Chemosphere 154 (2016) 408–415

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Responses of plant calmodulin to endocytosis induced by rare earth elements

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HIGHLIGHTS

- La(III) affected the expression of calmodulin in endocytosis of plants.
- Low-level La(III) interacted with extracellular calmodulin by electrostatic attraction.
- High-level La(III) was coordinated with cytoplasmic calmodulin.
- Low-level or high-level La(III) improved or destroyed calmodulin structure.

ARTICLE INFO

Article history:
Received 26 January 2016
Received in revised form
13 March 2016
Accepted 23 March 2016
Available online 12 April 2016

Handling Editor: Caroline Gaus

Keywords:
Calmodulin
Endocytosis
Lanthanum
Molecular basis

ABSTRACT

The wide application of rare earth elements (REEs) have led to their diffusion and accumulation in the environment. The activation of endocytosis is the primary response of plant cells to REEs. Calmodulin (CaM), as an important substance in calcium (Ca) signaling systems, regulating almost all of the physiological activities in plants, such as cellular metabolism, cell growth and division. However, the response of CaM to endocytosis activated by REEs remains unknown. By using immunofluorescence labeling and a confocal laser scanning microscope, we found that trivalent lanthanum [La(III)], an REE ion, affected the expression of CaM in endocytosis. Using circular dichroism, X-ray photoelectron spectroscopy and computer simulations, we demonstrated that a low concentration of La(III) could interact with extracellular CaM by electrostatic attraction and was then bound to two Ca-binding sites of CaM, making the molecular structure more compact and orderly, whereas a high concentration of La(III) could be coordinated with cytoplasmic CaM or bound to other Ca-binding sites, making the molecular structure more loose and disorderly. Our results provide a reference for revealing the action mechanisms of REEs in plant cells.

1. Introduction

In comparison with known heavy metal elements (such as lead, cadmium, chromium), rare earth elements (REEs) in the environment and living organisms have not yet attracted a great deal of attention (Migaszewski and Gałuszka, 2015). The large-scale and under-supervised mining, production and application of REEs have led to their diffusion and accumulation in the environment and living organisms (Gonzalez et al., 2014; Klaver et al., 2014; Li et al., 2014; Liang et al., 2014; Minganti et al., 2014; Wang and Liang, 2014; Wang et al., 2014b; Hao et al., 2015; Mayfield and Fairbrother, 2015; Migaszewski and Gałuszka, 2015; Strady et al., 2015). In China, Japan, Australia and Germany, the average level of REEs in the soils is 181.0 μg g^{-1}, 97.6 mg kg^{-1}, 104.3 mg kg^{-1} and 15.5 mg kg^{-1}, respectively (Hu et al., 2006; Wang and Liang, 2015). In the Guadiana River (Europe) and Sidaosha River (China), the REE level in sediment reaches 224.5 mg L^{-1} and 30.5 μg L^{-1} (Delgado et al., 2012; Liang et al., 2014). In Tokyo, the level of REEs in total

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http://dx.doi.org/10.1016/j.chemosphere.2016.03.106
0045-6535/

suspended particulates of atmosphere has been found to be as high as 88.6 μg m^{-3}, and in China, the level of REEs in inhalable particles of atmosphere is up to 612.5 ng m^{-3} (Suzuki et al., 2011; Liang et al., 2014). Thus, REEs have become important elements affecting living organisms (Censi et al., 2011; Gonzalez et al., 2014; Li et al., 2014; Yang et al., 2014). Studies on the action mechanism of REEs in plants date back to 1917, when the beneficial effects of cerium on plant growth were firstly documented (Chien and Ostenhout, 1917). Despite almost a century of research, some basic and frontline questions, such as the mode, site and mechanism of REEs acting on plant cells, which are the structural and functional units of living organisms, are still unknown because of the cell's complex structure (Wang et al., 2008; Guo et al., 2013; Liu et al., 2013; Turra et al., 2013; Dressler et al., 2014; Fu et al., 2014; Gonzalez et al., 2014; Thomas et al., 2014; Wang et al., 2014a, 2014c; Zhang et al., 2014; Yang et al., 2015).

A recent study in our group found that low doses of environmental REEs cannot enter plant cells but are anchored on the plasma membrane in the form of nanoscale particles, and that these behaviors activate endocytosis (Wang et al., 2014a). The nanoscale particles on the plasma membrane increased as the dose of environmental REEs increased, and these behaviors strongly activated endocytosis (Wang et al., 2014a). Thus, REEs can break down the endocytic inertness in plant leaves as the result of long-term evolution and subsequently activate endocytosis. This activation is the primary response of plant cells to the action of REEs, which induces a series of physiological and biochemical responses (e.g. a change in Ca^{2+} level, which is an intracellular second messenger), and then influences cell growth (Wang et al., 2014a). Therefore, the activation of endocytosis induced by REEs may be considered as the cellular basis of REE activities in plants. However, how the endocytosis activation signal transmits downstream and then controls the growth of plant cells remains unclear.

Cytosolic Ca^{2+}, the second messenger, can regulate all intracellular activities in plants (Batistič and Kudla, 2012), and this regulation must rely on calmodulin (CaM) because CaM is a Ca^{2+} sensor (Cheval et al., 2013). Many studies showed that CaM is located in extracellular and intracellular spaces (Ma et al., 1999; Cheval et al., 2013). There is one helix-loop-helix EF-hand motif and four Ca binding sites in a CaM molecule (Fig. 1), which can capture trace Ca^{2+} (Cheval et al., 2013). The binding of Ca^{2+} changes the conformation of CaM molecule, making it become an activator for some enzymes, such as adenylate cyclase, phosphodiesterase, phosphorylase kinase, glycogen synthase kinase, protein kinase and protein phosphatase, which are then involved in information transmission, glycogen synthesis and decomposition, protein phosphorylation and dephosphorylation, gene expression, and cell growth and division (Batistič and Kudla, 2012). The questions that arise are: what impact does the activation of endocytosis induced by REEs have on plant CaM levels, and how do REEs affect plant CaM levels? Answering these scientific questions is crucial to further reveal the mechanisms of REE actions in plants.

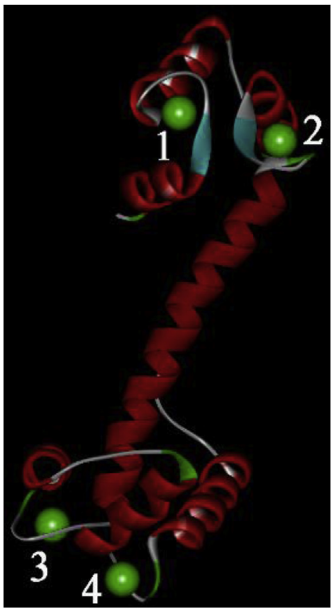

Fig. 1. The crystal structure of CaM. Green atoms are Ca-binding sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In this study, endocytosis in the model plant *Arabidopsis* after a treatment with or without trivalent lanthanum [La(III)] was observed by confocal laser scanning microscopy. Meanwhile, the effects of La(III) on the expression of CaM in *Arabidopsis* and horseradish (edible plant) were investigated using immunofluorescence labelling and confocal laser scanning microscopy. Moreover, the effects and mechanism of La(III) on the conformation of CaM molecule were investigated by circular dichroism (CD), X-ray photoelectron spectroscopy (XPS) and computer simulation. The work would provide references for revealing the mechanism of REEs acting on plants.

2. Materials and methods

2.1. Chemicals and experimental materials

$LaCl_3 \cdot 6H_2O$ (greater than 99.99% purity) purchased from Shanghai Pharmaceutical Co., Ltd., China. $LaCl_3 \cdot 6H_2O$ was dissolved in deionized water to give a concentration of 1 M, and then calibrated with EDTA standard solution. Cellulase (Cellulase R-10), pectinase (Pectase Y-23), mannitol and other common reagents were of analytical reagent grade. Freeze-dried CaM powder were purchased from Sigma-Aldrich Co. The simulated physiological solution was improved Krebs solution, including Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-, and its total ionic strength was 0.1 M.

The *Arabidopsis* and horseradish plants were cultured based on previous reports (Wang et al., 2014a; Zhou et al., 2015). At the seedling stage, the plants were sprayed with 30 or 80 μM $LaCl_3$ solution. The control plants were sprayed with deionized water. After12 h, the *Arabidopsis* leaves at similar leaf-position were collected for the observation of endocytosis, and after 7 days, the *Arabidopsis* and horseradish leaves at similar leaf-position were collected for the measurement of CaM immunofluorescence.

The 1.31×10^{-5} M CaM solution containing 0, 3.93×10^{-5}, or 1.31×10^{-4} M $LaCl_3$ in the simulated physiological solution was equilibrated for 12 h. Then the mixed solution of CaM and La(III) was used to CD and XPS measurements.

2.2. Endocytosis observation

After the *Arabidopsis* leaves were cut off, the hypodermis of the leaves were peeled and immersed into the solution of *N*-(3-triethylammoniumpropyl)-4-(4-diethylaminophenylhexatrienyl) (FM4-64) (10 μM) for 30 min. The samples were observed under laser confocal microscopy (Nikon Ti-E-A1R, 40×). The excitation wavelength was 514 nm, and the observation wavelength was 650 nm.

2.3. CaM immunofluorescence measurement

The protoplasts of *Arabidopsis* and horseradish leaves were