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ScienceDirect
JOURNAL OF RARE EARTHS, Vol. 32, No. 5, May 2014, P. 467
JOURNAL OF RARE EARTHS
www.re-journal.com/en/

Effects of lanthanum on POD expression and DNA methylation of purple pepper under salt stress

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Received 5 September 2013; revised 16 January 2014

Abstract: Pepper seedlings were hydroponically cultivated in 0–20 mg/L extraneous lanthanum chloride (La^{3+}) for different numbers of days to investigate its regulating effects under salt stress. The results showed that, depending on the salt stress of 0.75 g/L, a lower concentration of La^{3+} (1.0 mg/L) might result in the lowest death rate, increase POD activity and change the band type of the stem and root on the 8th day. Furthermore, a methylation analysis by the methylation-sensitive amplified polymorphism (MSAP) technique indicated that a lower concentration of La^{3+} (1.0 mg/L) triggered the lowest methylation level of 26.1%, of which the hypermethylation events were the primary type of methylation. In addition, the sequencing of 14 differentially expressed polymorphic fragments and the subsequent blast search revealed that La^{3+} could induce methylation events in salt-tolerance sequences.

Keywords: lanthanum chloride; pepper; salt stress; POD; DNA methylation; rare earths

In the world, nearly 20% of the cultivated areas and half of the irrigated lands are affected by salinity, which greatly influences the productivity and quality in agriculture[1,2]. Damage to plants because of salinity has been ascribed to ion toxicity, nutrient imbalance and osmotic stress. In addition, the damage also resulted in oxidative stress due to the accumulation of reactive oxygen species (ROS). ROS can interact with many molecules, resulting in DNA mutation, protein denaturation, lipid peroxidation, pigment co-oxidation, membrane destruction and so on[3]. To mitigate the damage initiated by ROS and maintain the normal function, plants have developed a complex antioxidative system, and this system includes low molecular weight antioxidants (e.g. α-tocopherol, β-carotenoids, glutathione and ascorbate) and enzymic antioxidants (e.g. peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT))[4]. It has been well established that the coordination activities of the multiple forms of POD, SOD and CAT in the different cell compartments, achieve a balance between the rate of formation and the scavenging of ROS[5].

As one of the rare earth elements (REEs), lanthanum has many useful applications in plants and has gradually become valued. Some studies have shown that the germination rate of seeds, the quality and yield of crops can be improved by the addition of an appropriate concentration of lanthanum[6,7]. Further studies have indicated that lanthanum may stimulate the plant defence system (POD, SOD and CAT), promote the accumulation of secondary metabolites (soluble sugars and proteins) of plant cells and prevent decline in the relative chlorophyll content and chlorophyll fluorescence parameters under different stresses[8–10]. Researchers also deduced that the probable mechanism of the effect of lanthanum on plants was its displacement of Ca^{2+} due to its similar properties and semidiameter[11] or because lanthanum appeared as a Ca antagonist through binding to surface-localised Ca^{2+} absorption sites and blocking Ca^{2+} channels[12]. However, to our knowledge, the molecular proof of the plant response to salt stress that is triggered by lanthanum remains scarce.

DNA methylation has been considered an underlying mechanism for temporary changes in plant phenotypes. DNA methylation is performed by DNA methyltransferases that catalyse the transfer of a methyl group from S-adenosyl-L-methionine to cytosine bases in DNA, and cytosine methylation primarily appears in both CpG and CpNpG sequences. Although its functions are not fully understood, studies have shown that DNA methylation is required for normal plant growth and plays an important role in regulating gene expression, cell differentiation, chromatin inactivation and genomic imprinting[13,14]. For example, stress or a virus has been shown to render changes in DNA methylation or to cause the demethyla-

Foundation item: Project supported by the Educational Commission Program of Anhui Province (KJ2013A074) and the National Xinghuo Plan (2012GA710028)

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DOI: 10.1016/S1002-0721(14)60095-8

tion of some genes[15,16].

Methods for detecting DNA methylation have been steadily improving along with the study of methylation. Recently, high performance liquid chromatography (HPLC), the bisulphite method and the methylation sensitive amplification polymorphism (MSAP) technique were used to analyse DNA methylation[17]. MSAP, which is combined with the appealing features of amplified fragment length polymorphism (AFLP), is based on restriction enzymes (*Hpa*II, *Msp*I and *EcoR*I) and PCR amplification and is highly efficient for the large-scale detection of cytosine methylation in the genome. Although both isoschisomers, *Hpa*II and *Msp*I, can recognise and cut the 5'-CCGG-3' sequence, these isoschisomers differ widely in their methyl residues at sites that are by cytosine; in particular, *Hpa*II is inactive if one or both cytosines are fully methylated (both strands methylated) and active with hemimethylated (one DNA strand) external cytosine (^{5m}CCGG), whereas *Msp*I cleaves internal cytosine (C^{5m}CGG) but not external cytosine (^{5m}CCGG)[11,14]. Thus, the combination of an enzyme of high cleaving frequency (*Hpa*II or *Msp*I) with an enzyme of low cleaving frequency, such as *EcoR*I, could result in different types of bands, which show much useful information.

Using the MSAP technique, Chakrabarty et al. (2003) assessed the extent and pattern of cytosine methylation during somatic embryogenesis in Siberian ginseng (*Eleuterococcus senticosus*) and demonstrated that global DNA methylation rates were significantly decreased in embryogenic calli than in non-embryogenic calli[18]. Lu et al. (2008) analysed DNA methylation in different maize tissues and detected that the MSAP ratio and full methylation level were the highest in the bracteal leaf and lowest in the tassel[19]. Zhao et al. (2010) studied the relations between DNA methylation and salt stress in the cotton genome and found that the number of CCGG sites with cytosine methylation in a high salt-tolerant cotton line was less than that in a low salt-tolerant line and that the demethylation positively contributed to salt tolerance[20]. MSAP has also been used by Rodríguez Lópeze et al. to detect and quantify tissue of origin in salmon and veal products[21].

In this study, we first used lanthanum, which is a representative of REEs, to investigate its regulating effects on the death rate and POD expression of pepper seedlings under salt stress and then analysed the change in DNA methylation using the MSAP technique. We also isolated, sequenced and verified some fragments that were differentially methylated. The major aim of this work was to explore the characterisation and regularity of DNA methylation in plants that are triggered by lanthanum addition under salt stress.

1 Experimental

1.1 Materials and treatments

Seeds of the purple pepper "7036" were provided by the Genetic Laboratory of Anhui Science and Technology University. Seedlings with two true leaves were cultivated for use. Salt stress was applied by adding different concentrations of NaCl in the Hoagland solution and the Hoagland nutrient solution was refreshed every 4 d. In total, 20 seedlings that were most similar in appearance were used for three replicates, and dead seedlings were numbered on the 8th and 20th days, respectively. An approximately 50% death rate of seedlings under salt stress of 0.75 g/L was selected as the criteria for testing its salt tolerance.

Then, based on the above result, different concentrations of La^{3+} were used in the nutrient solution with the same salt concentration of 0.75 g/L (Table 1). On the 8th and 20th days, dead seedlings were numbered. The leaves, stems and roots of live seedlings were prepared for POD electrophoresis. On the 20th day, the live seedlings were prepared and stored at –80 ºC for DNA extraction and for the MSAP assay.

1.2 POD electrophoresis

Leaves, stems and roots from different sampling days were homogenised separately in two times the volume (*v/m*) of the sample buffer (0.065 mol/L tris-citric acid, pH 8.2) using a mortar and pestle in an ice bath. After centrifugation at 8000 r/min for 10 min at 4 ºC, the supernatant was collected for use. Gel concentrations for condensing and separating were 3% and 10%, respectively, and the sample volume was 10 μL, with 2% bromophenol blue as the indicator. After electrophoresis for 2.5 h, the POD isozyme was stained using the modified benzidine method[22].

1.3 DNA extraction

DNA was extracted using the CTAB method described by Hu[23]. The quality and quantity of DNA were measured using gel electrophoresis and spectrophotometric analysis, and the last concentration of DNA was adjusted to 20 ng/μL.

1.4 MSAP assay

The MSAP assay was adapted from the protocol by

Table 1 Experimental design with La^{3+}

No.	Treatments
CK	Hoagland solution+0.75 g/L NaCl
T1	Hoagland solution+0.75 g/L NaCl+1.0 mg/L $LaCl_3$
T2	Hoagland solution+0.75 g/L NaCl+5.0 mg/L $LaCl_3$
T3	Hoagland solution+0.75 g/L NaCl+10.0 mg/L $LaCl_3$
T4	Hoagland solution+0.75 g/L NaCl+20.0 mg/L $LaCl_3$