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Expression Profiling of Certain MADS-box Genes in *Arabidopsis thaliana* Plant Treated with Silver Nanoparticles

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Abstract

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Silver nanoparticles (AgNPs) have been shown to enhance seed germination and plant growth. In this study, we examined the plant response to AgNP dosage using a model plant, *Arabidopsis thaliana.* Seedlings were treated with different concentrations of AgNPs. Seedling fresh and dry weights were measured for all treatments. The exposure of plants to 1 mg/l AgNPs resulted in a significant increase in seedling fresh and dry weights compared with control plants, although the exposure to lower and higher concentrations resulted in a decrease in fresh and dry weights. Expression profiling for 22 MADS-box genes was carried out using quantitative real-time PCR (qRT-PCR). Among the investigated MADS-box genes, eight genes were upregulated and four genes were downregulated at each of the AgNP concentrations, 0.1 and 1 mg/l. Revealing the effects of AgNPs on the expression of MADS-box genes will increase our knowledge of their role before implementing a large-scale agricultural utilization of AgNPs in the improvement of plant growth and development.

Keywords: AgNPs; molecular mechanisms; plant growth; qRT-PCR

The interactions of nanomaterials with plants have not been fully elucidated (Ma *et al*. 2010). Silver nanoparticles (AgNPs) are one type of nanomaterials which have an early impact in healthcare products because of their antimicrobial properties (Kumari *et al*. 2009). Nowadays, the effect of AgNPs on plant growth is under investigation (Nowack 2010). Recent studies suggested that AgNPs might be beneficial for enhancement of crops such as common bean, maize, castor, watermelon and zucchini (Salama 2012; Yasur & Rani 2013; Almutairi & Alharbi 2015). There are many reports indicating that the appropriate concentrations of AgNPs have an important role in enhancing seed germination (Barrena *et al*. 2009; Savithramma *et al*. 2012) and plant growth (Sharma *et al*. 2012; Kaveh *et al*. 2013). Agricultural researches have sought to ensure that using nanomaterials in increasing the crop productivity is safe and effective.

The available studies on the impact of AgNPs on vascular plants have consistently shown that AgNPs have detrimental effects on plant growth. Stampoulis *et al*. (2009) reported that *Cucurbita pepo* plants that were grown in hydroponic solutions amended with 100 and 500 mg/l of 100 nm AgNPs showed 41% and 57% decreases in biomass and transpiration rates, respectively, compared with control plants. Gubbins *et al*. (2011) reported that AgNPs could inhibit the growth of *Lemna minor*. Recent studies have reported that the plant response to AgNPs results in either enhancement or inhibition of growth depending on AgNP dosage. Of serial concentrations, exposure to specific concentrations of AgNPs could enhance plant growth compared with non-exposed plants, while higher and lower concentrations negatively affect plant growth (Kaveh *et al*. 2013; Qian *et al*. 2013). Of the tested AgNP concentrations (25, 50, 100, 200 and 400 ppm), 50 ppm was the optimal

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treatment for eliciting a growth response in *Brassica juncea* seedlings. Fresh weight, root and shoot length, and vigour index of seedlings are positively affected (Sharma *et al*. 2012). The treatment of *A. thaliana* plants with 1 and 2.5 mg/l of AgNPs increased seedling biomass, whereas treatment with higher concentrations decreased seedling biomass (Kaveh *et al*. 2013). Exposure to AgNP suspensions inhibited the elongation of *A. thaliana* seedling roots and demonstrated a linear dose-response relation within the tested concentration range (Qian *et al*. 2013).

MADS-box genes in plants are putative transcription factors involved in regulating numerous developmental processes, such as meristem and organ identity in inflorescences and in flowers. MADS-box genes are also involved in processes other than flower development, such as the establishment of developing embryos, seed coat and ultimately in root and fruit development (De Bodt *et al*. 2003; Kofuji *et al*. 2003). Moreover, the molecular mechanisms that mediate plant response to nanomaterials are still unclear because of the limited number of studies. Some previous studies have investigated the molecular response of *A. thaliana* to AgNPs through gene expression profiling and have indicated that exposure of plants to the AgNPs causes a number of responses at the cellular level (Kaveh *et al*. 2013; Qian *et al.* 2013; Syu *et al*. 2014; Kohan-Baghkheirati & Geisler-Lee 2015).

The impact of AgNPs on plant growth can be clearly examined through the expression profiling of MADS-box genes. These genes have been chosen in this study for their considerable functions in the regulation of plant growth and development. In this study, we highlight the molecular mechanisms associated with positive and negative effects of AgNPs on plant growth to understand the interaction between AgNPs and plant molecular response. Expression of MADS-box genes in *A. thaliana* plants and seedling fresh and dry weight will be measured after exposure to various concentrations of AgNPs. This study provides a demonstration of the interactions between plant responses to nanomaterials and regulation of plant developmental processes.

MATERIAL AND METHODS

Exposure of *Arabidopsis thaliana* plants to AgNPs. *Arabidopsis thaliana* seeds of the Columbia (COL-0) ecotype (Carolina, USA) were kept on wet filter paper at 4°C in the dark for 24 h. Seeds were then surface-sterilized with 95% (v/v) ethanol for 5 min, and 0.6% (w/v) sodium hypochloride for 5 min. Seeds were germinated under sterile conditions in 10 × 10 cm Magenta boxes filled with 100 ml of semisolid nutrient medium consisting of 0.5% strength Murashige and Skoog medium (MS) nutrient solution supplemented with 0.3% sucrose (w/v) and 0.8% Phyto Agar (w/v) (pH 7.2). The boxes were incubated at 25°C with a 16 h light/8 h dark photoperiod for six days before AgNP treatment. AgNPs (silver nanopowder, 99.99%, 20 nm) were purchased from U.S. Research Nanomaterials (Houston, USA). *A. thaliana* plant response to AgNPs was tested by cultivating seedlings in the presence of increasing concentrations of AgNPs (0.05, 0.1, 0.5, 1, 1.5, 2, 2.5 and 3 mg/l) which were added to the MS nutrient medium. Control plants were grown in nutrient medium (MS) only. To detect the effect of AgNPs on plant growth, seedling fresh and dry weights were measured after 15 days for all treatments and control plants. The significance of differences between treatments was evaluated using *t*-tests at the 0.01 and 0.05 significance levels. Based on seedling growth measurements, plants showing the highest and the lowest growth with respect to control were selected for further analysis.

Gene expression analysis using qRT-PCR. Quantitative analysis of gene expression was performed for selected MADS-box genes using qRT-PCR. Total RNA was extracted with TRIzol reagent (Sigma, St. Louis, USA) from seedlings after 15 days, then reverse-transcribed into cDNA. Twenty-two MADS-box genes responsible for *A. thaliana* plant growth were selected for qRT-PCR analysis. MADS-box gene sequences available in the *Arabidopsis* Information Resource database (TAIR; http://www.arabidopsis.org/) were used to design gene-specific primers using Table 1. The housekeeping gene *GAPDH* was used as an internal control for its stable expression in *A. thaliana* tissues according to Czechowski *et al.* (2005). Three biological replicates were run for each of the 22 genes in addition to the *GAPDH* gene. Each reaction (20 µl) consisted of the 2X SYBR ready mix (Qiagen, Valencia, USA), forward and reverse primers (0.3 µM each) and 3 µl of first-strand cDNA. The reaction conditions were as follows: 50°C for 2 min and 95°C for 15 min, and 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative quantity of the target gene expression level was determined using the comparative Ct (cycle threshold) method (Livak & Schmittgen 2001). ΔCt values for each

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MADS-box gene were calculated by subtraction of Ct value for the *GAPDH* gene in each treatment of the Ct value of MADS-box gene in the same treatment. ΔCt values were calibrated for each gene using its expression in non-exposed plants as a calibrator to obtain ΔΔCt, and then $2^{-\Delta\Delta Ct}$ was calculated using the equation below.

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{GAPDH}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{exposed plants}} - \Delta Ct_{\text{non-exposed plants}}$$

$$\text{Ratio} = 2^{-\Delta\Delta Ct}$$

$2^{-\Delta\Delta Ct}$ values for all MADS-box genes were compared for *A. thaliana* plants treated with 0.1 and 1 mg/l AgNPs vs non-exposed plants, using two-tailed Student's *t*-test at $P < 0.05$ and fold change between 0.5 and 2.

RESULTS AND DISCUSSION

The effects of AgNPs on seedling fresh and dry weights in *A. thaliana*. To determine the effect of AgNPs on *A. thaliana* growth, plants were grown in MS containing increasing concentrations of AgNPs, ranging from 0.05 to 3 mg/l. Figure 1 presents the average fresh and dry weight values of plants exposed for 15 days to AgNPs. The exposure of plants to 1 mg/l AgNPs resulted in a significant increase in fresh and dry weight compared with control plants. However, exposure to lower and higher concentrations resulted in a decrease in the fresh and dry weights.

Table 1. Primer combinations used for qRT-PCR analysis; twenty-two *A. thaliana* MADS-box genes and the internal control gene, *GAPDH*, were used; gene accession numbers in TAIR databases are illustrated with the size of PCR products

Gene	Forward primer	Reverse primer	Accesion No. in TAIR	Product size (bp)
AGL16	5'-GGGGCAAGATCGCGATTAAG-3'	5'-CTACCGGTGCTGGAGAAGAT-3'	AT3G57230.1	204
AGL17	5'-GCCAGCTCCAGTGTGAAATC-3'	5'-ACTCCCGTTAGTTGCCGATA-3'	AT2G22630.1	167
AGL21	5'-CTCCAAACGAAGAAAGGGCC-3'	5'-ACTTCTGATGCGGGGTTCAA-3'	AT4G37940.1	195
AGL24	5'-ATCTTCTCTGCCACCGGAAA-3'	5'-AAGTAGTTTCTCCAGCCGCT-3'	AT4G24540.1	255
AGL72	5'-CGCTAGCTCCGATATCAGGA-3'	5'-CCGGTTATGGACTTCAAGCA-3'	AT5G51860.1	160
SHP1	5'-TGGAGGAAGGTGGGAGTAGT-3'	5'-AGAAGATGACGAGGGCAACT-3'	AT3G58780.1	189
MAF2	5'-CACTTTCTCCAAACGACGCA-3'	5'-AGGCTTCAAGTTCATCAGCA-3'	AT5G65050.1	185
MAF4	5'-ATCATCTCTGCCACCGGAAG-3'	5'-AGCAACTCCTTGTGCGAAAG-3'	AT5G65070.1	152
MAF5	5'-GGAAGAGTGAAGCCATGGGA-3'	5'-CGATGAAAAGAGCGACGGAG-3'	AT5G65080.1	156
SVP	5'-TTTGTTCGTTGTGATGGCGA-3'	5'-CTCGAACAGTTTTCCGGTGG-3'	AT2G22540.1	181
SEP1	5'-TGGGAAGAGGAAGAGTAGAGC-3'	5'-GCATGTTTGAGGAGCTGCAA-3'	AT5G15800.1	189
SEP2	5'-GGGGTGAGGAAAGATGGGAA-3'	5'-ACGGTTGGAGAAGACGATGA-3'	AT3G02310.1	166
AP1	5'-AATGGGAAGGGGTAGGGTTC-3'	5'-TGGGAGAAGACAACAAGAGCA-3'	AT1G69120.1	150
PI	5'-CTCAGGCATTTGAAGGGAGA-3'	5'-TGGAAAGTGAGTTGCCGTTG-3'	AT5G20240.1	176
FLC	5'-CGGCGATAACCTGGTCAAGA-3'	5'-TGAGTTCGGTCTTCTTGGCT-3'	AT5G10140.1	239
FLM	5'-TGTTGTCGTCGTATCTGCCT-3'	5'-GCAGTCTCAAGTTGTTCCTCC-3'	AT1G77080.2	249
FUL	5'-GCCGAGACGTTTCACAAAGT-3'	5'-GGAGCGCAGATATGGATTCG-3'	AT5G60910.1	219
VIP5	5'-GTCGTCGATCTCTGCAACAC-3'	5'-AAGAACCCTCACGTCTCCTC-3'	AT1G61040.1	201
VDD	5'-ATGGGGAAGGTCATGGCAAG-3'	5'-CATCTCCGTACCATCTGCCT-3'	AT5G18000.1	181
PHE1	5'-AGCTACGTGATGAGAACCGT-3'	5'-GGGACAGATGCATCCACAAC-3'	AT1G65330.1	184
XAL1	5'-AACCCGGTTCACAGACAAGT-3'	5'-AAGAAGAACCACGACCACCA-3'	AT1G71692.1	208
RSB1	5'-GAGTTGGTGTCAGGAGGTGA-3'	5'-AACTTGTGTCTTGCGCTGTC-3'	AT2G45650.1	172
GAPDH	5'-TTGGTGACAACAGGTCAAGCA-3'	5'-AAACTTGTCGCTCAATGCAATC-3'	AT1G13440.1	209

bp – base pair

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The lowest fresh and dry weight values were observed with 0.1 mg/l AgNPs. Based on this result, 1 mg/l of 20 nm AgNPs nanopowder seems to be the optimal dose that enhances *A. thaliana* growth, whereas the lower and higher concentrations seem to be toxic to plant growth. This dissimilarity of AgNP effect on plant growth between enhancement and inhibition suggests a need for agricultural researchers to understand the interaction between nanomaterials and the plant physiological and molecular response. Plants exposed to 0.1 and 1 mg/l of AgNPs were selected for MADS-box gene expression analysis to understand the plant positive and negative response to AgNPs at the molecular level, for which the plants exposed to 1 mg/l present the positive effect, while the plants under 0.1 mg/l present the negative effect of AgNPs on plant growth. The comparison between gene expression profiling relevant to each of the negative and positive plant responses to AgNPs at the molecular level is important in order to characterize the regulation of gene networks on plants under exposure to nanomaterials. The enhancement of *A. thaliana* growth with 1 mg/l of AgNPs was also shown on poplar leaf fresh weight (Wang *et al.* 2013) and *Brassica rapa* ssp. *rapa* L. fresh seedling biomass with the same dose of AgNPs (Thiruvengadam *et al.* 2015). In contrast, 0.01–0.05 mg/l of 5 or 10 nm AgNPs increased *A. thaliana* root growth and shoot fresh weight, whereas 1 mg/l of 5 or 10 nm AgNPs inhibited *A. thaliana* growth (Wang *et al.* 2013). This opposite effect of 1 mg/l dose of AgNPs might be due to the difference in AgNP size used in this study (20 nm), which was larger than in Wang *et al.* (2013) study (5 or 10 nm AgNPs). Of the tested particle sizes, (5 and 10 nm polyethylene glycol-thiol-coated AgNPs, and 25 nm carbon-coated AgNPs) the smaller 5 and 10 nm AgNPs (with higher specific surface area) exerted higher toxicity to both *A. thaliana* and poplar than the 25 nm AgNPs at the same exposure concentration (Wang *et al.* 2013).

Expression patterns of MADS-box genes in response to AgNPs. To investigate expression patterns of the MADS-box genes in response to AgNPs, qRT-PCR was performed using cDNAs isolated from *A. thaliana* plants treated with 0.1 and 1 mg/l of AgNPs which revealed the lowest and the highest growth, respectively. The relative transcript levels of MADS-box genes are shown in Figure 2. Expression level changes more than 2-fold were considered to be upregulation, whereas expression level changes less than 0.5-fold were considered to be downregulation. The analysis of MADS-box gene expression revealed different expression patterns in response to AgNPs as evidenced by the $2^{-\Delta\Delta Ct}$ values. As shown in Figure 2, the $2^{-\Delta\Delta Ct}$ values were between 0.01 and 6.81-fold with 0.1 mg/l AgNPs, while with 1 mg/l AgNPs, the $2^{-\Delta\Delta Ct}$ values were between 0.01 and 8.7-fold. The *AGAMOUS-LIKE 72* (*AGL72*) gene showed the highest expression level at 0.1 mg/l, which was 6.81-fold, whereas the highest expression level at 1 mg/l (8.7-fold) was for the *MADS AFFECTING FLOWERING 2* (*MAF2*) gene. Among the investi-

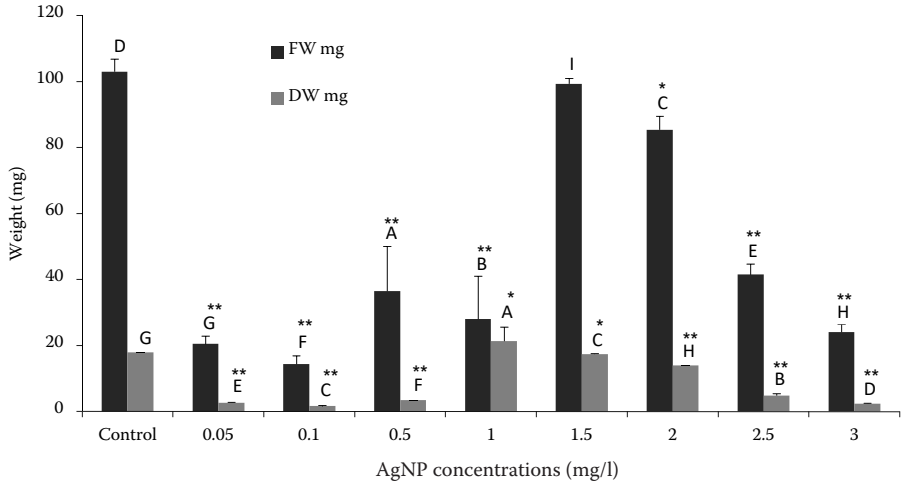

Figure 1. Influence of silver nanoparticles (AgNP) concentrations on seedling growth for *Arabidopisis thaliana* plants Vertical axis presents: mean values for seedling fresh weight (FW) and seedling dry weight (DW); different letters indicate a significant difference between treatments at the 0.01 and 0.05 levels by *t*-test; *indicates significant effects at a 0.05 probability level, and ** indicates significant effects at a 0.01 probability level

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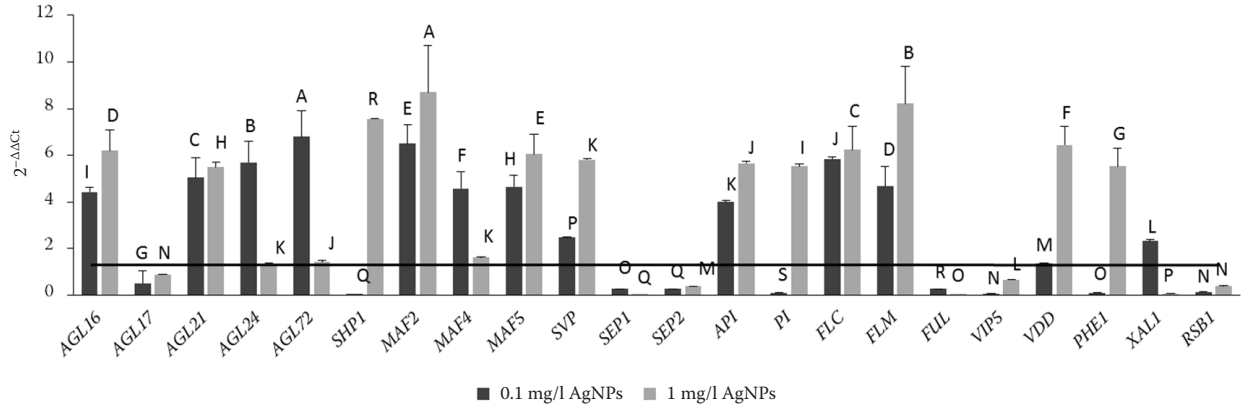

Figure 2. qRT-PCR analysis of 22 MADS-box genes in *Arabidopsis thaliana* plants treated with silver nanoparticles (AgNPs) Total RNA was extracted from whole plants after exposure to AgNPs for 15 days; the expression levels of MADS-box genes were calculated by the comparative Ct methods (ΔΔCt) and filtered for statistical significance (two-tailed Student's *t*-test *P*-value < 0.05); the *y*-axis shows fold changes ($2^{-\Delta\Delta Ct}$) in gene expression relative to the non-exposed plants, which present 1-fold $2^{-\Delta\Delta Ct}$ value and are illustrated by horizontal line; the $2^{-\Delta\Delta Ct}$ values from three independent experiments are presented; the *GAPDH* gene was used as an internal control; the graph shows MADS-box genes that are upregulated (fold change > 2) and downregulated (fold change < 0.50) at 0.1 and 1 mg/l of AgNPs; different letters indicate a significant difference between treatments at the 0.01 and 0.05 levels by *t*-test

gated MADS-box genes, eight genes, *AGL16*, *AGL21*, *MAF2*, *MAF5*, *SHORT VEGETATIVE PHASE* (*SVP*), *APETALA1* (*AP1*), *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS M* (*FLM*), were upregulated (> 2-fold increased) and four genes, *SEPALLATA1* (*SEP1*), *SEP2*, *FRUITFULL* (*FUL*) and *REDUCED SHOOT BRANCHING1* (*RSB1*), were downregulated (< 0.5-fold decreased) at both AgNP concentrations, 0.1 and 1 mg/l. Exposure to 1 mg/l AgNPs resulted in the upregulation of 12 MADS-box genes and downregulation of five genes, while exposure to 0.1 mg/l AgNPs resulted in the upregulation of 12 genes and downregulation of eight genes relative to the gene levels in non-exposed plants.

The MADS-box genes: *FLC*, *SVP*, *FLM, MAF2, MAF4* and *MAF5* genes repress the floral transition (Michaels & Amasino 1999; Scortecci *et al*. 2001; Ratcliffe *et al*. 2003; Li *et al*. 2008; Kim & Sung 2010). *AGL24*, *RSB1*, *AGL72*, *AGL17* and *XAL1* genes play a role in the regulation of flowering time as activators of flowering (Michaels *et al*. 2003; Hsu *et al*. 2003; Han *et al*. 2008; Tapia-López *et al*. 2008; Dorca-Fornell *et al*. 2011). *AGL16*, *AGL21*, *SHP1* and *XAL1* have specific functions during root morphogenesis with *AGL17* (Burgeff *et al*. 2002; Tapia-López *et al*. 2008; Moreno-Risueno *et al*. 2010). The MADS-box gene *FUL* is involved in meristem identity specification and fruit development (Ferrándiz *et al*. 2000). *SHP1* together with *SEP1 SEP2*, *VERDANDI* (*VDD*), *PHE1, AP1* and *PI* are necessary to ensure the proper development of petals, stamens and carpels and are involved in seed development (Pelaz *et al*. 2000; Köhler *et al*. 2005; Sundström *et al*. 2006; Matias-Hernandez *et al*. 2010).

The five MADS-box genes that are responsible for floral repression (*FLC*, *FLM*, *MAF2*, *MAF5* and *SVP*) revealed an overexpression in response to AgNPs. The floral activators, *AGL17* and *RSB1*, revealed expression level changes less than 0.87-fold upon exposure to AgNPs. However, the other floral activators, *AGL24*, *AGL72* and *XAL1*, were upregulated with 0.1 mg/l AgNPs. MADS-box genes that are responsible for the development of carpel, ovule and fruit differentiation: *SEP1*, *SEP2*, and *FUL* were downregulated under both AgNP exposure levels (0.1 and 1 mg/l), whereas *SHP1* was upregulated with 1 mg/l AgNPs. MADS-box genes that are responsible for root development: *AGL16*, *AGL17*, *AGL21*, *SHP1* and *XAL1* revealed different patterns of expression upon exposure to AgNPs. In contrast to *AGL16* and *AGL21,* which were upregulated upon exposure to both AgNP concentrations, *AGL17* revealed expression levels lower than in control plants. Moreover, the *SHP1* gene was upregulated only at 1 mg/l AgNPs, and *XAL1* was upregulated at 0.1 mg/l AgNPs. This variability in expression might be due to different roles of the *AGL17*, *XAL1* and *SHP1* genes when *AGL17* has an additive role in promoting flowering, whereas

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the *SHP1* gene is involved in the development of carpel, ovule and fruits, and *XAL1* is involved in the regulation of flowering. This alteration in gene expression for some MADS-box genes from up- to down-regulation under 0.1 and 1 mg/l of AgNPs might be due to the toxic effect of 0.1 mg/l AgNPs. Similarly, some studies reported that stress genes in *A. thaliana* experienced alterations in gene expression upon exposure to AgNPs (Kaveh *et al*. 2013; Qian *et al*. 2013; Syu *et al*. 2014; Kohan-Baghkheirati & Geisler-Lee 2015).

CONCLUSION

The role of nanomaterials in plant growth is unclear and complicated due to the lack of studies about the plant response to nanomaterials. In this study we aimed to understand the effect of AgNPs on plant growth in an early developmental stage at the molecular level. It is clear from the evidences that MADS-box gene expression was regulated by AgNPs, which were observed with either positive or negative effects on plant growth. Despite the limitations of the gene expression pattern approach, up- or downregulation of MADS-box genes has given insight into the molecular mechanisms responsible for the plant response to AgNP. This will improve our knowledge of the possible effect of utilization of AgNPs in increasing crop productivity.

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