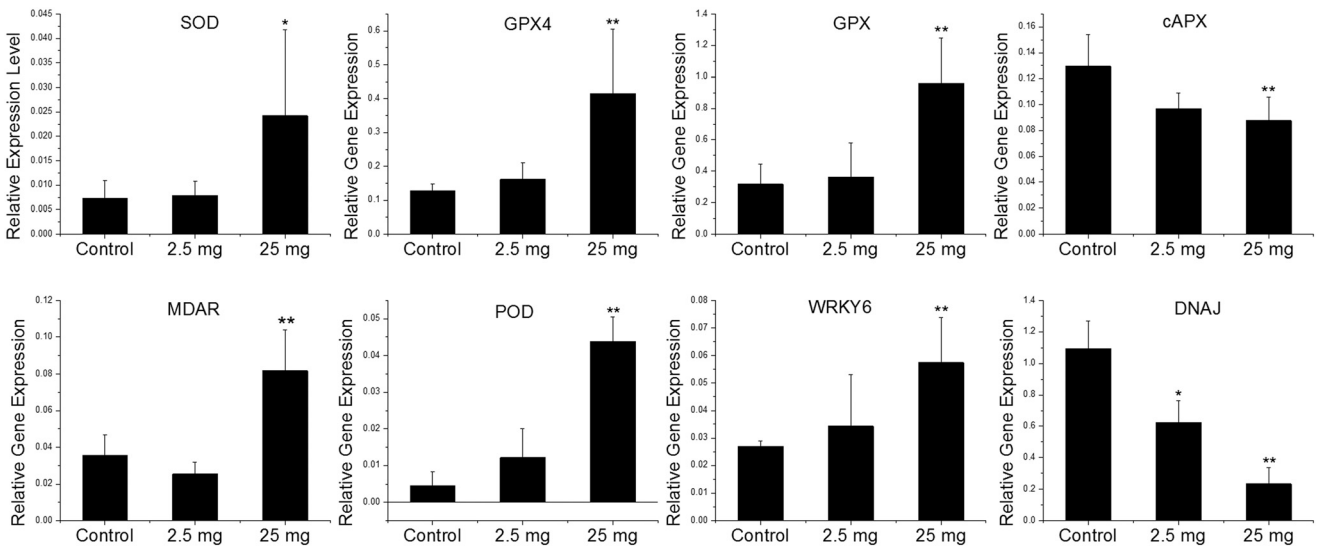


Fig. 3 Expression levels of eight significantly changed genes in cucumber leaves after foliar application of nanopesticide for 1 week at different doses (0, 2.5 and 25 mg as Cu). The data are the means of six replicates. Error bars represent the standard deviation. $*P < 0.05$, $**P < 0.01$, as compared to the control.

and *GPX* are primary scavenger enzymes involved in detoxifying ROS in mammalian systems.[40,44,45,48] *SOD* acts as the first line of defense against ROS by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2.[41] *GPX*, *APX*, *MDAR*, *CAT* and *POD* work to further convert H_2O_2 to nontoxic H_2O through different reactions (Scheme 1). *GPX* utilizes glutathione (*GSH*) as an electron donor to reduce ROS.[43] *APX* enzymes play a key role in catalyzing the conversion of H_2O_2 into H_2O using ascorbate as an electron donor.[47] *MDAR* helps to scavenge the monodehydroascorbate radical and generate dehydroascorbate

(*DHA*), the oxidized form of ascorbate.[50] *APX* activity generally increases along with the activities of *CAT*, *SOD* and *GSH* reductase in response to environmental stress.[49] Our results showed that *cAPX* (cytosol *APX*) was significantly decreased by 32% ($p < 0.01$) in response to 25 mg of $Cu(OH)_2$ nanopesticide (Fig. 3). Li *et al.*[46] also reported that the activity of *APX* was decreased in Chinese cabbage (*Brassica campestris* ssp. *chinensis Makino*) after copper treatment.

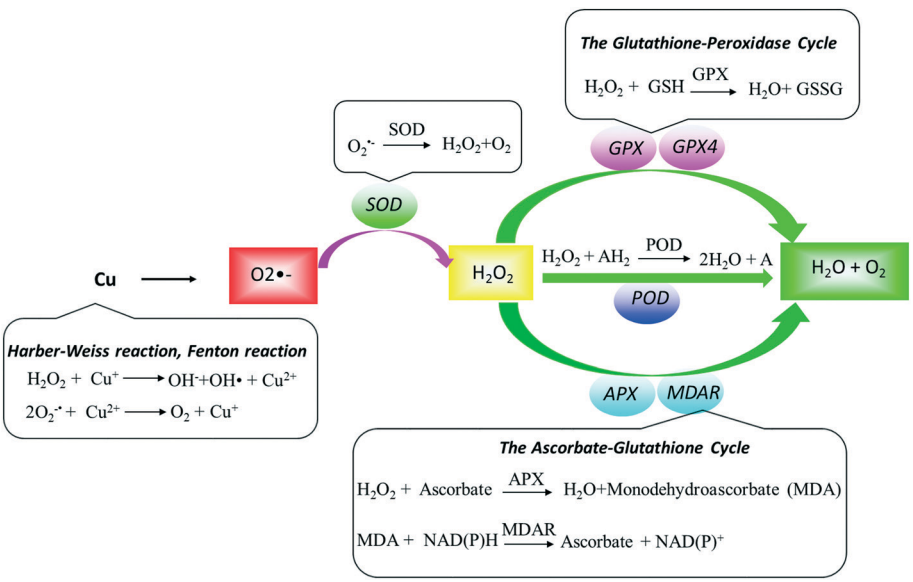
Peroxidase (*POD*) is another commonly reported defense-related enzyme gene, which has been reported to respond to Cu induced stress.[21] As shown in Fig. 3, the transcript levels of *POD* at a dose of 25 mg $Cu(OH)_2$ nanopesticide were increased almost 9-fold compared to the control ($p < 0.01$). *CAT* has been reported to directly decompose H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification.[42] However, the expression level of *CAT* was unchanged by the $Cu(OH)_2$ nanopesticide. In summary, the activation of some ROS scavenger enzyme genes provides evidence that the nanopesticide triggered excessive production of ROS and induced oxidative stress. The activation of genes related to antioxidation and detoxification suggests an active and positive response to oxidative stress generated by the nanopesticide.

Detoxification related genes (*WRKY* and *GST*). The *WRKY* gene family appears to play important roles in the regulation of transcriptional reprogramming associated with plant stress responses.[54] In this study, the induction of *WRKY6* gene transcripts was 1.1 times higher in leaves treated with 25 mg nanopesticide than in the control ($p < 0.01$) (Fig. 3). Previous studies have demonstrated that many *WRKY* genes behave strongly and rapidly induce expression in response to certain abiotic stresses, such as wounding, drought or salinity, indicating their regulatory function in these signaling pathways.[55,56] These results suggest that at higher doses plants up-regulate these detoxification-related genes to enhance tolerance to $Cu(OH)_2$ nanopesticide.

Glutathione S-transferases (*GSTs*) are ubiquitous enzymes that play key roles in detoxifying oxidative-stress metabolites.[51] *GSTs* are known for their detoxification of xenobiotics by catalyzing the conjugation of the reduced form of glutathione (*GSH*) to non-toxic peptide derivatives.[52] *GSTs* can also act as antioxidants by tagging oxidative degradation products for removal or by acting as a glutathione peroxidase to directly scavenge peroxides.[53] However, the expression level of *GST* was unchanged at either dosing of the $Cu(OH)_2$ nanopesticide.

Regulatory genes (*RBOH*, *MAPK1*, and *MAP3K3*). *RBOH*, *MAPK1*, and *MAP3K3* are regulatory genes. In this study, the expression levels of *RBOH*, *MAPK1* and *MAP3K3* were not significantly changed at any exposure dose (data not shown), although previous reports showed multiple functions of these genes. The *RBOH* gene family has been reported to play important roles in plant development, defense reactions and hormone signaling.[57] The tobacco *RBOH* gene is responsible for ROS production in cryptogein-elicited tobacco cells.[58] In addition, the *Arabidopsis* NADPH oxidase *RBOHD* mediates rapid, long-distance, cell-to-cell signaling, which can be triggered by diverse stimuli, including wounding, heat, cold, high-intensity light and salinity stresses.[59] *MAPK* plays an important role for cell survival under oxidative stress.[60]

Heat response genes (*HSP70* and *DNAJ*). Abiotic stress usually induces protein dysfunction. Heat-shock proteins (HSPs) play important functions in assisting protein refolding under stressful conditions; thus expression usually increases in response to stress.[61] In this study, both *DNAJ* (*HSP40*) and *HSP70* were not overexpressed when exposed to the $Cu(OH)_2$ nanopesticide. In contrast, *DNAJ* expression levels decreased in a dose-dependent fashion with the $Cu(OH)_2$ nanopesticide (Fig. 3), suggesting *DNAJ* expression was inhibited. Leng



Scheme 1 The generation of reactive oxygen species by Cu ions from the $Cu(OH)_2$ nanopesticide and antioxidant enzyme reactions.

et al.[62] investigated the transcriptome response of grapevine to copper stress using RNA-seq and found that high molecular weight *HSPs* (*HSP70*, *HSP90*, *HSP101*) were down-regulated, while *HSPs* 16–30 kDa were up-regulated.

Changes in gene expression in response to ionic Cu

Exposure to Cu^{2+} from $CuSO_4$ significantly changed the transcript levels of 14 genes (Fig. S9†). The expression levels of antioxidant (*SOD*, *CAT*, *GPX*, *MDAR*, *POD*) and detoxification (*GST*, *WRKY*) related genes were up-regulated when exposed to 10 mg Cu from $CuSO_4$, which is quite similar to the response to the $Cu(OH)_2$ nanopesticide at 25 mg Cu. The heat shock response gene (*DNAJ*) also decreased with increasing $CuSO_4$ dose, consistent with the nanopesticide results. *MDAR* transcript levels responded to ionic Cu even at very low doses (0.21 mg Cu). These results indicate that these transcriptome changes induced by the $Cu(OH)_2$ nanopesticide may be mainly due to the release of Cu^{2+}.

However, the abundance of c*APX* was significantly increased ($p < 0.01$) after exposure to 10 mg Cu, which is quite different from the response to exposure to the nanopesticide, which decreased c*APX*. In addition, the nanopesticide did not induce regulatory gene expression (*RBOH and MAPK*), while 10 mg Cu significantly induced the gene expression of *RBOH* and *MAP3K3* (Fig. S9†). *MAPK* cascades play pivotal roles in intra- and extra-cellular signaling of plant defense mechanisms.[63] It has been reported that hydrogen peroxide activates the *MAP3K ANP1*, *AtMPK6* and related *AtMPK3* genes.[64] Interestingly, in this study, exposure to different doses of $CuSO_4$ did not change the expression of *MAPK1* but increased the expression of *MAP3K3*, suggesting that ROS stress regulation in cucumber due to Cu^{2+} is through the *MAP3K3* pathway and not *via MAPK1*.

Low molecular weight antioxidant

In addition to the above-mentioned antioxidant enzymes, the antioxidant defense system includes non-enzymatic components. Previous studies reported that some low molecular weight molecules such as carotenoids, phenolic compounds, ascorbic acid, and glutathione (GSH) can scavenge ROS through donated electrons.[65] The $Cu(OH)_2$ nanopesticide did not affect the content of carotenoids (Fig. 2) or total phenolics (Fig. S10A†) at any of the exposure doses. However, ionic Cu dosed at 10 mg significantly decreased the levels of carotenoids ($p < 0.01$) and total phenolics ($p < 0.05$) (Fig. S6 and S10B†), which may indicate the impairment of the non-enzymatic antioxidant defense system by ionic Cu at higher doses. Dehydroascorbic acid, the oxidized form of ascorbic acid, was unchanged compared to the control (data not shown) when exposed to the $Cu(OH)_2$ nanopesticide. These data indicate that antioxidant enzymes play a more important role in dealing with $Cu(OH)_2$ nanopesticide induced oxidative stress compared to non-enzymatic components, which is different from the behavior of higher doses of ionic Cu that also affect the non-enzymatic components.

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