

EFFECTS OF MAGNESIUM ION DOPED CARBON QUANTUM DOTS ON ROOT GROWTH AND RELATED GENES OF *ARABIDOPSIS THALIANA* SEEDLINGS

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Abstract

As time progresses of the times, different kinds of nanomaterials affect the life cycle of plants as they are released into the environment. The purpose of this paper was to take Mg-doped carbon quantum dots (Mg-CDs) as an example to research the *Arabidopsis thaliana*. The effects of Mg-CDs on the growth and development of *A. thaliana* was examined. The absorption and distribution of Mg-CDs in *A. thaliana* roots and leaves and the relative expression levels of related genes (*E2Fa* and *Histone H4*) in the cell cycle of *A. thaliana* seedlings were also determined. Results showed that Mg-CDs could accelerate the growth of *A. thaliana* roots. They were widely distributed in the cell wall and cytoplasm of the roots and chloroplasts of the leaves, and the relative expression levels of *E2Fa* increased first and then decreased with the increase in Mg-CDs concentration, as did that of *Histone H4*. Further analysis indicated that Mg-CDs affected the cell cycle of *A. thaliana* seedlings, thus resulting in root elongation. These findings showed that Mg-CDs have a great application potential in biotechnology research.

Key words: Mg-doped carbon quantum dots; *Arabidopsis thaliana*; Absorption; Cell cycle-related gene.

Introduction

The increasing production of nanomaterials in recent years inevitably disperses into the ecological environment, and their ecological effects have come into focus in the past few years (Batley *et al.*, 2012). The effects of different nanomaterials on the growth of many different species of plants have been extensively studied (Tripathi & Sarkar, 2015). The variety of nanomaterials and plants may lead to different results, such as enhancement and inhibition (Prasad *et al.*, 2012; Chen *et al.*, 2020). Research findings showed that multiwalled carbon nanotubes (MWCNTs) stimulated the proliferation of tobacco (*Nicotiana tabacum* L.) cells, and they could be absorbed by tomato (*Solanum lycopersicum*) plants to accelerate the growth of tomato flowers and fruits (Khodakovskaya *et al.*, 2012, Khodakovskaya *et al.*, 2013). Fullerene (C₇₀) at 0.4 mg/mL and MWCNTs delay flowering time and reduce seed setting rates in rice (*Oryza sativa* L.), respectively (Lin *et al.*, 2010). Water-soluble carbon nanotubes (wsCNTs) increase the growth rate of roots, shoots, and branches of gram (*Cicer arietinum*) and promote the absorption of water in plants (Tripathi *et al.*, 2011).

Among the many nanomaterials, carbon quantum dots (CDs) are usually formed by modification or functionalization of organic or biological molecules (Luo *et al.*, 2014). CDs are a new type of carbon-containing nanoparticles that are dissolved easily in water. Furthermore, they exhibit excellent fluorescence properties (Roy *et al.*, 2015), low toxicity, biocompatibility (Ding *et al.*, 2014), long fluorescence lifetime, and wide fluorescence spectral regions, with great potential for bioimaging (Liu *et al.*, 2012). They have broad application prospects in the field of biological science and biotechnology (Yang *et al.*, 2009, Du *et al.*, 2015), thereby making CDs increasingly important. For some practical applications, Element-doped CDs could improve the quantum yield and change the optical properties

of CDs, which could improve their unique optical and physical properties, such as N (Edison *et al.*, 2016), S (Wang *et al.*, 2015), P (Li *et al.*, 2017) and Mg (Han *et al.*, 2018). Studies have found that the toxicity of CDs to plants are related to concentration, and C-dots could be absorbed in plants and transported from roots to leaves (Chen *et al.*, 2016). SWCNTs could enter plant cells through cell walls and membranes, transport different substances into different plant organelles, and allow nanoparticles to enter the cell through the cell walls and aggregate at specific subcellular locations (Liu *et al.*, 2009, Kurepa *et al.*, 2010). Some scholars have studied the internalization mechanism of MWCNTs penetrating plant protoplasts and targeting specific cell substructures (Serag *et al.*, 2011).

When cells proliferate or differentiate, the cells in the G1/S phase integrate signals (G0/G1) and activate cell division, allowing the genome to replicate (G1/S phase transition and S phase) and eventually divide the cells equally into two. Thus, the transition in the G1/S phase is essential in the cell cycle. Various internal and external environmental factors could regulate cell cycle, and cell cycle finally affect plant growth and development. Abnormal expression of cell cycle-regulated genes could result in abnormal cell proliferation under the different environmental factors (Wang *et al.*, 2009). *E2Fa* is the key gene controlling cell division, and the *E2Fa-DPa* transcription factor of *A. thaliana* positively regulates plant cell division, which is expressed in the S phase and participates in the transition of the G1/S phase; the differentiated cells re-enter the S phase, when *E2Fa* and *DPa* are heterotopically expressed in cells (Veylder *et al.*, 2002). *E2Fa* and *H4* are involved in the transformation of *A. thaliana* cell cycle G1/S phase (Cui *et al.*, 2017). The expression of G1/S transition-related genes *E2Fa* and *Histone H4* in *A. thaliana* seedlings is significantly reduced, and growth is inhibited under Cd stress and UV-B radiation (Jiang *et al.*, 2011, Cui *et al.*, 2017).

Due to few species and limited means of studying plants, the mechanism of action of CDs in plant cells is not clear. The study of Mg metal doping in CDs (Mg-CDs) is even rarer. Existing studies were only limited to the physiological level of plants, and little is known about gene expression levels, immunity, and genotoxicity in plant cell cycles at the molecular level. Therefore, the present work explored the effect of Mg-CDs on root growth by influencing cell cycle-related genes in *Arabidopsis thaliana* seedlings, thereby providing new evidence for elucidating the molecular mechanism of Mg-CDs accelerating plant growth.

Materials and Methods

Chemical reagent: Citric acid, urea and magnesium acetate were used in this work. All chemicals used were analytically pure.

Synthesis and Characterization of Mg-CDs: Citric acid (0.192 g), urea (0.12 g) and magnesium acetate (0.214 g) were added into 15 mL ultrapure water and stirred evenly. The dissolved mixture was heated in an autoclave with liner for 15 h (180°C). Wait until the sample temperature dropped to room temperature, centrifuged for 25 min (10000 r/min) and retain the supernatant. The sample was cooled to room temperature and centrifuged for 25 min (10000 r/min) to retain the supernatant, which was then filtered with 0.22 mm filter element. The filtrate was frozen under -20°C for 12 h and then freeze-dried. Transmission electron microscopy (TEM) was done to observe the morphology and X-ray photoelectron spectroscopy (XPS) to characterize the structure. Fourier transform infrared spectroscopy (FT-IR) was performed from on a Nicolet380 infrared spectrometer (Varian, USA).

Plant cultivation: *A. thaliana* (Col-0) were used in this paper. The seeds were immersed in sterilized water for 1 h, sterilized with 1.5% (v/v) NaClO for 10 min and washed five times with sterilized water, transferred to 4°C for 3 days. Then, the seeds were cultured on 1/2 MS medium (with 1.5% sucrose) with different concentrations of Mg-CDs (0 mg/mL, 0.05 mg/mL, 0.1 mg/mL and 0.15 mg/mL) and placed into a smart light incubator at 22°C.

Seed germination rate and growth: The seeds of *A. thaliana* were cultured directly on 1/2 MS medium Mg-CDs under different concentrations after vernalization and NaClO disinfection. After the seeds were cultured for 1 day, the germination rate was calculated. Culture was continued for 10 days, and the seedling root length was measured.

Confocal microscopy and fluorescence imaging analysis: Five-day-old seedlings were immersed in a Mg-CDs solution with 0.1 mg/mL to determine the distribution of Mg-CDs in root cell after being absorbed by *A. thaliana* seedlings. While the seedling was immersed for 6 h and 12 h, FV-1000 Confocal system (Olympus, Co.) was used to observe the root tip. FV-1000 software was used to obtain images. The leaves of the seedlings grown on the medium with 0.1 mg/mL Mg-CDs were

observed using Olympus laser confocal microscopy to determine whether Mg-CDs were transported to leaves after being absorbed by roots. The observation of root and leaf cells was repeated at least three times. The Mg-CDs were excited at 405 nm.

Gene expression: The total RNA of 10-day-old *A. thaliana* seedlings was extracted using TRIzol to determine the expression level of cell cycle-regulated genes. Reverse transcription of RNA was performed using a reverse transcription kit (TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix, Transgen, China) to obtain cDNA. The relative expression levels of cell cycle-regulated genes (*E2Fa* and *Histone4 H4*) in *A. thaliana* seedlings with different concentrations of Mg-CDs were detected using an RT-qPCR instrument (ABI 7500Fast Real-Time PCR System, China; the RT-qPCR kit purchased from ABI was PowerUp SYBR Green Master Mix). The reference gene was *ACT2*. Table 1 shows the primer sequences.

Table 1. Primer sequences for RT-qPCR.

Gene name	Forward primer(5'-3')	Reverse primer(5'-3')
<i>E2Fa</i> (Cui <i>et al.</i> , 2017)	ACCATCCACCG TCATCTC	GCTCCTGTCGT TATTATTACTG
<i>Histone4</i> (Cui <i>et al.</i> , 2017)	GATTCGTCGTC TTGCTCGTAG	CAGTCACCGTC TTCCTCCTC
<i>ACT2</i> (Cui <i>et al.</i> , 2017)	TCGTGGATTCC AGCAGCTTCC	CCGATGGGCAA GTCATCACG

Statistical analysis

All experiments were repeated three times. GraphPad Prism 5 software was performed for data statistical analysis. ANOVA was used to analyze the experimental data.

Result and Discussion

Structural characterization of Mg-CDs: HRTEM was used to characterize Mg-CDs, and their morphology and structure were investigated. The hydrothermal synthesis of Mg-CDs showed excellent water solubility. The HRTEM image (Fig. 1a), the lattice spacing of Mg-CDs was 0.23 nm, corresponding to the (100) crystal surface of graphite (Zheng *et al.*, 2015). The particle size distribution was approximately 4.45 nm. The main Mg-CDs functional groups were characterized via FT-IR (Fig. 1b). Due to the vibration of O-H and N-H bonds, the wide absorption bands appeared at 3412 and 3220 cm⁻¹. The vibration of C-O bond corresponded to the peak detected at 1580 cm⁻¹. C-O-C bonds were observed at 1055 cm⁻¹. Fig. 1b showed that the Mg-CDs surface had abundant hydrophilic functional groups (hydroxyl and carboxyl groups). XPS was also used to further investigate the surface structure of the Mg-CDs. The spectrogram (Fig. 1c) shows four peaks of C, N, O and Mg, demonstrating the presence of C1s (285.08 eV), N1s (400.08 eV), O1s (532.08 eV) and Mg1s (1304.08 eV) on the Mg-CDs surface. This finding was consistent with the elemental analysis results (Fig. 2).

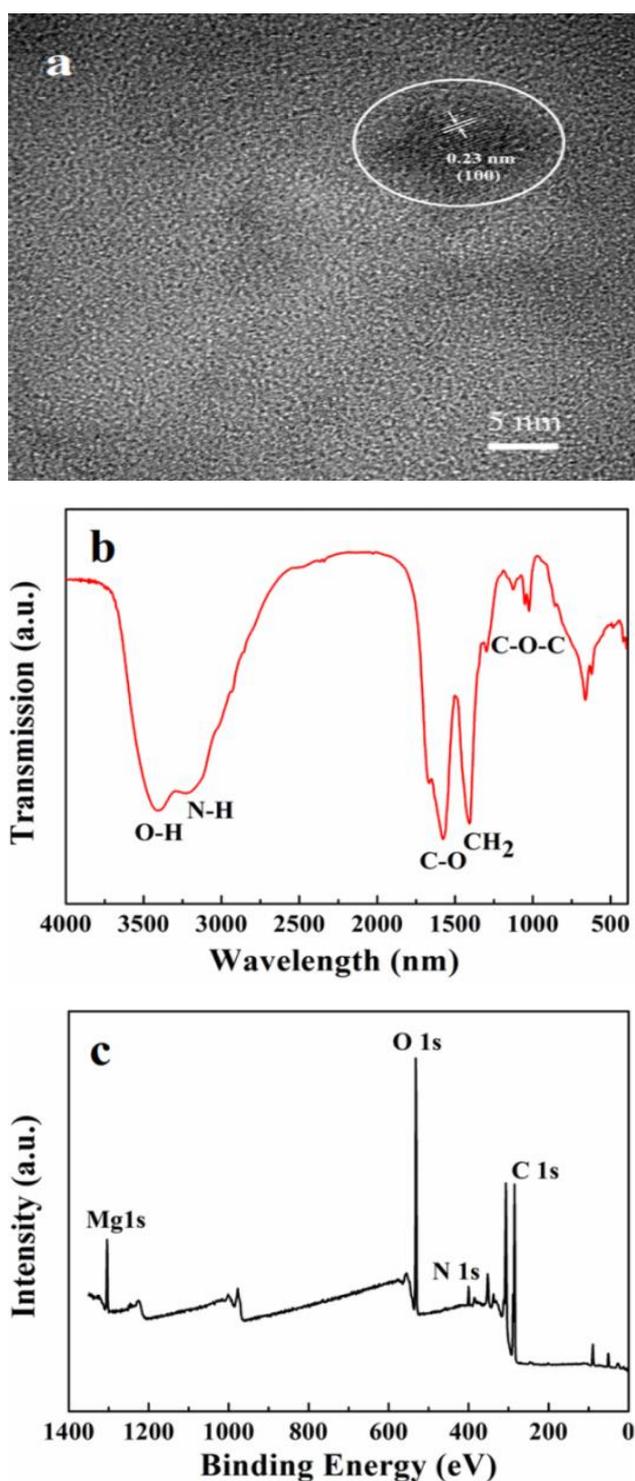


Fig. 1. (a) HRTEM image; (b) FT-IR spectra; (c) XPS spectrum.

Effects of Mg-CDs on plant growth: The model plant *A. thaliana* was selected for a series of experiments to investigate whether Mg-CDs affected plant growth and development. First, *A. thaliana* seeds were cultured on 1/2 MS medium with different concentrations of Mg-CDs (CK: 0 mg/mL). After seed germination was performed, the seed germination rate was counted. The 0.05 mg/mL treatment group showed significantly increased germination rate, but no significant change was found in the 0.1 and 0.15 mg/mL treatment groups (Fig. 3a). After 10 days of cultivation, *A. thaliana* seedlings were obtained and the root length of each treatment group was measured (Fig. 3b). Different

concentrations of Mg-CDs treatments showed different root lengths. Compared with the CK, the experimental groups exhibited significantly increased root length. The root length of the 0.05 and 0.1 mg/mL groups was approximately 35% longer than CK, and the root length of the 0.15 mg/mL group was 1.2 times longer than that of the CK. Thus, the elongation of *A. thaliana* roots was more significant in the 0.05 and 0.1 mg/mL groups, indicating that this promotion was related to Mg-CDs concentration. Previous reports showed that the growth of the roots of mung bean sprouts was improved after CDs treatment (Wang *et al.*, 2018), and water-soluble C-dots (wsCND) could also accelerate the growth of wheat roots (Tripathi & Sarkar, 2015). In the present study, the Mg-CDs facilitating *A. thaliana* root elongation was consistent with the reports of previous studies. Due to their physical and chemical properties, Mg-CDs was less toxic than other carbon nanomaterials. Thus, the seed germination is not inhibited by the increase in Mg-CDs concentration. Furthermore, Mg-CDs could promote *A. thaliana* root growth, possibly due to the changes in the metabolic rate of carbon nanoparticles entering the root cells, resulting in an increase in root auxin content (Lan *et al.*, 2018). In addition, because of the increase in root vigor, the water absorbance in the root system and plant growth increase (Wang *et al.*, 2018). In conclusion, Mg-CDs significantly facilitate *A. thaliana* root growth.

Uptake of Mg-CDs in *A. thaliana* root cells:

Fluorescence signal was detected in the treated group (Fig. 4d-l), but not in the un-treated group (Fig. 4a-c), indicating that Mg-CDs could be absorbed by cells through the cell wall of plants. Fluorescence was also observed at the root hairs, probably because Mg-CDs were absorbed by the root hairs as water molecules (Fig. 4d). When the Mg-CDs were treated for 6 h, they were mainly concentrated in the cell membrane, and weak fluorescence was detected in the cytoplasm and nucleus (Fig. 4g-i). When the Mg-CDs were treated for 12 h, they entered the cell aggregates in the cell wall and cytoplasm, and the fluorescence was strong. This finding indicated that the Mg-CDs were absorbed by the cells in large quantities but the fluorescence in the nucleus remained weak. When the Mg-CDs were treated for 12 h, they were enriched in the cell wall and cytoplasm, and the fluorescence was strong, indicating that the Mg-CDs were heavily absorbed by the cell, but the fluorescence in the nucleus remained weak (Fig. 4j-l). Early studies have shown that the results of *A. thaliana* cell uptake of CDs are consistent with animal cell imaging, that is, the CDs could be absorbed into the cell membranes and cytoplasm (Cao *et al.*, 2007, Chen *et al.*, 2013). Studies have also shown that CDs could enter the nucleus, but transport through the cell membrane is difficult. Even those who enter the nucleus need a long incubation time (Puvvada *et al.*, 2012). Plant cells are different from animal cells, a protective barrier exists outside the cell membrane of plant cells, that regulates the transport of substances through stomata (Kurepa *et al.*, 2010, Chen *et al.*, 2010). The cell-wall pore sizes of different plant species measured using different techniques are estimated to be approximately 10 nm (Kruk & Jaroniec, 1999, Berestovsky *et al.*, 2001). Given their small size (less than 5 nm), CDs could be absorbed through the cell walls of plants, and most of which are stained in the cell membranes and cytoplasm; in addition, a weak fluorescence is present in the nucleus (Dong *et al.*, 2012, Zhang *et al.*, 2013). Therefore, in the present study, the

distribution of Mg-CDs absorption by *A. thaliana* root was observed through laser confocal microscopy (FV-1000 Confocal system). The diameter of Mg-CDs was less than 5 nm. Thus, the Mg-CDs may be absorbed by the root system through the cell-wall pore size.

Transport of Mg-CDs in *A.thaliana* Roots: Laser confocal microscopy was used to confirm whether Mg-CDs were transported to leaf cells through the roots. The leaves of 5-day-old seedlings were cultured in a petri dish with 0.1 mg/mL concentration of Mg-CDs solution, as shown in Fig. 5. Fluorescence was not detected in (Fig. 5a-c), which is the control group. Blue fluorescence signal was observed in the cytoplasm of leaves in the treated group (Fig. 5d-f). Fluorescent signals could also be

observed in the chloroplasts (red arrow in Fig. 5d). Previous studies have shown that the SWCNTs entering leaf tissue through vascular infiltration are transported to intact chloroplasts and captured and localized into chloroplasts, and SWCNTs significantly increase photosynthetic conversion to allow biochemical sensing within plants (Wong *et al.*, 2016). CDs could penetrate the maize root epidermal cells into the cortex and vascular bundles of the roots and then transport the leaf cells through the vascular bundles; fluorescence signals are also observed in the leaves (Chen *et al.*, 2016). Nanoparticles could be absorbed by plant roots and transported to plant leaves through the transport of symbionts and exosomes (Kurepa *et al.*, 2010), which may also be a method for plant transport of Mg-CDs.

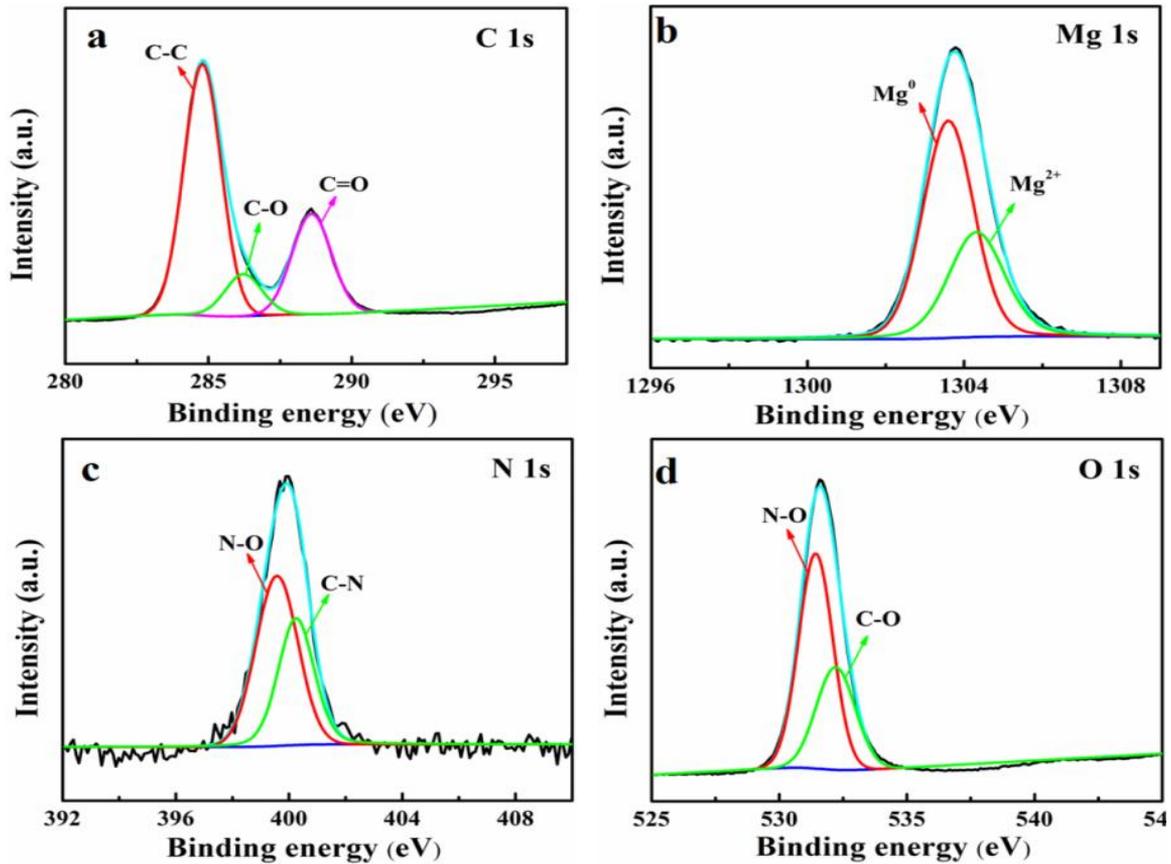


Fig. 2. The XPS spectrum of the Mg-CDs; (a) C 1s; (b) Mg 1s; (c) N 1s; (d) O 1s.

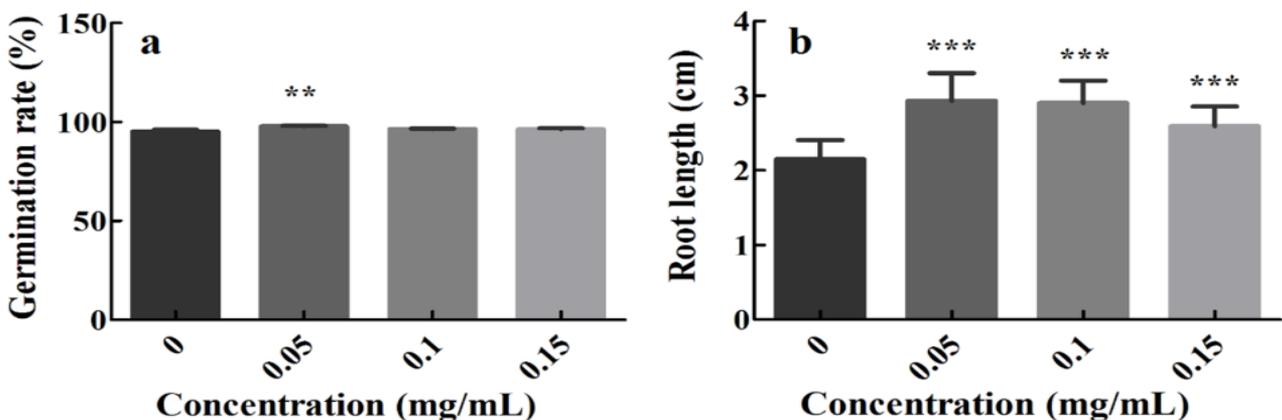


Fig. 3. Growth of *A. thaliana* seedlings for 10d in different groups. (a) Germination rate; (b) Root length. * indicates significant difference from CK. * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$).

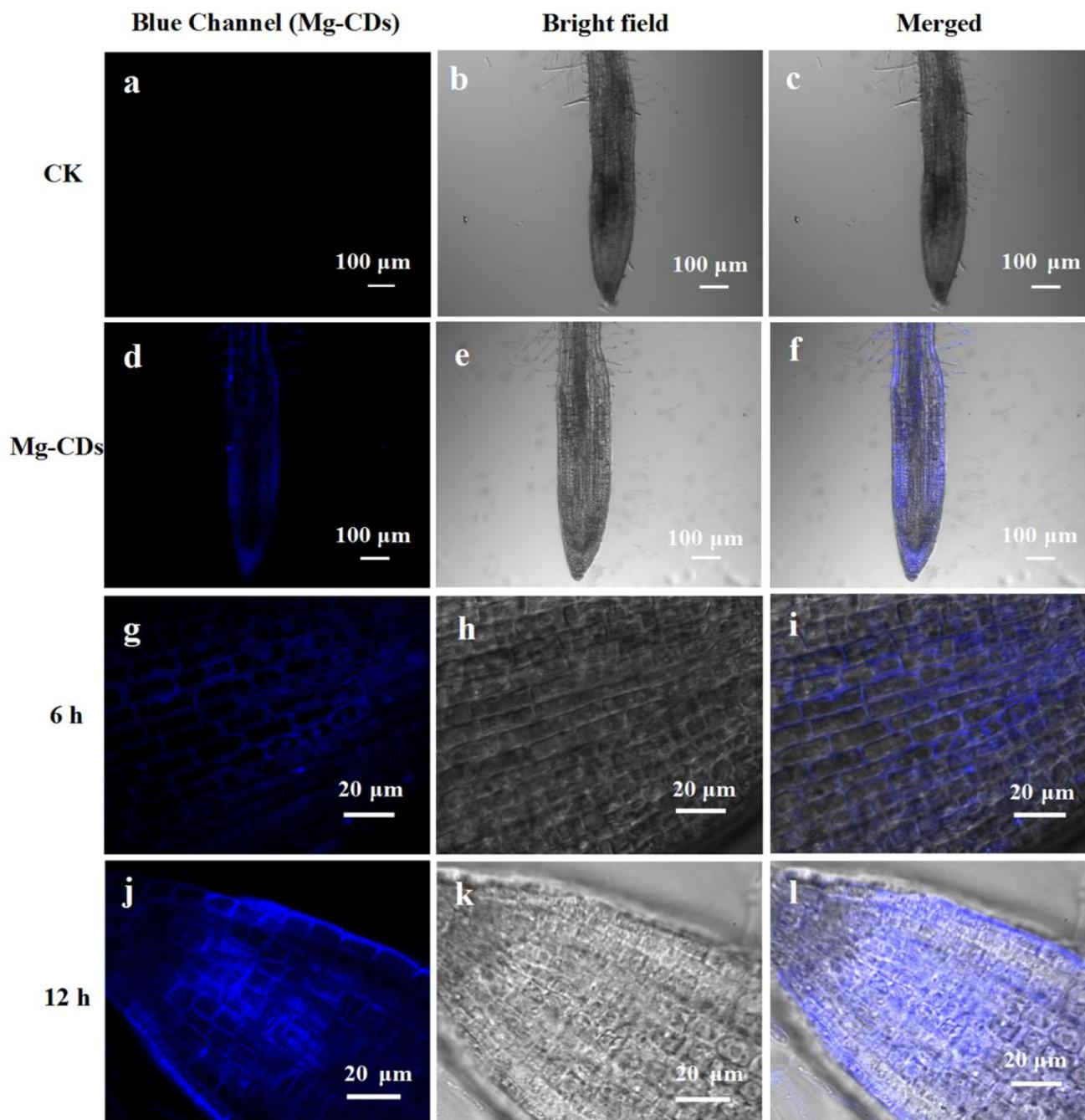


Fig. 4. Confocal imaging of Mg-CDs distribution in *A.thaliana* seedling. (a-l) Distribution of Mg-CDs in the roots of *A. thaliana* seedlings; (a, d, g, j) Blue Channel; (b, e, h, k) Bright field; (c, f, i, l) Merged.

Expression of cell cycle-regulation gene: The expression levels of cell cycle-related genes (*E2Fa* and *Histone H4*) in 10-day-old *A. thaliana* seedlings were examined using RT-qPCR to verify the effect of Mg-CDs on the molecular level of plants. *E2Fa* and *Histone H4* were the marker genes in G1/S transition, expressed mainly during the transition (Himanen *et al.*, 2002). It was observed that after Mg-CDs treatment with different concentration, the relative expression levels of *E2Fa* and *Histone H4* increased to varying degrees (Fig. 6). These expression levels increased first and then decreased with the change in concentration. The expression of *E2Fa* in the 0.05 and 0.15 mg/mL groups increased significantly, while the 0.1 mg/mL group reached extreme remarkable levels (Fig. 6a). CDs could act on the G1/S phase of eukaryotic cells to stimulate cell proliferation and influence cell cycle (Havrdova *et al.*, 2016). The cell

cycle normally goes through an interphase (G1, S, and G2) (Verbon *et al.*, 2012), followed by mitosis and cytokinesis, and enters the S phase when G1 cells have grown large enough (twice their own mass) (Collins *et al.*, 1997). A previous research has indicated that *E2Fa* is essential for cell proliferation and promotes cell growth by stimulating *A. thaliana* internal circulation (Sozzani *et al.*, 2006, Magyar *et al.*, 2012). *E2Fa* overexpression induces ectopic cell division in transgenic *A. thaliana* plants, while *E2Fa* overexpression binds AtDPa and induces intracellular replication or cell proliferation (Veylder *et al.*, 2002). Previous reports have demonstrated that silencing of *E2Fa* in *A. thaliana* leads to shortened roots (Magyar *et al.*, 2012). The above results indicated that Mg-CDs treatment could regulate the expression levels of *E2Fa* and *H4* in *A. thaliana* seedling cell cycle and might promote the hypothesis of cell proliferation.

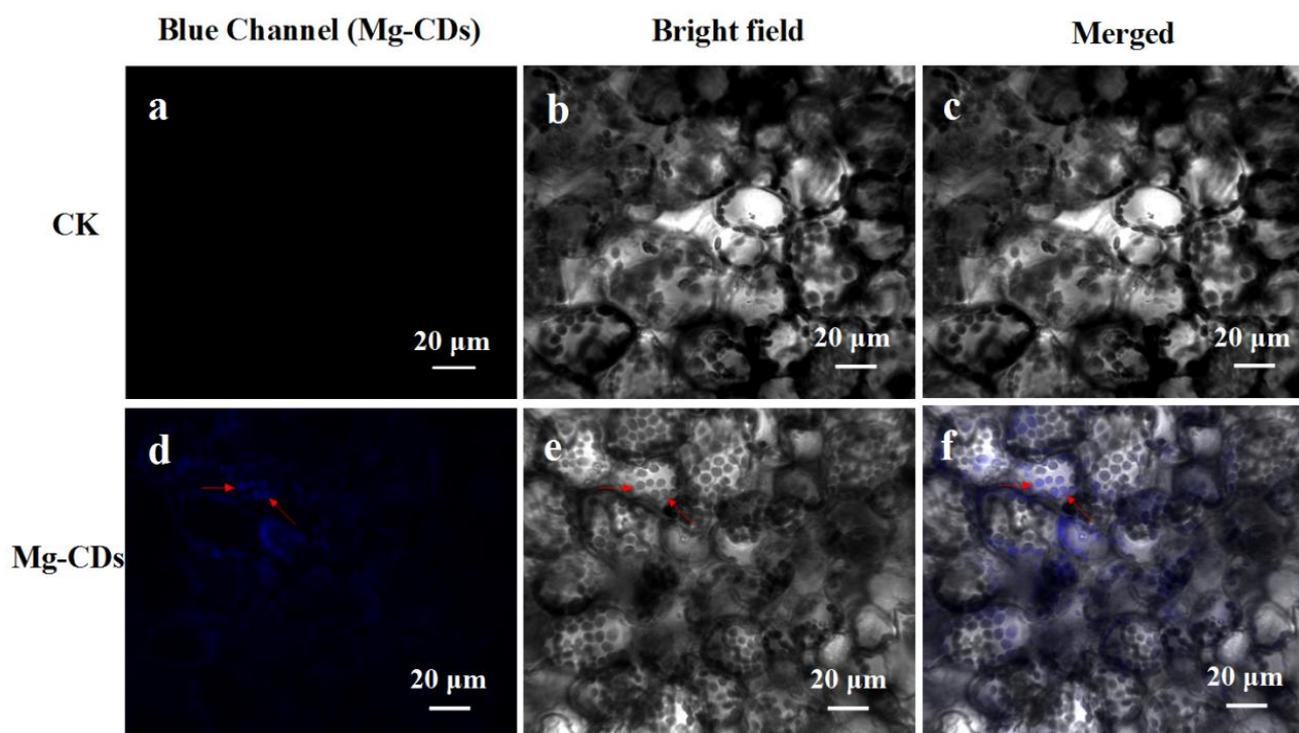


Fig. 5. Confocal imaging of Mg-CDs distribution in *A.thaliana* leaves. (a, d) Blue Channel; (b, e) Bright field; (c, f) Merged. The red arrows indicate chloroplasts.

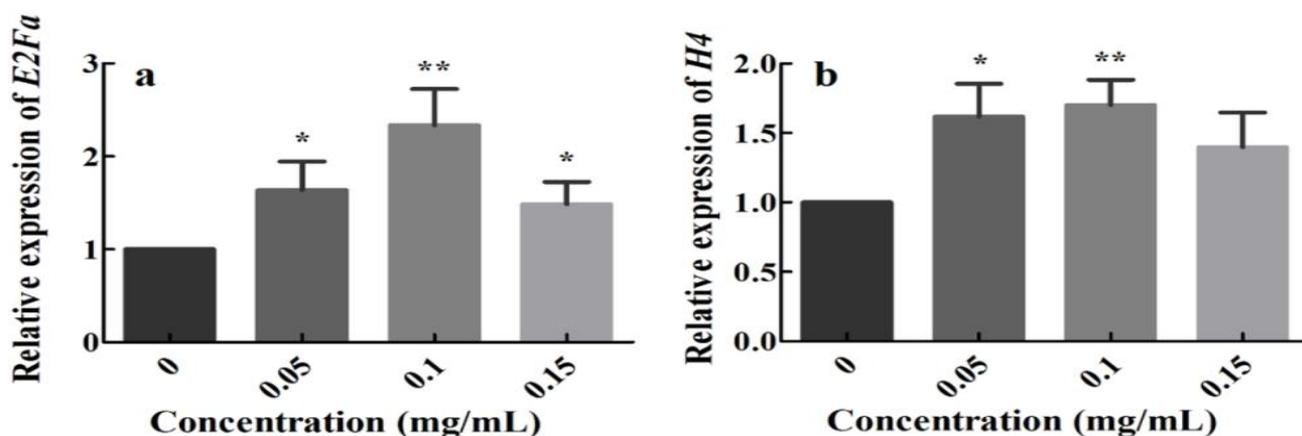


Fig. 6. Effects of Mg-CDs on gene expression in *A.thaliana* seedling for 10 d. (a) *E2Fa*; (b) *H4*. *AtACT2* was used as reference gene. * indicates remarkable difference from CK.

Conclusion

The effects of Mg-CDs on *A. thaliana* cell growth were investigated, including growth and cell cycle-related genes. First, Mg-CDs were characterized, and their absorption, distribution, and transmission in *A. thaliana* were observed using laser confocal microscopy. The changes in related genes in the cell cycle were also studied. The results showed that Mg-CDs had a dose effect on the root growth of *A. thaliana*, and they could facilitate root growth. They could also penetrate the cell wall of *A. thaliana* cells and transfer from roots to leaves. The expression levels of *E2Fa* and *H4* were upregulated after Mg-CDs treatment, thus accelerating root growth. These results indicated that the specific role of Mg-CDs in biotechnology was essential.

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