Journal of Proteomics 148 (2016) 113–125

Contents lists available at ScienceDirect

Journal of Proteomics

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

Proteomic analysis of soybean root exposed to varying sizes of silver nanoparticles under flooding stress

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form 22 July 2016
Accepted 22 July 2016
Available online 26 July 2016

Keywords:
Soybean
Flooding stress
Root
Silver nanoparticles
Proteomics

ABSTRACT

Silver nanoparticles (Ag-NPs) are excessively used as antibacterial agents; however, environmental interaction specifically with the plants remain uncertain. To study the size-dependent effects of Ag-NPs on soybean under flooding, a proteomic technique was used. Morphological analysis revealed that treatment with Ag-NPs of 15 nm promoted soybean growth under flooding compared to 2 and 50–80 nm. A total of 228 common proteins that significantly changed in abundance under flooding without and with Ag-NPs of 2, 15, and 50–80 nm. Under varying sizes of Ag-NPs, number of protein synthesis related proteins decreased compared to flooding while number of amino acid synthesis related proteins were increased under Ag-NPs of 15 nm. Hierarchical clustering identified the ribosomal proteins that increased under Ag-NPs of 15 nm while decreased under other sizes. *In silico* protein-protein interaction indicated the beta ketoacyl reducatse 1 as the most interacted protein under Ag-NPs of 15 nm while least interacted under other sizes. The beta ketoacyl reductase 1 was up-regulated under Ag-NPs of 15 nm while its enzyme activity was decreased. These results suggest that the different sizes of Ag-NPs might affect the soybean growth under flooding by regulating the proteins related to amino acid synthesis and wax formation.

Biological significance: This study highlighted the response of soybean proteins towards varying sizes of Ag NPs under flooding stress using gel-free proteomic technique. The Ag NPs of 15 nm improved the length of root including hypocotyl of soybean. The proteins related to protein metabolism, cell division/organization, and amino acid metabolism were differentially changed under the varying sizes of Ag NPs. The protein synthesis-related proteins were decreased while amino acid metabolism-related proteins were increased under varying sizes of Ag NPs. The ribosomal proteins were increased under Ag NPs of 15 nm. The beta ketoacyl reductase 1 was identified as the most interacted protein under varying sizes of Ag NPs. The mRNA expression level of beta ketoacyl reductase was up-regulated under Ag NPs of 15 nm while its activity was decreased. These results suggest that the Ag NPs of 15 nm improved the soybean growth under flooding stress by increasing the proteins related to amino acid synthesis and waxes formation.

1. Introduction

In recent years, the research on nanoparticles (NPs) gained much attention due to their electronic, optical, mechanical, magnetic, and chemical properties [1]. NPs are the materials with the characteristic size range from 1 to 100 nm [2]. The decreased size is responsible for the interaction of NPs with different materials that leads to their toxicological effects [3]. Ag-NPs are among the most extensively used nanomaterials due to their unique antimicrobial properties. The global production of Ag-NPs is increasing with the count of tons per year [4]. In the environment, Ag-NPs undergo different transformations indicating their variable fate within the biological systems causing differential effects [5,6]. The main toxicity mechanism of Ag-NPs is characterized by the surface oxidation, release of Ag ions, and interaction with biomolecules within the organisms [5,7,8]. The over-production of Ag-NPs resulted in increasing interaction with the crops, which is needed to be investigated.

Ag-NPs are extensively used as a component of agricultural products, such as pesticides and fertilizers [9]. Ag-NPs promoted the growth of flooding-stressed *Crocus sativus* roots by blocking ethylene signaling [10]. Gene expression profiling of *Arabidopsis* treated with Ag-NPs indicated that up-regulated genes were associated with metal response, oxidative stress, and thalianol biosynthetic pathway; whereas, down-regulated genes were associated with ethylene signaling pathway [11]. The NPs mediated oxidative stress depends on the NPs size, surface

Abbreviations: Ag, silver; LC, liquid chromatography; MS, mass spectrometry; NPs, nanoparticles.

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http://dx.doi.org/10.1016/j.jprot.2016.07.027
1874-3919/

area, composition, and presence of metals and also on the cellular functions [12]. Verano-Braga et al. [13] reported that the 20 nm Ag-NPs enter the cell and caused cellular stress including the generation of reactive oxygen species and protein carbonylation while 100 nm Ag-NPs indirectly generated the oxidative stress [13]. Ag-NPs with 45 and 2 nm caused the lowest and highest accumulation of superoxide dismutase, respectively. Along with this, Ag-NPs activated the expression of genes involved in cellular events including cell proliferation, metabolism, and hormone signaling pathways [14]. Ag-NPs with 10 nm was more available biologically compared to the larger sizes and biological effects of Ag-NPs are largely dependent on size [15]. NPs cause differential growth effects on plants depending on the size and surface area. Based on the variable growth effects caused by different sizes of NPs, the molecular mechanisms affected by varying sizes of NPs need intensive investigation.

Climatic trends around the world are fairly rapid in the past few decades that are responsible for the imbalance in the environment [16]. Among the abiotic stresses, flooding has devastating effects on crop growth and ultimately causes a reduction in crop production [17]. Growth and development of various plant species are impeded by the flooding in soil, which could lead to the plant death [18]. In soybean, the flooding tolerance mechanism has been summarized recently highlighting the possible strategies used to overcome the damages caused by the flooding stress [19]. Out of three different kinds of NPs interaction with soybean, the aluminum oxide and Ag-NPs promoted the plant growth under flooding stress compared to the flooded plants [20]. In the comparative analysis, aluminum oxide NPs ameliorated the soybean growth at the seedling stage; however, zinc oxide and Ag-NPs inhibited the plant growth under flooding stress [21]. Moreover, 2 ppm Ag-NPs of 15 nm enhanced the growth under flooding stress while 2 and 50–80 nm reduced the growth at the early stages of plant development [22]. Therefore, effects of NPs on the plants are dependent on the particle size and concentration. The flooding response mechanism has been evaluated for the one particular size of aluminum oxide and Ag-NPs; however, the comparative effects of varying sizes of Ag-NPs on soybean under flooding stress are yet to be explored.

Because of the tremendous interactions with the plants, the effect of NPs in plants needs to be explored at the molecular level. Previously, the effect of one particular size of Ag-NPs on soybean under flooding stress was evaluated where the 15 nm facilitated the soybean growth; however, the size-dependent effects are unclear. The purpose of this study was to evaluate the size-dependent effects of Ag-NPs and to understand that whether the positive effect is specific to the 15 nm or not, when the soybean is treated with the other sizes. To investigate the comparative effects of varying sizes of Ag-NPs on early-stage soybean under flooding stress, morphological and proteomic analyses were performed. In this study, 5 ppm Ag-NPs were used because 5 ppm Ag-NPs of 15 nm were reported to promote the soybean growth under flooding stress [20]; therefore, the effect of other sizes of Ag-NPs was evaluated at the same concentration. In the previous studies [20,22], the effects of one particular size of NPs on the soybean under flooding stress were explored; however, this study focused on the aspect that either the different sizes of Ag-NPs at one concentration caused the similar or different alterations at the molecular level compared to the 15 nm Ag-NPs. Moreover, the changes caused by the Ag-NPs at the sub-cellular level are also studied in detail. This study is the first report on the size-dependent effects of Ag-NPs on soybean plant. In addition, bioinformatic, mRNA expression, and enzyme activity analyses were used to confirm the proteomic results.

2. Experimental procedures

2.1. Plant material and treatments

Seeds of soybean (*Glycine max* L. cv. Enrei) were sterilized with sodium hypochlorite solution, twice rinsed in water, and sown on 500 mL silica sand with 150 mL water in a plastic case (180 mm × 140 mm × 45 mm). Soybeans were grown in a growth chamber illuminated with white fluorescent light (160 μmol m^{-2} s^{-1}, 16 h light period/day) at 25 °C. For flooding stress, 2-day-old soybeans were transferred to glass tubes (38 mm ID × 130 mm) with 120 mL of reverse osmosis water [23]. After covering the glass tube with plastic cap allowing air flow, the tubes were kept at 25 °C in the dark.

To study the effects of different sizes of Ag-NPs on the morphology of soybean, the Ag-NPs (2, 15, and 50–80 nm particle size, US Research Nanomaterials, Houston, TX, USA), at 5 ppm were used. Two-day-old soybeans were treated for 0, 1, 2, 3, and 4 days and length of root including hypocotyl was measured. To study the effects on the protein abundance, 2-day-old soybeans were flooded without or with 5 ppm Ag-NPs for 1, 2, and 3 days. After treatments, root including hypocotyl was collected. Three independent experiments were performed as biological replicates for all experiments (Fig. 1). Biological replicate means that the plants were sown on the different days. For each treatment point, 4 and 8 plants were used for morphological and proteomic experiments, respectively.

2.2. Protein extraction and digestion for proteomic analysis

Proteins were extracted from the collected samples and purified with methanol and chloroform according to the methods previously described [21,24]. Three independent biological replicates were used for each sample. Protein concentration was determined using the Bradford method [25] with bovine serum albumin as the standard. The purified protein fractions were digested and alkylated.

2.3. Mass spectrometry analysis

The resulting tryptic peptides were acidified and analyzed by liquid chromatography (LC) mass spectrometry (MS) as methods described previously [26,27].

2.4. Protein identification of acquired mass spectrometry data

Identification of proteins was performed using the Mascot search engine (version 2.5.1; Matrix Science, London, UK) and Proteome Discoverer software (version 1.4.0.288; Thermo Fisher Scientific, San Jose, CA, USA) against a soybean peptide database (54,175 sequences; Phytozome, version 9.1; http://www.phytozome.net/soybean) [28,29].

2.5. Differential analysis of the identified proteins

The relative abundances of peptides and proteins were compared using the commercial label-free quantification package SIEVE software (version 2.1.377; Thermo Fisher Scientific) by methods as previously described [22].

2.6. Bioinformatic analysis

To determine the functional role of the proteins identified in the MS analysis, functional categorization was performed using MapMan bin codes and MapMan software [30,31]. For soybean, the analysis was performed as previously described [22].

2.7. Cluster and in silico protein-protein interaction analyses using protein abundance

Protein abundance ratios at different time points of flooding stress with Ag-NPs treatments were used for the cluster analysis, which was performed with the Genesis software (version 17.6, http://genome.tugraz.at) [32]. Interacting proteins were identified by comparisons of changes in abundance ratios over time. Identified proteins were analyzed for *in silico* protein-protein interactions that were estimated by