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Gel-free/label-free proteomic analysis of wheat shoot in stress tolerant varieties under iron nanoparticles exposure

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ABSTRACT

Iron nanoparticles (Fe NPs) have stimulatory effects on the germination ratio and plant growth of wheat. To elucidate the effects of Fe NPs on shoot of drought tolerant Pakistan-13 and salt tolerant NARC-11, a gel-free/label-free proteomic technique was used. The weights/lengths of seedling, shoot, and root of wheat varieties were increased on 5 ppm Fe NPs exposure. The number of proteins related to photosynthesis and protein metabolism was decreased and increased in drought tolerant variety and salt tolerant variety, respectively, treated with Fe NPs compared to untreated plants. Differentially changed proteins in drought tolerant variety and salt tolerant variety were mainly related to photosynthesis. Out of photosynthesis related proteins, light reaction was enhanced in salt tolerant variety compared to drought tolerant variety on Fe NPs exposure. The abundance of ribulose bisphosphate carboxylase/oxygenase small chain in drought tolerant variety was higher than that in salt tolerant variety; however, in salt tolerant variety, it was increased 3 fold by Fe NPs exposure compared to untreated plant. These results suggest that Fe NPs improve the growth of wheat seedling, which might be associated with the increase of protein abundance in photosynthesis in salt tolerant variety.

1. Introduction

Advancement in nanotechnology provided the tool and technological platform to focus the effects and mechanism of nanoparticles (NPs) in plants [1]. Nanotechnology brought competitive advantages in the field of physical, biological, and engineering sciences [2]. NPs of 1–100 nm have unique properties than their corresponding bulk material and got attention from scientific community [3]. Metal oxide NPs are being increasingly used for commercial applications ranging from inclusion in self-cleaning coatings, topical sunscreens, and antimicrobial soaps [4]. With the large production of NPs for use in everyday consumer products, NPs contamination in the environment is becoming an important matter of concern [5]. The control of engineered NPs release into the environment has proven difficult due to the rapid growth of the nanomaterial industry and the usage of nanomaterials in a wide array of products [6]. However, current knowledge about the effects of NPs on environment is very limited and further studies are prerequisite.

NPs improved the seed germination and growth, plant protection, pathogen detection, and pesticide/herbicide residue detection [7]. The uptake efficiency and effects of various NPs on the growth and metabolic functions vary among plants [8]. Engineered NPs influenced the plant growth and seed germination in positive or negative way [9]. Titanium oxide NPs exposure increased root length while did not increase germination ratio and plant biomass of wheat and rapeseed [10,11]. However, aluminum oxide and zinc oxide NPs inhibited root elongation of corn, cucumber, soybean, cabbage, and carrot [12,13]. Keeping in view these previous studies, improvement in the growth conditions through novel techniques is still not clear.

Among the different metal NPs, iron (Fe) NPs have been used widely in multiple industrial, commercial, and biomedical applications to benefit society [14]. Because of their high reactivity and magnetic property, Fe NPs were used as remediation agents for environmental applications [15]. Germination ratio and plant growth were used in the studies to determine the impact of Fe NPs exposure to different types of plants, such as ryegrass, barley, lettuce, cattail, and poplar [16–18]. Root length was increased by inducing cell wall loosening in *Arabidopsis* under Fe NPs exposure [16]. High concentration showed toxic effect; while, low concentration enhanced plant growth [17], indicating concentration of Fe NPs determines the positive or negative effects. High concentration of Fe NPs inhibited germination while low

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; NPs, nanoparticles; Fe, iron; RuBisCO, ribulose bisphosphate carboxylase/oxygenase.

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concentration did not have any toxic effect in rye grass, barley, flax, and lettuce [18,19]. Based on these findings, comprehensive technique is useful for elucidation of molecular mechanism of NPs, which provides an insight in understanding the behavior of NPs towards plants.

NPs modified the agronomic characters of plants, which are a key factor for improving the wheat cultivation [20]. Alumina NPs significantly improved the length of root [21] and cesium NPs enhanced the growth, shoot biomass, and grain yield of wheat [22]. Zinc and titanium NPs reduced the biomass [23,24]; while, silver NPs reduced seedling growth [25] in wheat. Based on these reports, further studies are needed for elucidating the molecular mechanism of NPs on wheat. Fe NPs have stimulatory effects on the germination ratio and plant growth of peanut [26], *Arabidopsis* [16], lettuce [18], and wheat [27]. On the other hand, Fe NPs have inhibitory effects on shoot growth of rye grass, barley, and flax [19]. Titanium oxide NPs improved redox status of cold sensitive and tolerant chickpea plants [28]. Although effects of Fe NPs on wheat morphology have been explored [27], its response mechanism has not been elucidated. To elucidate the role of Fe NPs on drought tolerant Pakistan-13 and salt tolerant NARC-11, proteomic and morphological analyses were performed. Furthermore, immunoblot analysis was performed to confirm the proteomic results.

2. Experimental procedures

2.1. Plant materials and treatments

Seeds of wheat (*Triticum aestivum* L.) varieties Pakistan-13 and NARC-11 were sterilized in 3% sodium hypochlorite solution, twice rinsed in water, and soaked for whole night. On next day, seeds were sown in petri dishes (90 mm ID × 15 mm) containing three layers of filter paper and wet with water thoroughly. The seeds were treated at 4 °C for two days followed by dark treatment for two days at 25 °C. Plants were grown in a growth chamber illuminated with white fluorescent light (200 μmol m^{-2} s^{-1}, 16 h light period/day) at 25 °C. For morphological analysis, 5-day-old wheat plants were treated without or with 1, 5, 10, and 50 ppm Fe NPs (20 nm size, US Research Nanomaterials, Houston, TX, USA). Weights/lengths of seedling, shoot, and root were measured at 6 day of treatment. For proteomic analysis, shoot was harvested at 2 day of treatment without or with 5 ppm Fe NPs exposure. Twenty seeds were used for each treatment in each replication and 3 independent experiments were performed as biological replicates for all experiments. Biological replicate means that seeds were sown on different days.

2.2. Protein extraction

A portion (500 mg) of each sample was ground to powder in liquid nitrogen using a mortar and pestle, and transferred to an acetone solution containing 10% trichloroacetic acid and 0.07% 2-mercaptoethanol [29]. The resulting mixture was vortexed, sonicated for 10 min, and then incubated for 60 min at −20 °C. The suspension was centrifuged at 9000 x g for 20 min at 4 °C, the supernatant was discarded, and obtained pellet was washed twice with 0.07% 2-mercaptoethanol in acetone. The final pellet was dried using a Speed-Vac concentrator and resuspended in lysis buffer, consisting of 7 M urea, 2 M thiourea, 5% CHAPS, and 2 mM tributylphosphine, by vortexing for 1 h at 25 °C. The resulting suspension was centrifuged twice at 20,000 x g for 20 min at 25 °C and the supernatant was collected as total protein. Protein concentrations were determined using the Bradford assay with bovine serum albumin as the standard [30].

2.3. Protein purification and digestion for mass spectrometry analysis

Proteins (150 μg) were purified with methanol and chloroform to remove any detergent from the sample solutions [31]. Briefly, 600 μL of methanol was added to each sample and the resulting solution was mixed before the further addition of 150 μL of chloroform and 450 μL of water. After mixing, the samples were centrifuged at 20,000 x g for 10 min to achieve phase separation. The upper aqueous phase was discarded keeping the white disk unbroken and 450 μL of methanol was added slowly to lower phase. The samples were centrifuged at 20,000 x g for 10 min, supernatants were discarded, and pellets were dried. The dried pellets were resuspended in 50 mM NH_4HCO_3, proteins in the samples were reduced with 50 mM dithiothreitol for 30 min at 56 °C, and then alkylated with 50 mM iodoacetamide for 30 min at 37 °C in the dark. Alkylated proteins were digested with trypsin and lysyl endopeptidase (Wako, Osaka, Japan) at 1:100 enzyme/protein concentrations at 37 °C for 16 h. The resulting tryptic peptides were acidified by mixing with formic acid (pH < 3) and the resulting solution was centrifuged at 20,000 x g for 10 min. The obtained supernatant was collected and analyzed by nanoliquid chromatography (LC)-mass spectrometry (MS).

2.4. Nanoliquid chromatography-tandem mass spectrometry analysis

Peptides were loaded onto an Ultimate 3000 Nano LC system (Dionex, Germering, Germany) equipped with a C18 PepMap trap column (300 μm ID × 5 mm, Dionex). The peptides were eluted from the trap column and separated using 0.1% formic acid in acetonitrile at a flow rate of 200 nL/min on a C18 Tip column (75 μm 1D × 120 mm; Nikkyo Technos, Tokyo, Japan) with a spray voltage of 1.8 kV. The peptides were analyzed on a nanospray LTQ XL Orbitrap MS (Thermo Fisher Scientific, San Jose, CA, USA) operated in data-dependent acquisition mode with the installed Xcalibur software (version 2.0.7; Thermo Fisher Scientific). Full-scan mass spectra were acquired in the MS over 400–1500 *m/z* with a resolution of 30,000. A lock mass function was used to obtain high mass accuracy [32]. As the lock mass, the ions $C_{24}H_{39}O_4^+$ (*m/z* 391.28429), $C_{14}H_{46}NO_7Si_7^+$ (*m/z* 536.16536), and $C_{16}H_{52}NO_8Si_8^+$ (*m/z* 610.18416) were used. Values for ion isolation window were set as follows: activation type was collision-induced dissociation (CID), default charge state was 2, isolation width was 2.0 *m/z*, normalized collision energy was 35 eV, and activation time was 30,000 msec. Values for dynamic exclusion were determined as follows: repeat count was 2, repeat duration was 30 s, exclusion list size was 500, exclusion duration was 90 s, and exclusion mass width was ±1.5 Da. The ten most intense precursor ions above a threshold value of 500 were selected for collision-induced fragmentation. The acquired MS spectra were used for protein identification.

2.5. Protein identification from the mass spectrometry data

Proteins were identified using the Mascot search engine (version 2.5.1; Matrix Science, London, UK) and UniProt Knowledgebase SwissProt (http://www.uniprot.org). DTA files were generated from acquired raw data files and then converted to Mascot generic files using Proteome Discoverer software (version 1.4.0.288; Thermo Fisher Scientific). The parameters used in the Mascot searches were as follows: carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Peptide mass tolerance was set at 10 ppm, fragment mass tolerance was set at 0.8 Da, and peptide charges were set at +2, +3, and +4. An automatic decoy database search was performed as part of the search. Mascot results were filtered with the Mascot percolator to improve the accuracy and sensitivity of peptide identification [33]. False discovery rates for peptide identification of all searches were <1.0%. Peptides with a percolator ion score of >13 ($p < 0.05$) were used for protein identification. The Mascot search results were exported in msf format for differential analysis.