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Reduced Silver Nanoparticle Phytotoxicity in *Crambe abyssinica* with Enhanced Glutathione Production by Overexpressing Bacterial γ-Glutamylcysteine Synthase

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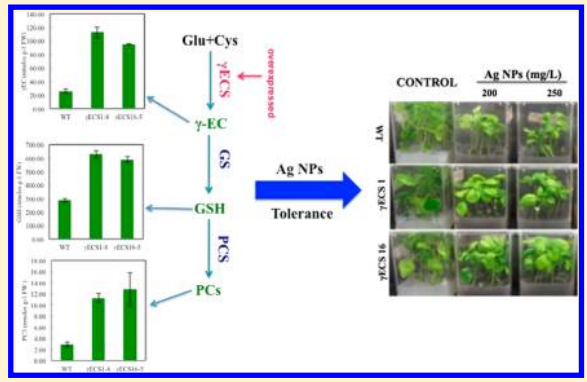
Supporting Information

ABSTRACT: Silver nanoparticles (Ag NPs) are widely used in consumer products, and their release has raised serious concerns about the risk of their exposure to the environment and to human health. However, biochemical mechanisms by which plants counteract NP toxicity are largely unknown. We have previously engineered *Crambe abyssinica* plants expressing the bacterial γ-glutamylecysteine synthase (γ-ECS) for enhancing glutathione (GSH) levels. In this study, we investigated if enhanced levels of GSH and its derivatives can protect plants from Ag NPs and $AgNO_3$ (Ag^+ ions). Our results showed that transgenic lines, when exposed to Ag NPs and Ag^+ ions, were significantly more tolerant, attaining a 28%−46% higher biomass and 34−49% more chlorophyll content, as well as maintaining 35−46% higher transpiration rates as compared to those of wild type (WT) plants. Transgenic γ-ECS lines showed 2−6-fold Ag accumulation in shoot tissue and slightly lower or no difference in root tissue relative to levels in WT plants. The levels of malondialdehyde (MDA) in γ-ECS lines were also 27.3−32.5% lower than those in WT Crambe. These results indicate that GSH and related peptides protect plants from Ag nanotoxicity. To our knowledge, this is the first direct report of Ag NP detoxification by GSH in transgenic plants, and these results will be highly useful in developing strategies to counteract the phytotoxicty of metal-based nanoparticles in crop plants.



1. INTRODUCTION

Nanotechnology has been applied in a diverse range of industries, including pharmaceuticals, cosmetics, electronics, and agriculture.[1−3] It is widely known that at nanoscale size dimensions (<100 nm), the physical and chemical properties of materials can change dramatically, and that much of this change is driven by the higher ratio of surface to volume. It is the usefulness of many of these unique size-dependent properties that has driven the exponential growth in nanotechnology.[4−7] However, it is also widely recognized that there is an insufficient understanding of nanomaterial fate, transport, and effects in the environment.[8,9] A number of recent published studies have demonstrated that upon nanoparticle exposure, toxicity to plants, microorganisms, and animals may occur.[10−15] Clearly, further work (particularly at the mechanistic and molecular scale) is necessary to fully characterize the risk of nanomaterial use and exposure.

Silver nanoparticles (Ag NPs) are among the most widely used nanomaterials in consumer products,[16] largely due to the observed antimicrobial activity; as such, concerns over the impacts of Ag NPs on nontarget biota have increased. Zheng et al. demonstrated that Ag−SiO_2 shell nanoparticles displayed antimycotic activity to phytopathogenic fungi at doses as low as 0.5 ppm.[17] Savithramma et al. observed that Ag NPs synthesized from *Shorea tumbuggaia* were highly effective at inhibiting both bacterial and fungal growth.[18] Similarly, *Escherichia coli* exposed to Ag NPs experienced growth inhibition, but importantly, the sulfidation of Ag NPs, as may be common in wastewater treatment, lessened the observed nanotoxicity.[19]

Although numerous reports have recently been published on the fate and toxicity of nanomaterials to terrestrial plants species, critical knowledge is still lacking. The mechanisms of nanoparticle toxicity remain elusive, but due to the increased reactivity and small size, nanoparticle entry into and the

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accumulation within plant cells may be significant. A growing body of evidence has demonstrated that Ag NPs at a wide range of concentrations can result in oxidative stress, leading to observed phytotoxicity, although low-exposure doses of Ag NPs from 0.01 to 0.1 mg/L could stimulate *Arabidopsis thaliana* growth.[20] Ravindran et al. and Stampoulis et al. noted that Ag NPs in a wide range of exposure doses from 10 to 1000 ppm inhibited seed germination, root elongation, biomass and growth, and transpiration rate in tomato (*Lycopersicum esculentum*), corn (*Zea mays*), and summer squash (*Cucurbita pepo*).[21,22] Upon exposure to Ag NPs (20 and 100 nm) for 14 days, duckweed (*Lemna minor L.*) exhibited a linear dose−response relationship; as exposure doses increased, both the frond number and the relative growth rate of duckweed were significantly decreased.[23] Lee et al. evaluated the phytotoxicity of Ag NPs to mung bean (*Phaseolus radiates*) and sorghum (*Sorghum bicolor*) under both agar and soil conditions.[24] Interestingly, the phytotoxicity of Ag NPs in soil was markedly less, presumably to the lower bioavailability of the particles in natural media. It is noteworthy that the majority of the NP phytotoxicity data is confined to physiological end-points, and that few studies have addressed toxicity at the molecular level. Kaveh et al. demonstrated that transcripts involved in the thalianol biosynthetic pathway (one plant defense mechanism) were highly up-regulated in Ag-NP-treated *A. thaliana*.[25] Panda et al. described the genotoxicity of Ag NPs to onion (*Allium cepa*) compared to that of other Ag forms, noting that the NPs caused cell death and DNA damage by inducing reactive oxygen species (ROS) generation.[26]

Crambe abyssinica, a member of *Brassicacea*, is naturally tolerant to abiotic stresses such as cold, salt, and heavy metals.[27] Crambe is a high-biomass, high-oil-content (35−40%) crop with a short life cycle, making it an ideal industrial crop for both biofuel production and phytoremediation.[28] Previously, we engineered Crambe to overexpress the *E. coli* γ-glutamylcysteine synthetase (γ-ECS) gene, which yields high levels of glutathione (GSH) as compared to levels in wild type (WT) Crambe.[27] GSH is widely recognized as one of the most important redox buffers in living cells for the detoxification of oxidative stress and damage caused as a result of high ROS levels and production under stress conditions. In this study, two independent γ-ECS transgenic Crambe lines were exposed to Ag NPs and Ag^+ ions to evaluate the potential tolerance of genetically modified Crambe to NP and ionic Ag. The measured physiological parameters included biomass, transpiration rate, and chlorophyll content, as well as Ag shoot and root content. In addition, to gain a perspective on toxicity and tolerance, three main thiol compounds involved in the entire GSH metabolic pathway (along with soluble nutrient elements) were evaluated. To our knowledge, this study represents the first report of the simultaneous physiological and biochemical effects of nanotoxicity on genetically engineered plants.

2. MATERIALS AND METHODS

Seed Sterilization. *C. abyssinica* cultivar BelAnn was transformed with the *γ-ECS* gene as described by Chhikara et al.[27] A total of two transgenic lines (γ-ECS1 and 16) showing high transgenic expression were selected for this study.[27] γ-ECS16 showed a higher expression level of γ-ECS transcripts as well as γ-ECS protein level compared to those of γ-ECS1.[27] The seeds of WT and two independent homozygous transgenic Crambe lines were surface-sterilized with 70% ethanol for 10 min, twice soaked in 25% (v/v) commercial bleach solution for 15 min, and washed five times with autoclaved deionized water (DI H_2O) for 5 min of rinsing.[29] The seeds were inoculated on half-strength Murashige and Skoog medium (1/2× MS medium: 2.22 g of MS basal medium with vitamins, 20 g of sucrose, and 8 g of phytoblend in 1 L of DI H_2O at pH 5.7) in magenta boxes.[30] The plants were incubated in a growth chamber at 22 °C with a 16 h/8 h light/dark cycle; the light intensity was 250 μmol $m^{-2}s^{-1}$.

Determination of Inhibitory Ag Concentrations. An initial screening study was conducted to characterize the dose-dependent nature of toxicity and to enable the determination of optimum Ag exposure concentrations for additional physiological and biochemical studies. The initial concentrations were 25, 50, 100, and 200 mg/L Ag NPs (particle size 20 nm; purchased from US Research Nanomaterials, Inc.); 500, 1000, 2000, and 3000 mg/L for bulk Ag (particle size: 44 μm; Strem Chemicals); and 25, 50, 100, and 200 μM for Ag^+ ions (Fisher Scientific). Ag NPs were twice dispersed by ultrasonic treatment for 30 min each time prior to storage in the dark at room temperature overnight.[11] The hydrodynamic diameters of Ag NPs in DI water and Hoagland's solution were measured by dynamic light scattering (DLS) (Figure S1 in the Supporting Information). For each treatment, 15 WT Crambe seeds per box were planted into Ag-amended 1/2× MS medium. All experiments were conducted in triplicate. These magenta boxes were maintained in a growth chamber as described above for 25 days prior to biomass determination.

On the basis of the preliminary dose−response assay (Figure S2 in the Supporting Information), 200 and 250 mg/L were selected as the Ag NP exposure levels, and 200 and 250 μM were chosen for Ag^+ ions. However, no significant difference of fresh biomass was found among bulk Ag treatments (even at a 3000 mg/L exposure dose) as compared to the results for Ag NP and Ag^+ ion treatments. Thus, additional work in this study will exclude bulk Ag treatment. Using identical exposure conditions, both WT and transgenic Crambe were exposed to Ag for 25 days.

Phytotoxicity of Ag NPs to *Crambe abyssinica* in Hydroponic Systems. To determine the impact of Ag exposure on transpiration and metal uptake, we germinated WT and transgenic Crambe in a magenta box with 1/2× MS medium containing phytoblend as described above. After 25 days, the plants were carefully removed from the solid medium and were rinsed gently with DI water to remove the attached media on the roots. Both the WT and transgenic Crambe were transferred to half-strength Hoagland's solution (Hoagland's modified basal salt mixture, purchased from Phyto Technology Laboratories) and allowed to acclimatize for 7 days prior to 5 days of exposure to the Ag NP and Ag^+ ion treatments (Figure S3 in the Supporting Information). The transpiration was measured by the volume of solution lost; fresh solution without Ag was added daily to maintain a consistent volume. After exposure, plant tissues were harvested to evaluate Ag content, GSH, and nutrient levels.

Measurement of Chlorophyll Content. Chlorophyll content was determined by a modified protocol as described in Lichtenthaler.[31] Briefly, 50 mg of fresh tissue was harvested, cut into pieces (<1 cm), and added to 15 mL centrifuge tubes amended with 10 mL of 95% ethanol. The tested tubes were kept in the dark for 3−5 days, and the chlorophyll content was measured by a UV−vis spectrophotometer (Spectronic Genesis 2). Chlorophyll a, chlorophyll b, and total chlorophyll were determined by the following equations: Chla = $13.36A_{664.2}$ −

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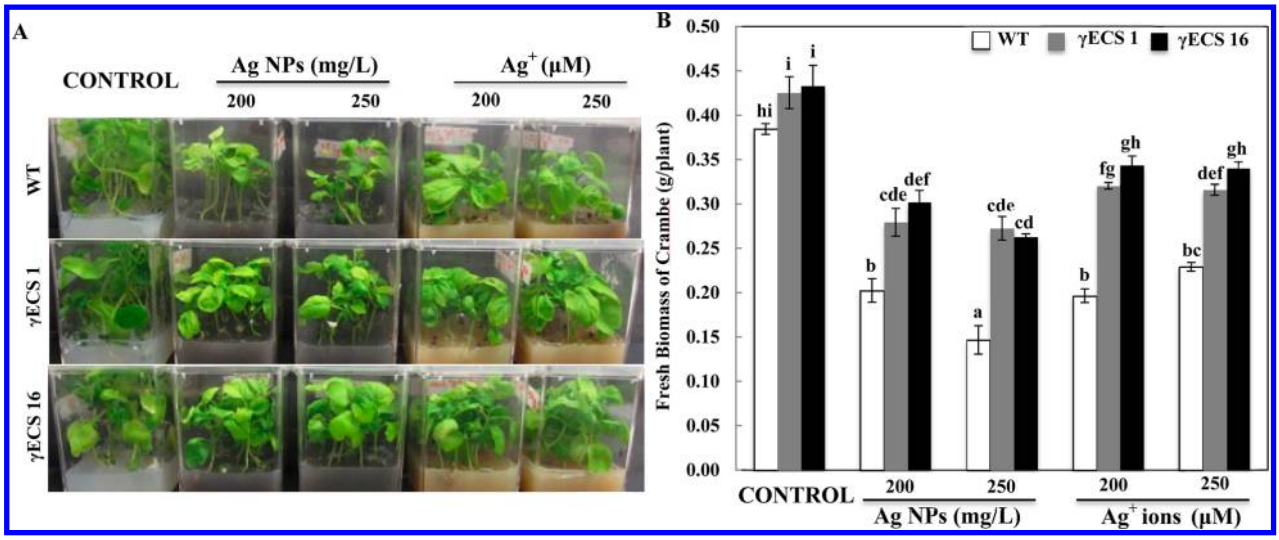

Figure 1. Growth analysis of WT and two γ-ECS lines treated with Ag NPs and Ag^+ ions. Plants were exposed to the indicated concentrations of Ag NPs and Ag^+ ions, respectively, for 25 days. (A) Images of WT and γ-ECS lines grown on Ag-NP- and Ag^+-ion-amended 1/2× MS media after 25 days of growth. (B) Total fresh biomass of Ag-NP- and Ag^+-ion-treated WT and two γ-ECS lines. Data are mean ± standard error of three replicates of 15 plants each. Values of the total fresh biomass followed by different letters are significantly different at $p \leq 0.01$.

$5.19A_{648.6}$; Chlb = $27.43A_{648.6} - 8.12A_{664.2}$; total chlorophyll = Chla + Chlb.

Measurement of Ag Accumulation and Nutrient Uptake in Crambe. Harvested root tissue was rinsed with DI H_2O three times to remove the surface retained Ag. All shoot and root samples were oven-dried at 65 °C for 3 days, and then 30 mg of tissue was transferred to 15 mL centrifuge tubes amended with 3 μL of HNO_3. The samples were digested at room temperature for 48 h. Samples of 500 mL of H_2O_2 were then added to complete the tissue digestion. The digests were diluted 35-fold with DI H_2O prior to the determination of Ag content by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7500ce).[32] Ag NPs- and $AgNO_3$-amended 1/2× Hoagland's solution was centrifuged at 5000 rpm for 1 h. Supernatant was passed through a 0.45 μm filter and then used for the determination of Ag content.

For the extraction of the soluble fraction of nutrient, 200 mg shoot- and root-tissue samples were extracted in 1 mL of 5% perchloric acid (PCA). Samples were again frozen and thawed three times and kept frozen at −20 °C until analysis.[33] For the quantitation of nutrients, PCA extracts were diluted 100-fold and analyzed using a simultaneous axial inductively coupled plasma emission spectrophotometer (ICP-AES, Vista CCD, Varian, Palo Alto, CA, USA) and Vista Pro software (version 4.0). The National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) standards for Eastern white pine needles (SRM 1575A) and apple leaves (SRM 1515) were used because no standards are available for fresh foliar tissues. We did use an in-house ground wood reference sample for quality control and assurance. For all samples, a standard curve was repeated after every 20 samples, and check standards were run after every recalibration and after every 10 samples.

Measurement of Lipid Peroxidation in Crambe. Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content in shoot and root samples of Crambe. Samples of 200 mg of plant tissues were homogenized in 4 mL of 0.1% (w/v) tricholoroacetic acid (TCA). The extracts were used for measuring MDA content following the method described in Jambunathan (2010).[34] Details are provided in the Supporting Information.

Measurement of Levels of Cysteine, γEC, GSH, and Phytochelatins in Crambe. A pool of approximately 200 mg of fresh tissue (a homogeneous mix of shoot and root tissue) was collected in a 1.5 mL Eppendorf tube containing 1 mL of extraction buffer (6.3 mM diethylenetrianmine pentacetic acid, DTPA, mixed with 0.1% trifluoroacetic acid, TFA). The extracts were used for the derivatization and analysis of thiol compounds (cysteine, γEC, GSH, and phytochelatin 3 (PC3)) as described in Minocha et al.[35] The specific procedures for thiol compound measurement are provided in the Supporting Information.

Statistical Analysis. For each assay, the means are averaged from four to five replicates, and the error bars correspond to the standard error of mean. A one-way analysis of variance (one-way ANOVA) followed by a Duncan multiple comparison test was used to determine the statistical significance of each parameter among the treatments. The values of each assay followed by different letters are significantly different at $p \leq 0.01$ or 0.05.

3. RESULTS AND DISCUSSION

3.1. Analysis of Fresh Biomass in Ag-Exposed Crambe. As described in the Materials and Methods section, transgenic γ-ECS and wild type (WT) plants were exposed to 200 and 250 mg/L Ag NPs and 200 and 250 μM Ag^+ ions. After 25 days of Ag exposure, the biomass (roots and shoots) of WT and two independent γ-ECS transgenic Crambe lines was determined at harvest. There were no significant differences in the biomass of WT and transgenic plants in the nonexposed control. Ag exposure in any form significantly reduced the biomass of all plant types (Figure 1). However, across all four Ag-exposure scenarios, γ-ECS Crambe lines achieved significantly higher biomass than did the respective WT plants. The WT Crambe biomass was 28−33% and 44−46% less than that of the transgenic lines with 200 mg/L and 250 mg/L Ag NPs, respectively. Similarly, transgenic Crambe lines attained significantly greater biomass under Ag^+ ion exposure as compared to that of the WT; at 200 and 250 μM, the WT mass was reduced by 39−43% and 27−33%, respectively, relative to γ-ECS Crambe. Collectively, Ag NP exposure

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resulted in more damages to both the WT and the transgenic Crambe as compared to the results from Ag^+ ion treatment; nanoscale effects might be the main reason, beyond the toxicity caused by Ag^+ ions.

The exposure dose for Ag NP and Ag^+ ions in our study is higher than that used in a number of other studies, including annual ryegrass (*Lolium multiflorum*) (0−40 ppm) in solutions,[36] wheat (*Triticum aestivum L.*) (2.5 ppm) in sand matrix,[37] *P. radiatus* and *S. bicolor* (0−40 ppm) in an agar test,[24] and rice (*Oryza sativa L.*) (0−60 ppm) in N6 growth cultivation media.[38] Transgenic Crambe lines exhibited greater tolerance and were noticeably healthier than WT after exposure to Ag NPs and Ag^+ ions (Figure 1). In accordance with a number of recent studies, Ag NPs caused greater phytotoxicity than did the corresponding bulk and ion treatments. Hawthorne et al. reported significantly less zucchini biomass upon exposure to 250 ppm of Ag NPs relative to an equivalent bulk Ag control.[10] Dimpka et al. illustrated that the inhibition of wheat root and shoot tissue was significantly greater for Ag NPs (2.5 mg/kg) than was an equivalent amount of Ag^+ ions.[37] In addition to Ag, other metal NPs such as CuO,[39−41] CeO_2 (0−4000 ppm),[42] and Al_2O_3[43] have been reported to induce oxidative stress and reduce the biomass of plants such as *Z. mays*, cucumber (*Cucumis sativus*), dotted duckweed (*Landoltia punctata*), and tobacco (*Nicotiana tabacum*).

3.2. Analysis of Chlorophyll Content in Ag-Exposed Crambe. Because chlorophyll is the critical photosynthetic pigment, chlorophyll levels can be a significant indicator of toxicity to plants. In the control plants, there were no significant differences in the chlorophyll content between the WT and the transgenic Crambe (Figure 2). The chlorophyll

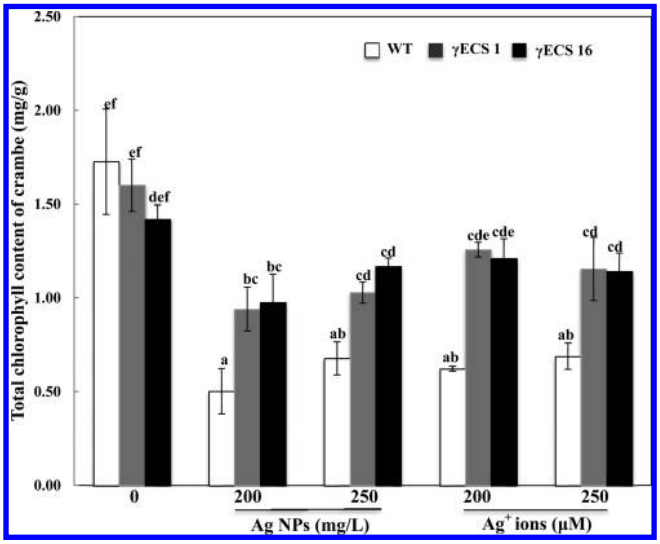

Figure 2. Analysis of total chlorophyll content in WT and γ-ECS lines treated with Ag NPs and Ag^+ ions. Data are mean ± standard error of three replicates of 15 plants each. Values of the total chlorophyll content followed by different letters are significantly different at $p \leq 0.05$.

content of all plants was decreased upon Ag exposure, regardless of the concentration and Ag types. However, similar to the biomass data above, across all four Ag-exposure scenarios, the chlorophyll levels in the γ-ECS Crambe lines were significantly greater than the levels in the respective WT plants. WT Crambe chlorophyll content was 47−49% and 34−42% less than that of the transgenic lines with 200 mg/L and 250 mg/L Ag NPs, respectively. Similarly, transgenic Crambe produced a significantly greater chlorophyll content under Ag^+ ion exposure as compared to that of WT; at 200 and 250 μM, the WT mass was reduced by 47−49% and 39−40%, respectively, relative to γ-ECS Crambe.

Similarly, Jiang et al. reported a time-dependent decrease in giant duckweed (*Spirodela polyrhiza*) chlorophyll content upon exposure to 0−10 ppm Ag NPs.[44] Oukarroum et al. investigated the impact of 0−10 ppm Ag NPs exposure on green algae (*Chlorella vulgaris* and *Dunaliella tertiolecta*) at two temperatures. The study not only reported dose-dependent decreases in chlorophyll content but also described a nanoparticle-induced disruption of photosynthetic electron transport.[45] Although other metal-oxide nanoparticles such as CuO NPs[41] have also been shown to decrease chlorophyll production, it is worth noting that both TiO_2[46] NPs and Au NPs[47] were reported to increase the production of the pigment in *T. aestivum spp.* and *Brassica juncea*. Additional studies are being planned to characterize the mechanism of Ag-NP-induced decreases in chlorophyll levels, which may occur by inhibition of chloroplast formation or by direct interaction with and degradation of chlorophyll. From the aspect of the molecular response to Ag-NP-treated *A. thaliana*, the down-regulation of the transcription levels of protochlorophyllide reductases, which are responsible for chlorophyll sysnthesis, were observed.[25] This result could further lead us to understand the mechanism of the chlorophyll degradation that occurred in the presence of Ag NPs.

3.3. Analysis of Ag Exposure on Transpiration in Crambe. To understand the effects of Ag NPs on plant transpiration rate, we set up a hydroponic system as shown in Figure S3 in the Supporting Information. The transpiration rate was determined by calculating the water loss by volume for each replicate over a 24 h interval for a period of 5 days (Figure 3). Similar to biomass and chlorophyll, the transpiration rate after 5 days of exposure was reduced by Ag NP exposure, regardless of concentrations, but both γ-ECS Crambe lines consistently transpired more solution than the respective WT individuals. The transpiration rates of WT Crambe treated with 250 mg/L Ag NPs were significantly reduced by 35−46% relative to the transgenic lines (Figure 3B). Similar results for the transpiration rate were obtained when plants were exposed to 200 mg/L Ag NPs (data not shown). Interestingly, the exposure to Ag^+ ions at a 250 μM concentration had no effect on solution transpiration rates, regardless of plant types (Figure 3C). Also, at a lower concentrations of 200 μM Ag^+ ions, no difference in transpiration rate was observed (data not shown).

Our findings are in agreement with Stampoulis et al., in which Ag NPs decreased *C. pepo* transpiration volume by 75% as compared to that of bulk-exposed and untreated plants.[22] Conversely, transpiration rate was not impacted in corn and radish plants grown in CeO_2-NP-amended soil.[48,49] Another study demonstrated that TiO_2 NPs could notably elevate transpiration rate in elm trees (*Ulmus elongata*) hydroponically.[50] This finding is highly significant in that, in spite of the well-known phytotoxicity of Ag NPs, the γ-ECS Crambe lines yielded significantly greater growth (biomass and transpiration) at equivalent exposures, strongly implicating GSH metabolism in the defense of NP-induced abiotic stress.

3.4. Analysis of Lipid Peroxidation in Ag-Exposed Crambe. Membrane integrity in both WT and transgenic Crambe was assessed by MDA formation (Figure 4). The MDA

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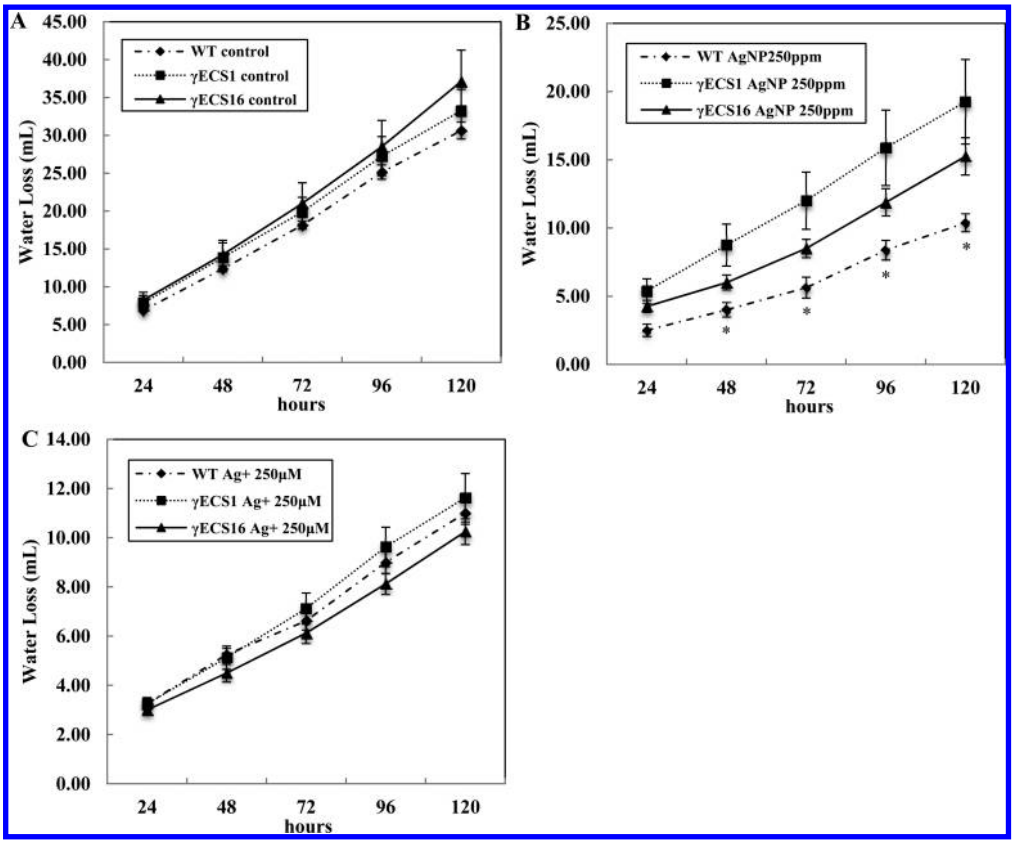

Figure 3. Analysis of the transpiration rate between WT and transgenic Crambe treated with 250 ppm Ag NPs and 250 μM Ag^+ ions for 5 days. Water loss was calculated in 24 h intervals. Data are mean ± standard error of five replicates. Values of transpiration rate marked with asterisks (*) are significantly different at $p \leq 0.05$.

content in the shoot tissue of Crambe showed that a significantly high level of MDA was produced in WT Crambe compared to that of both transgenic lines regardless of the Ag forms. With 200 mg/L Ag NPs and 200 μM Ag^+ ions, MDA content in both γ-ECS Crambe was reduced by 27.3−32.5% and 20.6−33.6%, respectively, relative to the WT. Similarly, in the root of both the WT and the transgenic Crambe, significantly low MDA content in both transgenic lines indicated that γ-ECS-engineered plants showed high tolerance to metal stresses, regardless of the metal form. Other NPs could also induce excess amounts of MDA production in plants, indicating that oxidative stress occurred in plants in the presence of metal-based NPs. As exposure doses of CuO NPs increased, the MDA content in root of mung beans was significantly elevated.[51] A high level of MDA was measured in CeO_2-NP-treated *A. thaliana*.[52] However, CeO_2 NPs could not induce lipid peroxidation in either corn plants or kidney beans.[53,54] The up-regulation of the relative expression of genes, including glutathione *S*-transferase, peroxidase, and superoxide dismutase (all of which can scavenge excess amounts of ROS) were evident in Ag-NP-treated *A. thaliana*.[25]

3.5. Analysis of Cysteine, γEC, GSH, and PC3 in Ag-Exposed Crambe. Cysteine is the primary precursor molecule in glutathione biosynthesis in plants. In the unexposed plants, cysteine levels were similar for the WT and the transgenic Crambe. However, upon exposure to Ag NPs and Ag^+ ions, cysteine production was significantly greater for the γ-ECS transgenic Crambe lines (Figure 5A), clearly demonstrating the enhanced GSH metabolic pathway enabled by γ-ECS gene overexpression. γ-EC and GSH play essential roles in metal detoxification through thiol group (−SH) chelation. In Figure 5B, it is shown that transgenic plants produced significantly higher levels (up to 4-fold) of γ-EC under the catalysis of γ-ECS, regardless of Ag presence. Glutathione synthetase (GS) converts γEC into γ-Glu−Cys−Gly (GSH), and in Figure 5C, significantly greater GSH synthesis is evident in both transgenic lines without any treatments. Notably, the GSH levels in WT and transgenic plants were decreased after being exposed to Ag NPs and Ag^+ ions compared to levels in the respective untreated control groups (Figure 5C). PC synthetase (PCS) can effectively convert GSH into phytochelatins (PCs) ($(\gamma$-Glu−Cys$)_{2\text{-}8}$-Gly), which results in depleting GSH levels. As shown in Figure 5D, the PC3 level in both transgenic lines was overproduced compared to the level in WT upon exposure to 250 mg/L Ag NPs.

Cysteine is an effective antioxidant and could counteract Ag-induced stress because of the thiol group (−SH). Li et al. found that a significant increase in cysteine could be induced through the overexpression of γ-ECS in *Arabidopsis* upon arsenic treatment because of the great demand to synthesize GSH.[55] In addition, cysteine can be converted into GSH rapidly under the catalysis of γ-ECS and GS. Under the abiotic stress caused by Ag, reactive oxygen species can be produced in plants, especially superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2).[26] Due to the direct toxicity of ROS in plant cells, GSH redox cycles are frequently activated for plant defense. In the GSH-mediated defense pathway, GSH peroxidase (GPx) can detoxify H_2O_2 by oxidizing GSH to GSSG (oxidized GSH).[56] GSH, also through its redox activity, can directly reduce Ag^+ ions through the conversion of GSH to GSSG.[57] This may

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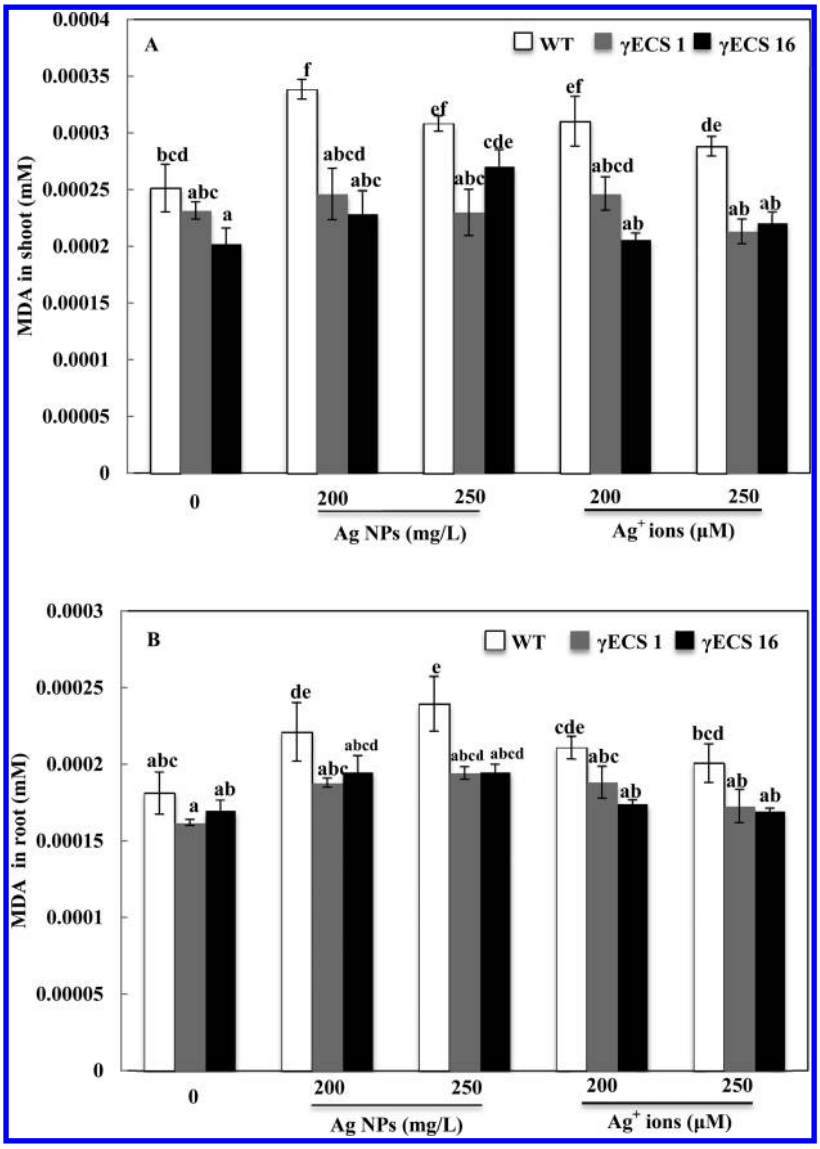

Figure 4. Lipid peroxidation in shoot and root tissue of WT and γ-ECS Crambe. MDA contents in shoot (A) and root (B) tissue of WT and transgenic Crambe treated with 250 mg/L Ag NPs and 250 μM Ag^+ ions, respectively, for 25 days. Data are mean ± standard error of four replicates of 15 plants each. Values of MDA content followed by different letters are significantly different at $p \leq 0.05$.

explain the reduction in overall GSH levels upon Ag NP and Ag^+ ion treatment as compared to that of the control group. Dimkpa et al. demonstrated that GSSG in *T. aestivum* was increased significantly after treating with 2.5 ppm Ag NPs and 2.5 ppm Ag^+ ions.[37] Highly induced transcription levels of glutathione synthase were evident in both In_2O_3- and CeO_2-NP-treated *Arabidopsis*.[52] Another reasonable pathway by which to explain the GSH depletion is through PC biosynthesis.[58,55] GSH is the precursor for the synthesis of PCs catalyzed by phytochelatin synthase (PCS).[58] It has been reported that PCS in plants can be activated by metal ions such as Cd^{2+}, Hg^{2+}, Ag^+, Zn^{2+}, and Cu^{2+},[59] which could yield high levels of PCs for the detoxification of toxic metals. Li et al. observed that in transgenic *Arabidopsis*, by overexpressing AtPCS1, Cd exposure enhanced PC levels several-fold over the control group levels.[60] Our results agree with these findings in that Ag NPs treatment increased PC3 levels in both WT and transgenic Crambe (Figure 5D). Another possible pathway for GSH depletion is that GSH can directly bind and complex with Ag via the −SH group.

3.6. Ag Distribution and Nutrient Displacement in Crambe. The Ag content in each of the non-Ag exposed Crambe plants was below 0.5 μg/kg (not shown in the figure). The shoot Ag content of transgenic Crambe under Ag NP treatment was significantly higher than that of WT. Notably, the Ag content in the γ-ECS 16 shoots reached 50.5 mg/kg, which is 5-fold higher than that of WT plants (Figure 6A). Under Ag^+ ion exposure, the shoots of γ-ECS transgenic Crambe also contained significantly higher Ag levels than did the WT plants, although the size of the increase was not as dramatic as that observed with the NP treatment. Contrary to the shoot data, Ag content in the roots of both γ-ECS 1 and γ-ECS 16 Crambe exposed to Ag NPs were lower than WT. However, the Ag content of plant roots exposed to Ag^+ ions did not vary significantly among the γ-ECS and WT Crambe plants (Figure 6B). Additional studies will be conducted to explore the decreased Ag content in transgenic root tissue. The shoot-to-root translocation factor (TFs; shoot concentration divided by root concentration) is shown in Figure 6C. Regardless of Ag type, the transgenic Crambe translocation had significantly

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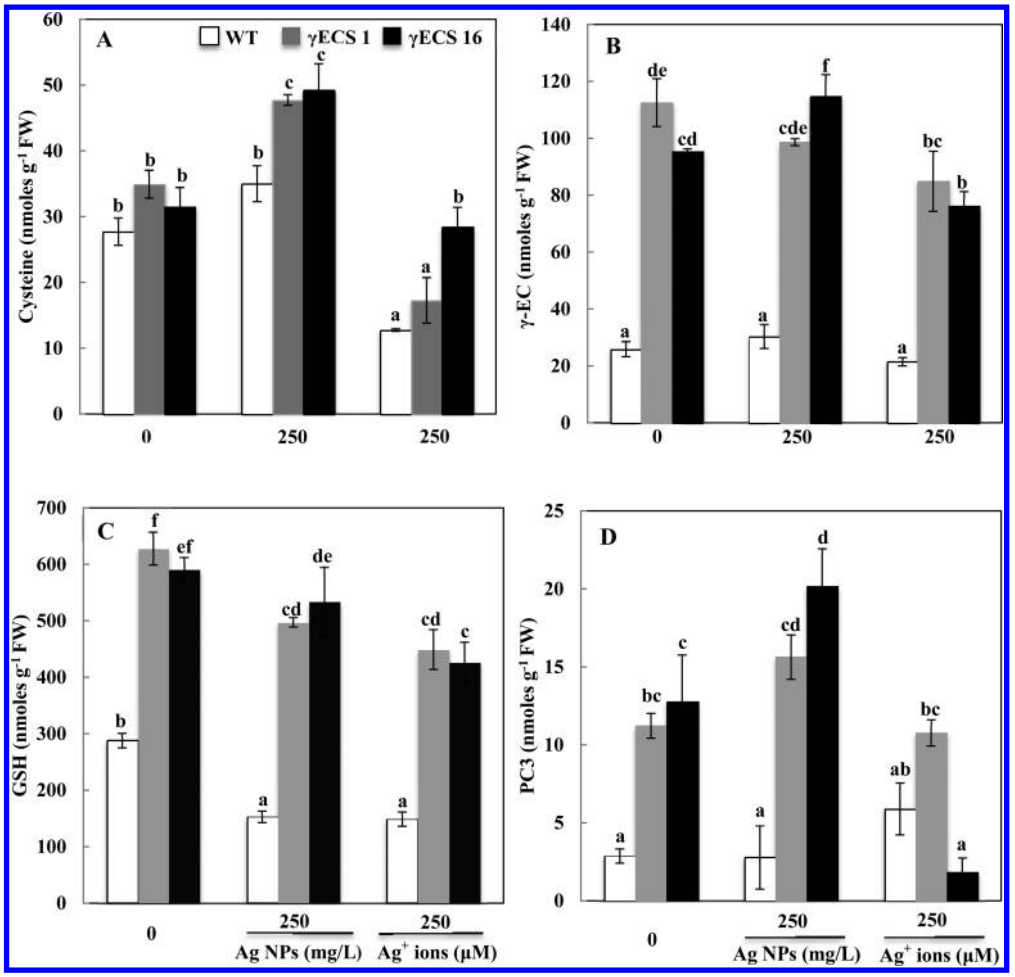

Figure 5. Analysis of total cysteine, γ-EC, GSH, and PC3 levels in WT and γ-ECS Crambe treated with 250 mg/L Ag NPs and 250 μM Ag^+ ions. The individual components above were extracted from a homogeneous mix of shoots and roots tissues. Panels A–D represent different levels of cysteine, γ -EC, GSH, and PC3 between WT and transgenic Crambe, respectively. Data are mean ± standard error of four replicates. Values of each component followed by different letters are significantly different at $p \leq 0.05$.

greater quantities of the element in the shoot tissue than did WT. The centrifuged Hoagland's solution supernatant of the $AgNO_3$-exposed plants had similar Ag levels across all plant types (Figure 6D). Similarly, for the γ-ECS 16 Crambe, the solution Ag content of the Ag NP treatment did not vary from that of the WT. However, for unknown reasons, the γ-ECS 1 treated with 250 ppm Ag NPs had nearly 3-fold higher Ag levels in the supernatant than did the WT. Notably, the root Ag content of these plants did contain significantly lower Ag levels than did the WT.

This observation is particularly noteworthy given the markedly decreased phytotoxicity observed with the γ-ECS Crambe, but it may have been a function of the significantly greater transpiration exhibited by the transgenic plants relative to WT. This observation may suggest that the higher levels of GSH and PC3 synthesis in transgenic plants may result in greater chelation of Ag or potentially differential storage in vacuoles or the cell wall. The findings of higher metal NP translocation than equivalent ions agree with the work of De L Torre-Roche et al., where soybean (*Glycine max*) and *C. pepo* accumulated more shoot Ag under NP exposure (500 and 2000 ppm) relative to Ag bulk or ions.[32] Similarly, Dimpka et al. reported that Ag levels in *T. aestivum* shoots were greater under 2.5 ppm Ag NPs treatment than under the equivalent bulk metal.[37] Others have reported on a dose-dependent accumulation of Ag NPs in organisms such as *L. multiflorum*[36] and *Chlamydomonas reinhardtii*.[61] In fact, similar dose-dependent nanoparticle accumulation has been noted for various concentrations of CuO, ZnO, and Au NPs in *C. sativus*,[39] *Z. mays*,[62] and *B. juncea*.[47] Although our study only investigated a limited concentration range of Ag NPs and Ag^+ ions in WT and transgenic Crambe, the data clearly suggest the phytochelation of Ag^+ with PC3, GSH, and cysteine, along with subsequent transport from roots to shoots. In related work, we observed that engineered *A. thaliana* overexpressing the γ-ECS gene could also accumulate higher levels of cadmium and mercury than did the WT.[58]

Along with Ag distribution, nutrient elements were also determined in both WT and transgenic Crambe (Table S1 in the Supporting Information). The levels of most soluble nutrient elements, such as Ca, K, Mg, Zn, and Mn, were similar regardless of Ag presence and plant types, indicating that either Ag NPs or Ag^+ ions could not disrupt nutrient transport in Crambe. However, the Fe level in WT Crambe, as one exception, was significantly lower in either Ag NP or Ag^+ ions treatment than was the WT control group. A similar phenomenon was not found in γ-ECS Crambe lines, as the Fe levels in these lines were higher than those of WT plants under Ag NPs or Ag^+ ions. In addition, our results showed that the P level in Ag^+-ion-treated transgenic Crambe was

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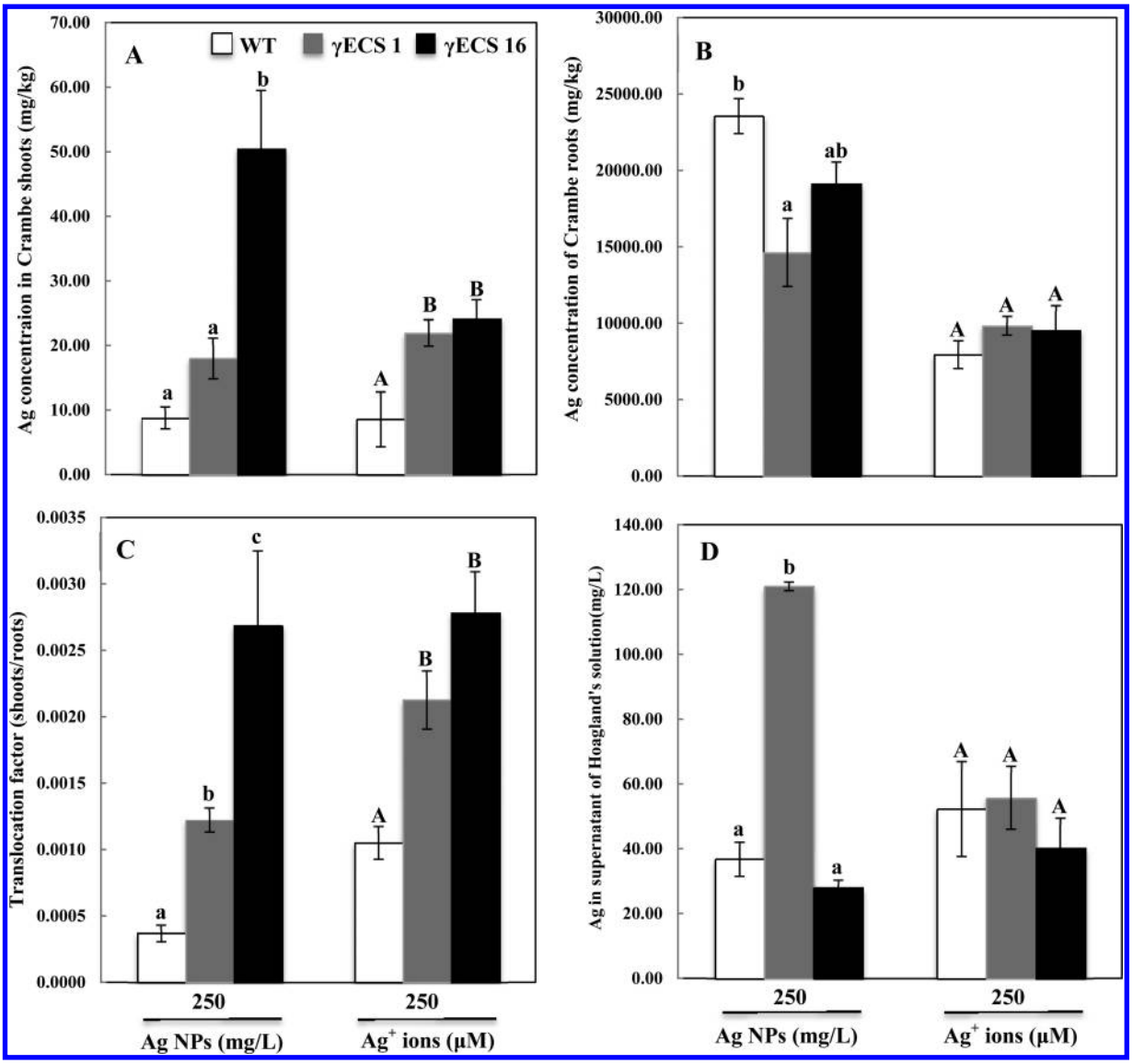

Figure 6. Ag uptake and distribution in WT and γ-ECS lines treated with 250 mg/L Ag NPs and 250 μM Ag^+ ions. Panels A–D represent Ag content in shoots, roots, TFs (shoots and roots), and supernatant of 1/2× Hoagland's solution, respectively. Data are mean ± standard error of four replicates. Values of Ag concentration in Ag NPs and Ag^+ ions treatment followed by different lowercase and uppercase letters, respectively, are significantly different at $p \leq 0.05$.

significantly elevated compared to that of the untreated transgenic controls, although the difference between levels in WT and transgenic lines treated with Ag^+ ions was not significant. Both the Zhao et al. and the Gao et al. studies suggested that CeO_2 and TiO_2 NPs could not disrupt nutrient levels in corn and *U. elongata*, respectively.[48,50] Further studies are necessary to understand how plants respond to nutrient element uptake in the presence of NPs and whether there is a defense mechanism (such as stomatal closure or transpiration rate reduction) in plants to avoid excess element uptake, which subsequently causes nutrient displacement.[63]

In conclusion, we showed that an enhanced level of GSH in transgenic Crambe expressing γ-ECS is involved in protecting plants from phytotoxicity caused by Ag NPs and Ag^+ ions. Furthermore, exposure to Ag NPs caused a significant decrease in Fe uptake only in wild type plants and not in γ-ECS lines. Because Fe is the most deficient nutrient for plant growth, our results holds significant importance in preventing crop yield loss as a result of Ag-NP- and Ag^+-ion-induced phytotoxicity and Fe deficiency. This study is highly helpful for understanding the fate, transport, and toxicity of NPs in plants and the role of GSH in counteracting the Ag NP phytotoxicity, which could prove useful for mitigating the threat of NPs in the food chain and the environment.

■ ASSOCIATED CONTENT

Supporting Information

Additional information includes the details on the measurement of lipid peroxidation and levels of cysteine, γEC, GSH, and PC3 in Crambe; figures showing the hydrodynamic diameter of Ag NPs in solution, phenotypes of WT Crambe versus the different forms of Ag, and the inhibitory concentration selection of different forms of Ag; photos of the hydroponic setup; and a table of nutrient uptake. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02007.

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Notes

The authors declare no competing financial interest.

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