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Comparison of *MAPK* and *thioredoxin* expression in wheat seedlings exposed to silver nitrate and silver nanoparticle

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ABSTRACT

The extensive use of heavy metals and nanoparticles (NPs) has led to their release into the environment that might have negative impacts on both organisms and the environment. In this study, the molecular responses of wheat seedlings to silver nitrate and silver nanoparticles (AgNPs) were assessed by transcript accumulation analysis of genes coding for products potentially involved in heavy metal tolerance. A quantitative Real-time PCR experiment was performed with *MAPK* (Mitogen-activated protein kinase) and *thioredoxin* genes using RNA isolated from wheat shoots treated for 0, 2, 6, 12 and 24 h with $AgNO_3$ and AgNPs at 100 $mg^{-1}L$ concentration. Results indicated that stressful conditions led to the antioxidant responses of wheat seedlings that could be reflected as changes in *MAPK* and *thioredoxin* gene transcripts. The gene expression patterns were slightly different. The expression of these genes in response to both treatments was high at the beginning of the stress and was decreased with time. Our results showed the effects of $AgNO_3$ treatment were faster than AgNPs. We found that wheat seedlings might develop different strategies to cope with $AgNO_3$ and AgNPs toxicity with change in the expression of *MAPK* and *thioredoxin* heavy metal-related genes.

Keywords: Real Time PCR; Protein kinase; Reactive Oxygen Species (ROS); Heavy metals

Introduction

In recent years, heavy metal toxicity has become a global challenge to all life forms: plants, animals and eventually humans. The unwanted growth of toxic heavy metals, mainly due to different anthropogenic activities leads to heavy metal pollution that can have terrible and unexpected effects on ecosystems (1-6). Heavy metals are one of the major abiotic stresses that limit crop productivity and plant growth through damage to normal metabolism and ion imbalances. Plants have evolved diversity of defensive strategies that permit them to adapt to undesirable environments for continued survival, growth, and development (3, 7, 8). Nanoproducts have now been applied in every field of our life. Design, production, optimization and application of nanoparticles (NPs) are attractive areas of investigations (9-12). The rising production of nanoparticles has led to concerns over the potential adverse impacts of these marital on the environment (13-17). Existing investigations showed that there is

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not a complete understanding of the potential risks of nanoparticles on the health of organisms and environment (18). In fact, despite the increasing quantity of research on the toxicity of nanoparticles in animal and bacteria, some studies are existing in higher plants (19, 20). The negative implications of AgNPs and Ag ions in plant are enormous, for example: degradation of the plasma membrane and changes in membrane permeability, failing of the proton motive force and inhibition of the ATP synthesis, inhibition of enzyme activity by binding to –SH groups of amino acids, denaturation of ribosome and inhibiting protein synthesis, creation of reactive oxygen species (ROS) and damaging to vital macromolecules. Silver nanoparticles reveal novel properties, which are observed neither in molecules nor in bulk metals (20-23). In all eukaryotes, *MAPK* pathways play an essential role in signal transduction involved in the regulation of growth, differentiation, development, programmed cell death, proliferation, and stress responses. In plant, over-expression of *MAPK* genes in responses to a range of abiotic and biotic stresses, including drought, cold, heat, salinity, heavy metal, UV, and pathogen attack, leads to stress adaptation (24, 25). *Thioredoxin* is a small protein with a redox active disulfide bridge that has a main role in redox regulation of protein function in plant metabolism. This ubiquitous protein is found in all living organisms from prokaryotes to higher eukaryotes. *Thioredoxin* initially was recognized as regulatory proteins in the reversible light activation of key photosynthetic enzymes, which have been found in the cytoplasm and mitochondria (26-29). Up to now, limited studies are available about the effects of NPs on gene expression of plants. Thus, having a good understanding related to the effects of heavy metal ions and nanoparticles on molecular responses of plants, is required. Both silver ions as toxic heavy metals and AgNP as one of the most common, widely used nanomaterials, can be released into the environment (30, 31) and affect the living organisms including plants (32, 33). Therefore, in the present work, comparison of gene expression of *MAPK* and *thioredoxin* genes in wheat exposed to silver nitrate and, silver nanoparticle are analyzed by transcript accumulation of the genes using Real-time PCR.

Materials and methods

Plant materials and growth conditions

Seeds of wheat (*Triticum aestivum* L. Var. Chamran) were obtained from Zarghan Agricultural Research Center, Iran and kept in the dark at 4°C for later use. Seeds were surface sterilized by soaking in 5% (w/v) sodium hypochlorite for 10 minutes. They were washed three times with distilled water and air-dried on filter papers. Seeds were allowed to germinate in the dark at 25ºC on moist filter papers. Twenty of five-day old seedlings were transferred into small plastic containers filled with perlite and Hoagland nutrient solution (pH 6.2). Wheat seedlings were grown in the growth chamber set at 16 h/8 h light-dark periods. Three replicates were used for each treatment.

Silver nitrate and silver nanoparticles treatments

Dry powder of AgNPs with average sizes of 20 nm and 99.99% purity was purchased from US Research Nanomaterials, lnc. (USA). Using Hoagland nutrient solutions as solvent, 100 $mg^{-1}L$ concentration of silver nanoparticles was prepared. The dissolved particles were dispersed by a high-power probe-type sonicator (Misonix, Qsonica LLC, Newton, USA) for 30 mins. Silver nitrate (99.9%) was used as $AgNO_3$ and obtained from Sigma-Aldrich (Sigma-Aldrich). The Hoagland nutrient solution was used as a control. After 2, 6, 12 and 24 h of the beginning of treatment, the leaves of control and treated wheat seedlings were harvested and stored at -80ºC until RNA extraction.

RNA isolation and cDNA synthesis

Total RNA was extracted using an RNA isolation kit (DENA Zist kit, Iran) according to the manufacturer's instructions. Purified RNA was kept in -80°C until DNase treatment used. Extracted RNA was quantified using Nano-Drop ND 1000 Spectrophotometer (Wilmington, USA). The quality of total RNA was evaluated by the ratios OD_{260}/OD_{280} and OD_{260}/OD_{230}. RNA integrity was verified on a 2% agarose gel; three bands corresponding to ribosomal RNA (28S, 18S and 5S) were apparent. DNase treatment was carried out using Fermentas (Fermentas, Hanover, MD) DNase

Kit as instructed in the manufacturer's protocol. First strand cDNA was generated using reverse transcriptase (Fermentas Cat Num: EN0531).

Primer design

Primers used for the amplification of target cDNAs were designated according to gene sequences of wheat available at the National Center for Biotechnology Information (NCBI) and used to design gene specific Real-time primers by Allele ID 7.8 software. Two primer pairs (whose were relatively short sequenced; approximately 100 bp and suitable for Real-time quantitative PCR) for each of the genes, were designed. The wheat *18s rRNA* gene were used as housekeeping genes by the specific primers as the internal control (whose expression proved not to be influenced by heavy metal stress) for data normalization. The sequence of primers (target and housekeeping genes) was presented in Table 1.

Table 1. Primers used for Real-time PCR amplification, resulting product length and Tm

Name of genes	Primer Sequence 5' → 3'	Product length (bp)	Tm (ºC)
MAPK	F: GCCACAAGAAGAACATAA R: AGCAACTACTCCATAACT	107	56
Thioredoxin	F: CTGAAGTCCATTGCTGAG R: CAGTTCCTCCTTGATAGC	108	58.8
18s rRNA	F: CGCTCCTACCGATTGAATGG R: CCTTGTTACGACTTCTGCTTCC	127	56

Real-time quantitative PCR

The reactions were prepared using the SYBR Green (Takara, Japan) according to the manufacturer's protocol. The first strand cDNAs were diluted 5X with RNase-free water. Quantitative PCR was performed in 20 µl reactions using gene specific primers, 4 µl of cDNA as a template. Gene expression was indirectly assessed using the SYBR Green dye in a line GeneK thermal cycler (Bioer, China) with initial denaturing of 10 min at 94ºC, followed by 40 cycles each consisting of 94ºC for 10 sec, 57-63ºC for 15 sec and 72ºC for 30 sec. After 40 cycles, the specificity of the amplifications was checked based on melting curves resulted by heating the amplicons from 50 to 9ºC. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 2% agarose gel. All amplification reactions were repeated twice under identical conditions, in addition to a negative control and four standard samples. The abundance of targeted gene transcripts was normalized to *18s rRNA* and set relative to control plants (no heavy metal exposure) according to the $2^{-\Delta\Delta CT}$ method (34).

For quantitative Real-time PCR data, the relative expressions for *MAPK* and *thioredoxin* were calculated based on the threshold cycle (CT) method. The mean relative levels of amplification of the target genes and standard deviations were calculated based on CT values. The CT for each sample was calculated using the Line-gene K software, where ΔCT was determined by subtracting the corresponding internal control CT value from the specific CT of the targets (*MAPK* and *Thioredoxin*), and ΔΔCT was obtained by subtracting the ΔCT of each experimental sample from that of the control sample. Copy numbers of genes under stress treatments were determined by using standard curves.

Statistical analysis

The experimental designs were randomized complete block, and each value reported is the average of three repeats. The raw data was imported into Microsoft Excel 2007 and Graph-Pad Prism 5 programs for calculations and graphic representation. SPSS (version 16.0) software was used for analysis of variance. Quantitative changes of parameters were evaluated through analysis of variance (one-way ANOVA),

with Duncan's multiple range tests at P≤0.05 to find out significant differences among treatments. All results are presented as the means ± standard deviation (SD).

Results

PCR and sequencing

The identity of each Real-time PCR product was determined using direct cDNA sequencing. PCR products were sequenced at the Macrogen Company sequencing facility using Applied Biosystems 3730 mXL automated DNA sequencer (Macrogen, Korea). For identification, sequence homology searches were carried out using the BLAST search facility available through the NCBI (Table 2).

Analysis of MAPK gene expression

Real-time PCR was performed to explore *MAPK* expression in response to AgNPs and $AgNO_3$. The results showed that the *MAPK* gene was significantly up-regulated at 2, 6, and 24 h (5.3, 1.7, and 1.3 fold, respectively) after $AgNO_3$ treatment compared with the control at the same times (Table 3 and Figure 1). In response to AgNPs, *MAPK* expression increased 2.5, 8, and 1.5 fold, respectively at 2, 6, and 24 h (Table 3 and Figure 1). Significant reduction in *MAPK* expression at 12 h after treatment in both AgNPs and $AgNO_3$ treatments and even control compared with the treatments of 0, 2, 6 and 24 h was observed. *MAPK* expression gradually increased at 2 and 6 h after the beginning of the treatment in wheat seedlings then it eventually decreased to a level that was slightly lower than the level in the non-treated control after 12 and 24 h.

Table 2. ***MAPK*** **and** ***Thioredoxin*** **cDNA sequences derived from wheat seedlings exposed to AgNPs and $AgNO_3$.**

Gene	Sequence
MAPK	GGGACCTACCAGTAGAATAGTCTGTGGCAGCCAGTACCAATATCCAGGAAGTTGTTGGCAAGGGGAGTTATGGAGTAGTTGCTA
Thioredoxin	CGGGTCAGCATACCACCGTTCCTGTTCATGAGGAGGAGACGTCAAGGACAGGGTTGTCGGAGCTATCAAGGAGGAACTCGA

Table 3. ***MAPK*** **and** ***Thioredoxin*** **expression in the wheat seedlings exposed to AgNPs and $AgNO_3$ concentration (control, and 100 $mg^{-1}L$) and different times after treatment (0, 2, 6, 12, and 24 h)**.

Thioredoxin	***MAPK***	**Concentration (mg^{-1} L)**
1.00 ± 0.11^{e}	1.00 ± 0.11^{f}	Control 0 h
0.85 ± 0.08^{e}	0.98 ± 0.12^{f}	Control 2 h
5.40 ± 0.29^{a}	5.21 ± 0.13^{b}	$AgNO_3$ 2 h
1.76 ± 0.20^{d}	2.47 ± 0.21^{c}	AgNPs 2 h
0.87 ± 0.08^{e}	0.86 ± 0.12^{f}	Control 6 h
1.79 ± 0.24^{d}	1.50 ± 0.10^{d}	$AgNO_3$ 6 h
5.05 ± 0.56^{ab}	6.90 ± 0.23^{a}	AgNPs 6 h
0.31 ± 0.04^{f}	0.41 ± 0.03i	Control 12 h
0.28 ± 0.02^{f}	0.43 ± 0.08^{hi}	$AgNO_3$ 12 h
0.28 ± 0.01^{f}	0.58 ± 0.04^{h}	AgNPs 12 h
1.01 ± 0.11^{e}	0.79 ± 0.07^{g}	Control
2.71 ± 0.20^{c}	1.08 ± 0.05^{f}	Control 24 h
4.08 ± 0.52^{b}	1.25 ± 0.06^{e}	AgNPs 24 h

Analysis of thioredoxin gene expression

Real-time PCR was performed to examine *thioredoxin* gene expression in response to AgNPs and $AgNO_3$. The results showed that the *thioredoxin* gene was significantly up-regulated at 2, 6, and 24 h (6.3, 2, and 2.6 fold, respectively) after $AgNO_3$ treatment compared with the control at the same times (Table 3, and Figure 2). In response to AgNPs, *thioredoxin* gene expression increased at 2, 6, and 24 h (about 2, 5.8, and 4 fold, respectively) after AgNPs treatment compared with non-treated wheat seedlings at same times (Table 2 and Figure 2). However, at 12 h after AgNPs and $AgNO_3$ treatments, there was no significant change compared with a control.

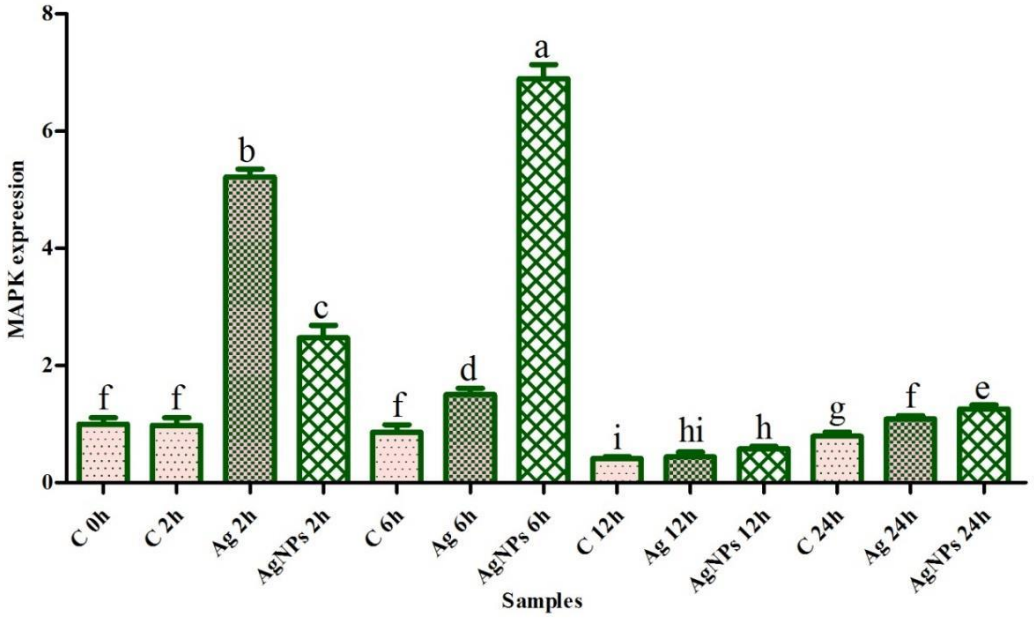

Figure 1. Analyses of the expression of the *MAPK* gene in the wheat seedlings exposed to AgNPs and $AgNO_3$ concentration (control, and 100 $mg^{-1}L$) and different times after treatment (0, 2, 6, 12, and 24 h).

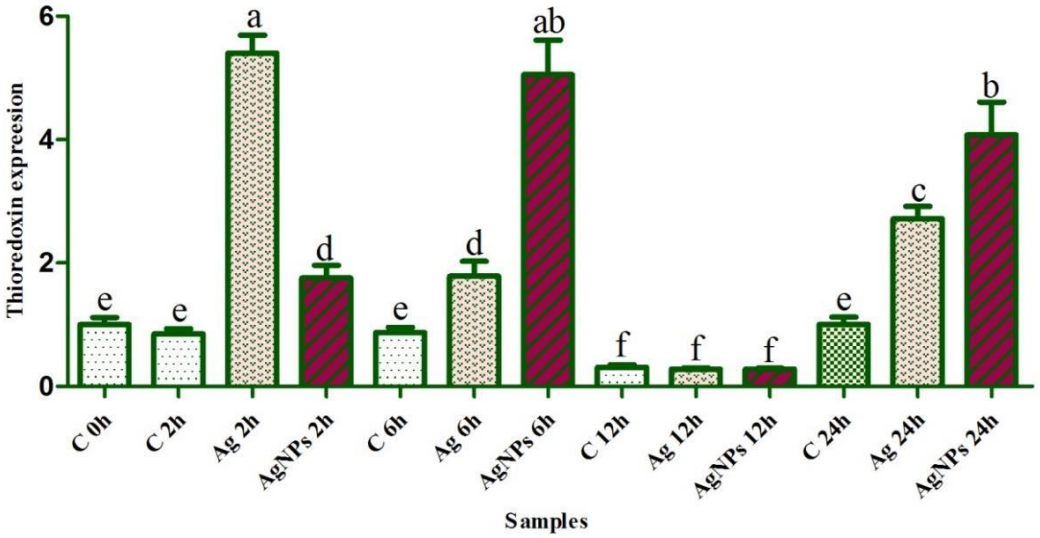

Figure 2. Analyses of the expression of *thioredoxin* gene in the wheat seedlings exposed to AgNPs and $AgNO_3$ concentration (control, and 100 $mg^{-1}L$) and different times after treatment (0, 2, 6, 12, and 24 h).

Discussion

MAPK is involved in the signal transduction pathways associated with environmental stress responses in all eukaryotes that induced at the mRNA level by drought, low temperature, high salinity, and heavy metal stress. Our results supported the results of other studies that showed molecular responses of stress on some plants (35-38). As it is clear from the results, rapid expression of this gene in the early hours indicates that the *MAPK* is a rapid response gene. The highest expression level of the *MAPK* gene belonged to $AgNO_3$ treatments at 2 h and AgNPs treatments at 6 h. Entering the plant into the dark phase of the circadian rhythms may be led to decrease in the *MAPK* expression in response to $AgNO_3$ and AgNPs treatments at 12 h. Previous studies showed expression of *MAPK* gene can control by light and biological clock (39, 40). In addition, promoter analysis of *MAPK* gene showed that it contains motifs that are responsive to the light and circadian rhythm.

Thioredoxin is the main disulfide reductase responsible for keeping proteins in their reduced state. The main role of *thioredoxin* proteins in AgNPs and $AgNO_3$ tolerance is detoxification of free radicals (28, 41). As results clearly showed, expression of *thioredoxin* gene in responded to both $AgNO_3$ and AgNPs treatments shortly after the start of treatment occurred. The expression of the *thioredoxin* gene responded to $AgNO_3$ treatments at 2 h was maximum (6.3 fold) then at 6 h (2 fold) decreased to reach to the control level at 12 h after the stress. Finally, at 24 h, expression of *thioredoxin* gene slightly increased. Also, the expression of the *thioredoxin* gene responded to AgNPs treatments at 2 h increased (2 fold) and at 6 h reached its maximum (5.8 fold) subsequently decreased to reach at a control level at 12h after the stress.

Finally, at the 24 h expression of *thioredoxin* gene, increased (4 fold). As was observed in the *MAPK* gene, entering the plant into the dark phase of the circadian rhythms may be led to decrease in the *thioredoxin* expression in response to $AgNO_3$ and AgNPs treatments at 12 h. According to the results of previous investigation, it was known that the expression of *thioredoxin* gene affected by light and biological clock (42, 43). In addition, promoter analysis of *thioredoxin* gene shows that it contains motifs that are responsive to light and the circadian clock.

It has been recognized that plants have developed molecular protection responses to biotic and abiotic stresses over evolutionary time as a survival mechanism. Regulation of gene expression plays a key role in response almost in all stresses. In this study, we compared the transcriptome profiles of *MAPK* and *thioredoxin* genes in wheat seedling control and exposed to $AgNO_3$ and AgNPs. Results showed that approximately, both genes had a short time response and in early onset of stress had the highest expression. Comparing the gene expression in control samples in all tested intervals showed that gene expression of *MAPK* and *thioredoxin* genes (Table 3) in dark phase (at 12 h after treatment) o was decreased. The result showed that the expression of *MAPK* and *thioredoxin* genes significantly was induced by heavy metal and nanoparticle stresses that can be serve benefit acknowledgments for future studies and suggested further study about the biological clock and the circadian cycle. Although silver ions and nanoparticles have several positive aspects in life, but overuse and lack of knowledge about the environmental impacts can damage in the environment. Therefore, to better understand regarding the toxicity effects of Ag ions and AgNPs on human, animals and plant health, further experiments should be performed. Results from this study, can provide new insights into the molecular mechanisms of plant response to AgNPs and $AgNO_3$.

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