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Full Length Research Paper

Analysis of the effects of cerium on calcium ion in the protoplasts of *Arabidopsis thaliana* with confocal microscopy

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The laser-scanning confocal microscopy has become a routine technique and indispensable tool for cell biological studies. In this study, the probe Fluo-3 AM was used to research the instantaneous changes of calcium ion (Ca^{2+}) in the protoplasts of *Arabidopsis thaliana*. The laser-scanning mode of confocal microscope is XYT, and the time interval between two images is 10.635 s. 30 optical sections were acquired with the laser-scanning confocal microscope, and the different concentrations of trivalent cerium (Ce^{3+}) (0.1, 0.5 and 1.0 mmol/L) were added after four optical sections were scanned, respectively. Furthermore, the fluorescence intensity of Ca^{2+} in the 30 optical sections was quantified with Leica Confocal Software. The quantitative data was exported and the trend chart was made with Excel software. The results indicate that Ca^{2+} concentration in the protoplasts of *A. thaliana* declined after 0.5 and 1.0 mmol/L Ce^{3+} was added. However, Ca^{2+} concentration slightly increased in the protoplasts treated with 0.1 mmol/L Ce^{3+}. The data indicate that the appropriate amount of Ce^{3+} can inhibit intracellular Ca^{2+} concentration in the protoplasts of *A. thaliana*. Since Ca^{2+} participates in signal transduction and plays a crucial role in regulating plant growth and development under environmental stresses, Ce^{3+} may regulate plant metabolism, growth and development via the changes of Ca^{2+} concentration.

Key words: Cerium, calcium ion, protoplast, confocal microscope.

INTRODUCTION

The rare earth elements (REEs) enriched fertilizers have been used in China since the 1980s (d'Aquino et al., 2009), and the effects of REEs on physiological responses have been reported in different plant species (Fashui et al., 2000; Chen et al., 2001; Hu et al., 2002, 2004). REEs-based fertilizers, mainly in their nitrate form, were obtained by extracting REEs from the ores using nitric acid (Hu et al., 2004; Xu et al., 2002). It has been found that low concentrations of REEs promote growth and productivity of some crops (He and Xue, 2005; Wu et al., 1983; Chang, 1991). REEs can be absorbed into plant cells, which are the basis for interpreting physiological and biochemical effects of REEs on plant cells (Gao et al., 2003). Trivalent europium (Eu^{3+}) could directly enter or be carried by the artificial ion carrier into plant cells via the channel of calcium ion (Ca^{2+}), and Eu^{3+} treatment could activate Ca^{2+}-ATPase on the plasma membrane (Zeng et al., 2003; Gao et al., 2003). The results of energy-dispersive X-ray microanalysis indicate that Eu^{3+} and trivalent lanthanum (La^{3+}) can be absorbed into plant cells and bound to the membranes of protoplasm, chloroplast, mitochondrion, cytoplast, and karyon (Gao et al., 2003).

Ca^{2+} is not only an essential microelement required for growth and development of vascular plants, but also a second messenger in responses to most external stimuli in plants (Bush, 1995; Webb et al., 1996a; 1996b; McAinsh et al., 1990; 1996; Irving et al., 1992). Numerous studies have indicated that Ca^{2+} participates

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in signal transduction and plays a crucial role in regulating plant growth and development under environmental stresses (Hepler and Wayme, 1985; Poovaiah and Reddy, 1993; Bush, 1995). Transient increase or decline of intracellular Ca^{2+} and Ca^{2+} oscillations have been observed in plant cells under various physiological conditions (McAinsh et al., 1997).

Some studies have indicated that REEs affect the plant physiological mechanism by regulating the Ca2+ level in plant cells. Most REEs show inhibitory effect against Ca^{2+} influx in the tobacco cells (Lin et al., 2006). The results of kinetic analysis indicate that La^{3+} can function as a mixed-type competitor for Ca^{2+}, and the effects of La^{3+} on ion absorption is similar to Ca^{2+} (Ono, 2000; Zeng et al., 2000). Since ionic radii is smaller than Ca^{2+}, La^{3+} shows higher affinity for the Ca^{2+} site than those ions with larger radii. La^{3+} can substitute Ca^{2+} at Ca^{2+}-site on the properties of the oxygen evolving center of photosystem II (Ono, 2000). In addition, Eu^{3+} might replace Ca^{2+} in the Ca^{2+}/calmidulin dependent phyto-chrome signal transduction system and plays important roles in plant development by promoting Ca^{2+} trans-portation across plasma membrane (Zeng et al., 2003). Recently, confocal microscopy has become a routine technique and indispensable tool for cell biological studies. The aims of this study were to investigate the effects of trivalent cerium (Ce^{3+}) on the instantaneous changes of Ca^{2+} in the protoplasts of *Arabidopsis thaliana* with the laser-scanning confocal microscopy.

MATERIALS AND METHODS

Seeds of *A. thaliana* were surface sterilized by soaking in 75% alcohol for 60 s and followed by 2.0% sodium hypochlorite for 10 min. The seeds were then rinsed five times in sterilized water prior to culture. The Murashige and Skoog medium (0.6% agar, 3.0% sucrose) was used for seed germination and as basal medium and the pH of the medium adjusted to 5.8 before agar was added. Plants were grown at 23.0 ± 2℃ using a 14/10 h light/dark cycle in a growth chamber under a light intensity of 6,000 lx. Plants were cultured in the Murashige and Skoog medium for 15 days and transferred to nutritional soil (57.0% nutrition soil, 29.0% vermiculite, and 14.0% perlite).

Protoplast isolation

The leaves of *A. thaliana* were collected at the 30th day, surface-sterilized with 1.0% hypochlorite and 0.1% Tween 20 for 10 min, and rinsed three times with sterile water. The leaves of *A. thaliana* were cut into small pieces (2 to 3 mm^2) and incubated in a solution with 2.0% cellulase, 1.0% pectinase, 50 mmol/L 2-(N-Morpholino) ethanesulfonic acid (MES, pH 5.8), 0.2 mmol/L $CaCl_2$, and 0.6 mol/L mannitol at 28℃ in the dark for 12 h. The protoplasts were filtered through a 48 µm pore size sieve and precipitated by swinging bucket centrifugation at 600 r/min for 5 min. Protoplasts were washed three times in washing solution containing 0.2 mmol/L $CaCl_2$ and 50 mmol/L sorbitol. The number of protoplasts was estimated under light microscope with neubauer chamber and the volume was adjusted to reach 1.5×10^6 viable protoplasts per millilitre.

Ca^{2+} visualization

Fluo-3 AM can assay free Ca^{2+} in cells (Kao et al., 1989; David and Stephen, 1993). To analyze Ca^{2+} level, protoplasts were loaded with 10 µmol/L Fluo-3 AM (Molecular Probes, Inc; diluted in 0.2 mmol/L $CaCl_2$ and 50 mmol/L sorbitol) at 20℃ for 30 min. Excess dye was eliminated by washing the protoplasts three times with 0.2 mmol/L $CaCl_2$ and 50 mmol/L sorbitol.

Laser scanning confocal microscopy

For microscopy, a 35 mm Petri dish was used, which has a sketched hole at bottom. The sketched hole was glued with a cover glass. The cover glass was coated with 0.1% poly-D-lysine, which enhance cell binding to polystyrene surfaces for certain cell-based assays and procedures. Then the protoplasts were cultured in the Petri dish for 10 min. The Petri dish was put under an inverted microscope (Leica TCS NT, Germany). A laser-scanning confocal microscope (Leica TCS SP2, Germany) with an air-cooled, argon-ion laser as the excitation source at 488 nm was used to view the sites of Ca^{2+} in the protoplasts of *A. thaliana*. Images were obtained with a HCX PLAN Apo 40.0×0.7 PH2 objective lens. Ca^{2+} in the protoplasts was detected in the red channel. The channel settings of pinhole, detector gain, amplification offset, and zoom were adjusted to provide an optimal balance of fluorescent intensity of protoplasts.

The laser-scanning mode is XYT, and the time interval between two images is 10.635 s. 30 optical sections were acquired and the different concentrations of $Ce(NO_3)_3$ was added into 35 mm Petri dish after four optical sections were scanned. The final concentration of Ce^{3+} was 0.1, 0.5 and 1.0 mmol/L, respectively. Three representative protoplasts were chosen and the fluorescence intensity of Ca^{2+} in the 30 optical sections was quantified with Leica Confocal Software. Data were collected by a computer attached to the instrument, stored on the hard drive, processed with a Leica TCS Image Browser, and transferred to Adobe Photoshop 6.0 for preparation of figures. The quantitative data was exported and the trend chart was made with Excel software.

RESULTS AND DISCUSSION

As a second messenger, Ca^{2+} plays important roles in regulating plant metabolism, growth, and development (Bush, 1995; Zhu, 2002; Sanders, 1999). Transient changes of intracellular Ca^{2+} have been observed in plant cells under various physiological conditions. Numerous studies have indicated that intracellular Ca^{2+} concentrations can be induced under various environmental stresses. La^{3+}, which is known as a Ca^{2+} channel blocker, inhibits a great range of Ca^{2+} channels by competing with Ca^{2+}. It has been found that La^{3+} indirectly affects intracellular Ca^{2+} homeostasis by inhibiting K^+ outward rectifiers (Messerli and Robinson, 1997; Ding and Pickard, 1993; Herrmann and Felle, 1995; Zhang et al., 1999).

In this study, it was found that Ca^{2+} concentration was slightly induced by 0.1 mmol/L Ce^{3+} in the protoplasts of *A. thaliana* (Figure 1). However, Ca^{2+} concentration was decreased in the protoplasts treated with 0.5 and 1.0 mmol/L Ce^{3+} (Figures 2 and 3). Moreover, the intracellular distribution of Ca^{2+} in the protoplast of *A. thaliana* was imaged with confocal microscopy. There was more

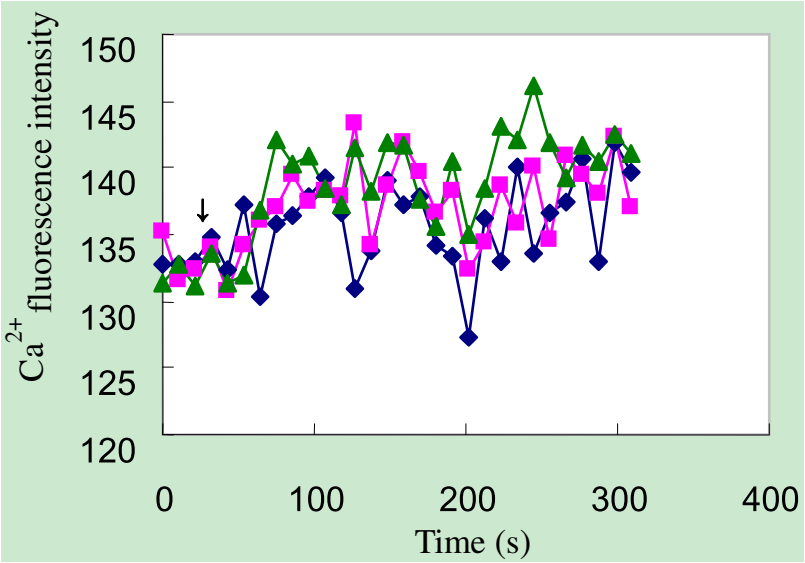

Figure 1. Effects of 0.1 mmol/L Ce^{3+} on Ca^{2+} in the protoplasts of *A. thaliana*. The changes of Ca^{2+} fluorescence intensity in three protoplasts of *A. thaliana* were quantified with Leica confocal software. 0.1 mmol/L Ce^{3+} was added at 31.906 s (The arrow indicates the time that Ce^{3+} was added).

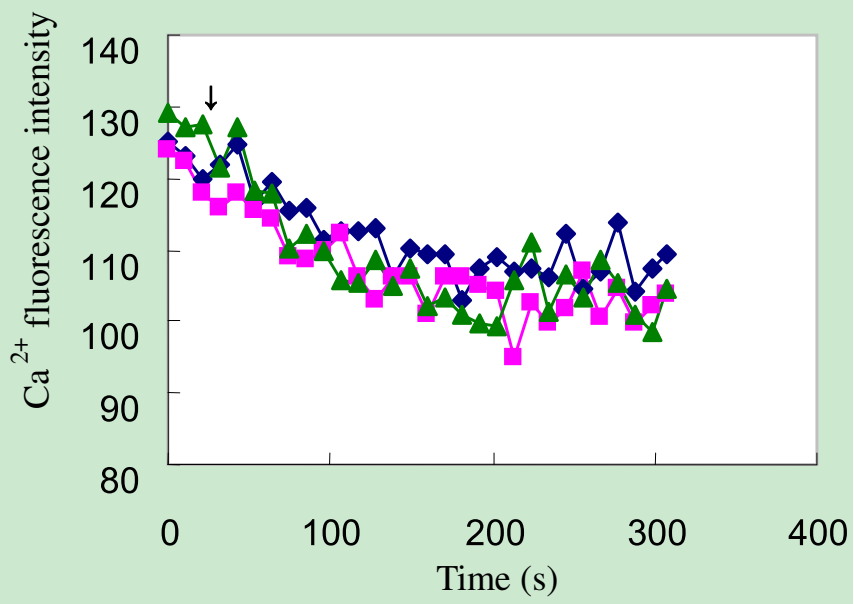

Figure 2. Effects of 0.5 mmol/L Ce^{3+} on Ca^{2+} in the protoplasts of *A. thaliana*. The changes of Ca^{2+} fluorescence intensity in three protoplasts of *A. thaliana* were quantified with Leica confocal software. 0.5 mmol/L Ce^{3+} was added at 31.906 s (The arrow indicates the time that Ce^{3+} was added).

distribution of Ca^{2+} in the control group than the group treated with 1.0 mmol/L Ce^{3+} (Figures 4 and 5). Although, the intracellular Ca^{2+} concentration was inhibited in the protoplasts treated with higher concentration of Ce^{3+}, the low concentration of Ce^{3+} could slightly induce Ca^{2+} concentration in the protoplasts of *A. thaliana*. The results indicate that the appropriate amount of Ce^{3+} could inhibit intracellular Ca^{2+} concentration in the protoplasts of *A. thaliana*, which was consentaneous with the data of Lin et al. (2006). Similar with the data of McAinsh et al. (1997), Ca^{2+} oscillations were also found in the present study (Figures 1, 2 and 3). Ce^{3+} may regulate plant metabolism,

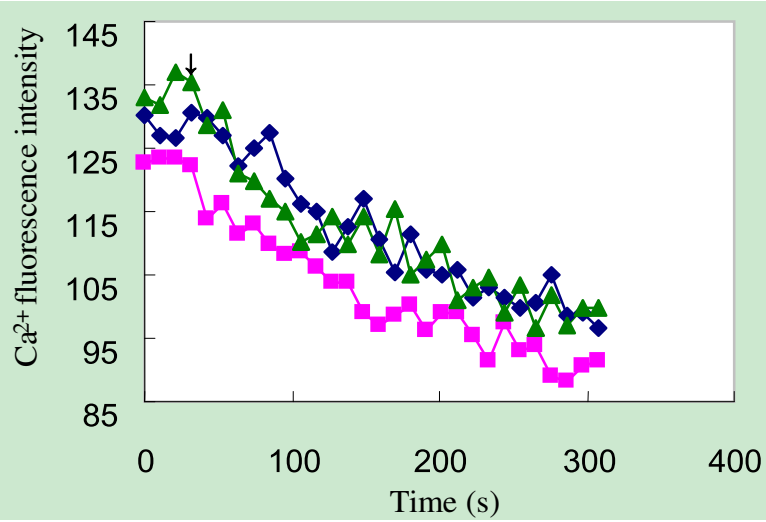

Figure 3. Effects of 1.0 mmol/L Ce^{3+} on Ca^{2+} in the protoplasts of *A. thaliana*. The changes of Ca^{2+} fluorescence intensity in three protoplasts of *A. thaliana* were quantified with Leica confocal software. 1.0 mmol/L Ce^{3+} was added at 31.906 seconds (The arrow indicates the time that Ce^{3+} was added).

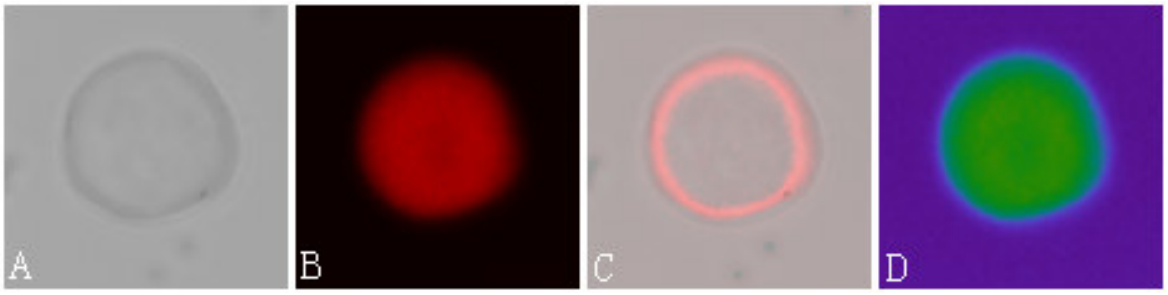

Figure 4. Intracellular localization of Ca^{2+} in the protoplast of *A. thaliana* (the control). (A) Phase contrast image of protoplast; (B) red channel, showing Ca^{2+} fluorescence in the protoplast; (C) A and B combined; (D) the distribution of Ca^{2+} in the protoplast.

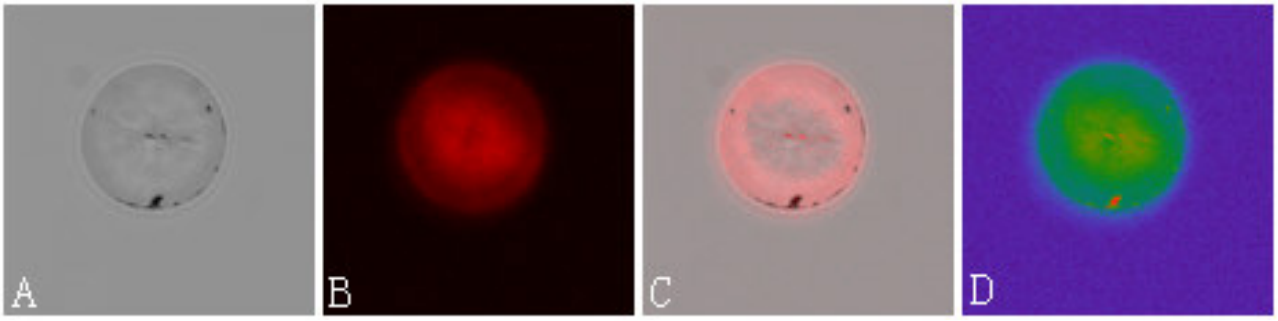

Figure 5. Intracellular localization of Ca^{2+} in the protoplast of *A. thaliana* (treated with 1.0 mmol/L Ce^{3+}). (A) Phase contrast image of protoplast; (B) red channel, showing Ca^{2+} fluorescence in the protoplast; (C) A and B combined; (D) the distribution of Ca^{2+} in the protoplast.

growth and development via the changes of Ca^{2+} concentration. It appears that the different concentration of Ce^{3+} has a different physiological mechanism on regulating Ca^{2+} concentration in the protoplasts, which

needs to be further researched in future.

Conclusion

In summary, the results indicate that Ca^{2+} concentration in the protoplasts of *A. thaliana* was declined after 0.5 and 1.0 mmol/L Ce^{3+} was added. However, Ca^{2+} concentration was slightly increased in the protoplasts treated with 0.1 mmol/L Ce^{3+}. The data indicate that the appropriate amount of Ce^{3+} can inhibit intracellular Ca^{2+} concentration in the protoplasts of *A. thaliana*. Since Ca^{2+} plays a key role in signal transduction and regulating plant growth and development under environmental stresses, Ce^{3+} may regulate plant metabolism, growth and development via the changes of Ca^{2+} concentration.

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