Journal of Plant Physiology 171 (2014) 1142–1148

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Phytotoxic and genotoxic effects of silver nanoparticles exposure on germinating wheat seedlings

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ARTICLE INFO

Article history:
Received 3 February 2014
Received in revised form 7 May 2014
Accepted 7 May 2014
Available online 17 May 2014

Keywords:
Triticum aestivum
Silver nanoparticles
Proteomics
AFLP
TEM

ABSTRACT

We investigated the effects of 1 and 10 mg L^{-1} AgNPs on germinating *Triticum aestivum* L. seedlings. The exposure to 10 mg L^{-1} AgNPs adversely affected the seedling growth and induced morphological modifications in root tip cells. TEM analysis suggests that the observed effects were due primarily to the release of Ag ions from AgNPs.

To gain an increased understanding of the molecular response to AgNP exposure, we analyzed the genomic and proteomic changes induced by AgNPs in wheat seedlings. At the DNA level, we applied the AFLP technique and we found that both treatments did not induce any significant DNA polymorphisms. 2DE profiling of roots and shoots treated with 10 mg L^{-1} of AgNPs revealed an altered expression of several proteins mainly involved in primary metabolism and cell defense.

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Introduction

Engineered silver nanoparticles (AgNPs) are widely used in personal care products, clothing, food, building materials and medical equipment. AgNPs enter natural ecosystems and persist for extended periods of time (Dobias and Bernier-Latmani, 2013). The toxicity of AgNPs has been demonstrated in various prokaryotic organisms and mammalian cell lines (Marambio-Jones and Hoek, 2010). In higher plants, different and conflicting effects of AgNPs have been reported, depending on the intrinsic AgNP properties (size and shape, aggregation state, and surface coatings), experimental system, plant species and developmental stage (Remédios et al., 2012).

AgNPs are able to induce DNA damage in plants, causing the formation of chromatin bridges, stickiness, disturbed metaphase and multiple chromosomal breaks (Kumari et al., 2009; Panda et al., 2011; Patlolla et al., 2012). Recently, a whole-genome cDNA microarray analysis and a proteomic study have provided new insights into the response of *Arabidopsis thaliana* (Kaveh et al., 2013) and *Eruca sativa* (Vannini et al., 2013) to AgNPs and Ag^{+}.

In the present study, we combined proteomic, amplified fragment length polymorphism (AFLP), light and transmission electron microscopy (LM, TEM) analyses to characterize the molecular and morphological effects of AgNPs on the early phases of wheat (*Triticum aestivum* L.) germination, an important and vulnerable stage in the angiosperm life cycle. The differences in protein abundance reflect differences in metabolic activity better than differences in gene transcript levels because the latter are not always reflected at protein levels. AFLP is a robust, highly informative DNA fingerprinting method (Vos et al., 1995) that has been successfully employed to measure genotoxic activity caused by environmental pollutants (Muller et al., 2004; Labra et al., 2007; Aina et al., 2007).

At the genome level, AFLP did not reveal an appreciable genotoxic effect of 10 mg L^{-1} AgNPs on root and shoot cells. However, proteomic and morphologic data indicate that 10 mg L^{-1} AgNPs affects the levels of several proteins involved in metabolism, protein synthesis/folding, and stress responses in multiple cellular compartments. Furthermore, microscopy analysis suggests that the overall toxicity of AgNPs is driven primarily by the release of Ag ions from these nanoparticles.

Materials and methods

AgNP suspensions and characterization

All experiments were carried out using 10 nm PVP-AgNPs (Biopure AG10, Nanocomposix, San Diego, CA). All experimental concentrations were prepared by diluting the AgNP stock solution

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http://dx.doi.org/10.1016/j.jplph.2014.05.002
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(1 g L^{-1}) in deionized water. All dilutions were freshly prepared before use.

The shape and the size of PVP-AgNPs were determined by TEM as previously described (Vannini et al., 2013).

Seed treatment

The seeds (*Triticum aestivum* L. cv Blasco) were surface sterilized with 10% sodium hypochlorite solution for 10 min and then rinsed with distilled water. For each treatment, 100 seeds were soaked for 4 h in 32 mL of 1, 10 mg L^{-1} of PVP-AgNPs. Two sets of control samples were grown: in deionized water only and in deionized water supplemented with 1 or 10 mg L^{-1} of PVP. A filter paper moistened with 5 mL of test solutions was put into each 100 × 15 mm sterilized Petri dish. Fifteen seeds per dish were transferred onto the filter paper. Each concentration point of the treatments was performed six times. All treatments were conducted in triplicate. Dishes were placed for 5 days in the dark under controlled temperature (25 ± 1 °C). At the end of the exposure, seedlings were washed three times with 0.1 M EDTA and then with MilliQ-water. Length and weight of roots and shoots were measured, separated and immediately frozen at −80 °C.

Plant Ag content determination

Ag content was determined by flame atomic absorption spectroscopy (F-AAS; Thermo-Electron Atomic Absorption Spectrometer, Vannini et al., 2013).

Light and transmission electron microscopy (LM and TEM) analysis

Control and treated root samples (2 mm long) were treated as described in Vannini et al. (2013).

AgNPs were enhanced with QH silver (Nanoprobes) for 4 min as described by the manufacturer. Five plants were analyzed for each type of treatment. All treatments were conducted in triplicate.

DNA extraction and AFLP analysis

DNA was extracted using the Plant Genomic DNA Miniprep Kit (Sigma–Aldrich), starting from single plantlets treated with 1 and 10 mg L^{-1} PVP-AgNPs. For each treatment, 5 individuals were analyzed: root and shoot were separated from each plantlets and used for DNA extraction.

For each sample a total of 50 ng of DNA was digested (2 h) with EcoRI (1 U) and MseI (1 U). The DNA fragments were ligated (with T4-DNA ligase) to EcoRI (2.5 pmol) and MseI (25 pmol) adapters in a final volume of 20 μL. Ligation reaction was performed at 22 °C for 2 h. This mixture was used as the template in the preamplification reaction containing DNA primers E00 and M00 (Table S1) complementary to the core of the EcoRI and MseI adapters, respectively. The amplification was performed as described in Labra et al. (2003). The PCR products were amplified in a second round with primers containing selective bases (Table S1); this was carried out using primer pairs E32–M38, E32–M40, E38–M38, E38–M40, E38-M42. The EcoRI-primer was labeled by using fluorescent 6-carboxy fluorescein (6-FAM) on the 5 nucleotide. The temperature profile for this step was the following: one cycle of 5 min at 94 °C, followed by 10 cycles of 30 s at 94 °C, 1 min at 65 °C (for the first cycle, subsequently reduced each cycle by 1 °C for the next nine cycles), and 1 min at 72 °C, followed by 25 cycles of 30 s at 94 °C, 1 min at 56 °C and 1 min at 72 °C. The PCR was terminated with a final incubation step of 10 min at 72 °C. The amplified fragments were fractionated and detected with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with the data collection software 3.0 (ABI). AFLP fragment analysis was performed with GeneScan Analysis Software 4.0 (ABI) and the data were assembled in binary format. Fragments were resolved using capillary electrophoresis. The percent of polymorphisms among analyzed samples and control ones was estimated.

Supplementary Table S1 can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2014.05.002.

Protein sample preparation and two-dimensional IEF/SDS-PAGE

Roots and shoots from seedlings exposed to 10 mg L^{-1} PVP-AgNPs were homogenized by using mortar and pestle in liquid nitrogen with an addition of sand quartz. Total protein extraction and two-dimensional electrophoresis were performed as previously described (Marsoni et al., 2008). Three independent extractions and three gel replicas for each experimental condition were performed. Protein detection and image analysis were conducted according to Vannini et al. (2013). Only the spots with a fold change of ±1.5 and ANOVA p-value ≤0.05 were accepted as differentially expressed.

Mass spectrometry analysis

Selected spots were excised from the 2-D gels, digested and tryptic fragments were analyzed by LC–ESI–MS/MS (liquid chromatography–electrospray ionization tandem mass spectrometry) (Marsoni et al., 2010). Spectra were compared with in situ database by SEQUEST algorithm incorporated in BIOWORKS BROWSER 3.3 software (ThermoFisher Scientific Inc.): search was performed against the Triticum subset database (31,852 entries), obtained from NCBI-nr database (http://www.ncbi.nlm.nih.gov/) by FASTA database utilities tool of BIOWORKS, and, in case of mismatch, against full NCBI-nr database (25,877,237 entries). The searches were carried out as described in Marsoni et al. (2008).

If needed the name of unknown proteins was annotated by protein similarity search performed by alignment analysis against the NCBI-nr database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For predicting the subcellular localization we used EukmPLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi/, Chou and Shen, 2010).

Statistical analysis

All results were presented as mean of the replicates ± standard deviations (SD). Differences between treatments for the different measured variables were tested by one-way variance (Anova), followed by Tukey's HSD post hoc test when significant differences were found ($p \leq 0.05$).

Results

Effects of AgNPs on wheat seedlings

All experiments were carried out using commercially manufactured 10 nm AgNPs coated with PVP to avoid NP aggregation. The mean size of the AgNPs calculated by TEM was 13.2 nm with 70% of the particles ranging from 5 to 15.5 nm (data not shown).

Phytotoxicity was assessed by measuring the effects on germination, shoot and root growth and biomass accumulation relative to unexposed controls. Germination of control samples was <90%; AgNP treatments did not show any significant effect on the percentage or rate of germination (data not shown). As shown in Fig. 1A and B, the root growth was slightly reduced at 1 mg L^{-1} of AgNPs whereas, the root length of wheat seedlings exposed to 10 mg L^{-1} of AgNPs were 63% of the control ($p < 0.05$). Root tip light browning was observed after 10 mg L^{-1} treatment. The presence of 10 mg L^{-1}

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