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Mineral nutrient imbalance, DNA lesion and DNA-protein crosslink involved in growth retardation of *Vicia faba* L. seedlings exposed to lanthanum ions

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Abstract

Effects of mineral nutrient imbalance, DNA lesion and DNA-protein crosslink on growth of *Vicia faba* L. seedlings hydroponically cultivated in concentrations of extraneous lanthanum (La) for 20 days were investigated in the present experiment. The results showed that contents of La, Cu or K elements in roots generally changed synchronously with those in leaves, while Ca, Fe, Zn, Mg, Mn or P in the roots altered inversely to those in the leaves. Thus, the extraneous La led to redistribution and imbalance of mineral nutrient elements in the roots and leaves. DNA lesion and DNA-protein crosslink were investigated by single cell gel electrophoresis (SCGE) and sodium dodecyl sulfate/potassium (SDS/K^+) precipitation methods, respectively. The results demonstrated that the increasing La induced DNA break and DNA-protein crosslinks (DPCs) in the seedlings. These results suggested that mineral nutrient imbalance, DNA lesion and DNA-protein crosslink were involved in the growth retardation and growth alteration of the seedlings, which may help to understand the mechanisms of rare earth elements (REEs) on plant growth.

Key words: rare earth elements; mineral nutrient; single cell gel electrophoresis; DNA lesion; DNA-protein crosslinks
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Introduction

Over the past thirty years, applications of rare earth elements (REEs) have been rapidly expanded in industrial, agricultural, forestry sectors and animal husbandry (Ni, 1995; Hu et al., 2004; Wang et al., 2004; Liu et al., 2006; Wang et al., 2007). REEs were released into the environment from mining activities (Sabbioni et al., 1984). Thus, more and more REEs have entered the environment due to human activities. The bioaccumulation and bioaugumentation of REEs through food chain eventually pose risks to human beings. In spite of positive effects of REEs on plants and animals, adverse effects were also confirmed in occupational and environmental exposure to REEs (Yang et al., 2006; Zhu et al., 1996). Consequently, concerns about the toxicological effects and mechanisms of REE(s) application on organisms have been rising in recent years, which need to be further investigated.

REEs were reported to regulate plant growth by affecting distribution and contents of mineral elements (such as Ca, Fe, Cu, K, P, Mg) in plants (Hu et al., 2004; Wang et al., 2008b, 2011). REEs have similar chemical properties as Ca^{2+} in respect to ionic radius, coordination chemistry and preferences for oxygen donor groups, and exert similar physiological effects on organisms, especially in regulation of plant growth (Hu et al., 2004; Lai et al., 2006). REEs can displace biometals in metallobiomolecules of membrane, metallic proteins and enzymes, leading to mineral nutrient imbalance, membrane permeability, modification of active conformation in biomolecules, and even disorder of biological functions (Zeng et al., 2006; Qiu et al., 2005). REEs also have potential capability to interfere with some metallic enzymes involved in cell proliferating and extending activities.

REEs have stronger affinity to non-metallic elements than calcium in biomolecules (Qiu et al., 2005), which may underlie the binding of REE(s) with DNA molecules. Previous studies showed that REE(s) induced genotoxic effects on human lymphocytes and rat embryos, which were detected by single cell gel electrophoresis (SCGE) or comet assay (Yongxing et al., 2000; Zhou et al., 2003; Paiva et al., 2009). Ce^{4+} was reported to induce DNA fragmentation and apoptosis in *Taxus cuspidate* cells (Ge et al., 2002).

Generally, DNA lesion includes single strand breaks, double strand breaks, alkali labile sites (primarily apurinic

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and apyrimidinic sites), incomplete excision repair sites, DNA-DNA crosslinks, and/or DNA-protein crosslinks (DPCs). DPCs can be created when a protein becomes covalently bound to DNA. In normal cells, DPCs are usually induced at basic level. DPCs may anchor chromatin and prevent from self-remodeling. DPCs are also expected to act as bulky helix-distorting adducts and physically block the progression of replication, transcription, DNA incision or repair during gene expression (Barker et al., 2005). Thus, DPCs may be involved in cell proliferation and extension. However, whether REE ions induce DPCs and thus interfere with plant growth has been little reported yet.

La was demonstrated to interfere with cell proliferating cycle phases in root tips of *Vicia faba* seedlings in our previous study (Wang et al., 2011). However, the related mechanisms of La on cell proliferation and growth remain far from clear. In the present experiment, La is selected as a representative of light rare earth elements. The objectives are to investigate the changes of mineral nutrients and potential genotoxicity, and thus explore the possible inhibition of DNA-protein crosslink on cell proliferation and growth of *V. faba* seedlings exposed to La solution.

1 Materials and methods

1.1 Main reagents

Low melting point agarose (LMP), normal melting point agarose (NMP), ethidium bromide, proteinase K, and Hoechst 33258 were purchased from Sigma Chemical (USA). $La(NO_3)_3$, Na_2EDTA, NaOH, Tris, SDS, KCl, etc. were purchased from Shanghai Chemical Reagent (China). All chemicals were of analytical grade and Milli-Q (Z00QSV001, USA) water was used throughout the experiments.

1.2 Plant material and La treatment

Seeds of *V. faba* were surface sterilized with 0.1% (m/V) sodium hypochlorite solution for 10 min and rinsed thoroughly in distilled water. After germination at 22–24°C, six uniform seeds were selected and transplanted into 1.2-L containers filled with equal Hoagland solution (Lucretti et al., 1999). One half mmol/L of ammonium dihydrogen phosphate was directly sprayed over the seedlings every day instead of dissolution in the solutions. Extraneous La concentrations were 0, 1, 2, 4 and 8 mg/L in the solutions by appropriate dissolution of $La(NO_3)_3$. The solutions were replaced every two days and pH were measured between 5.3 and 5.5. The containers were placed in a growth chamber under controlled conditions (15-hr photoperiod with active radiation of 220 μmol/(m^2·sec), 75% relative humidity, and 23°C/19°C day/night regime), and aerated consecutively. Three containers were prepared in each treatment. Roots and leaves were harvested for chemical analysis and biological measurements after 20 days of the treatments.

1.3 Measurement of root lengths and shoot heights

Lengths between apical leaves and stem base were measured denoting as heights of seedlings. Root lengths were measured from stem base to primary root tips.

1.4 Measurement of mineral nutrient contents by ICP-OES

Fresh roots were rinsed with 1 mol/L HCl and then with deionized water. Digestion of samples was performed according to the previous protocol (Wang et al., 2008a). Elemental contents were detected by Inductively Coupled Plasma Optical Emission (ICP-OES) (Optima 5300, Perkin-Elmer SCIEX, USA) and expressed as μg/g dry weight (dw). Certified standard samples (GBW07429) and triplicates of all samples were used to ensure accuracy and precision.

1.5 Determination of DNA damage in cells of root tips and apical leaves

The comet protocols were performed according to methods of Gichner et al. (2004) with minor modification. Microscope slides were precoated with 1% (m/V) normal melting point agarose (NMP) and allowed to dry overnight at room temperature. Nearly 50 root tips (5 mm in length) or 6 pieces of apical leaves were collected from each container, spread with equivalent cold Galbraith buffer (Galbraith et al., 1983) and sliced gently with a fresh razor blade under dim light on ice. Nuclear suspension was collected and filtered through nylon mesh of 25 μm pore size to remove gross particles and centrifuged at 700 ×g for 10 min at 4°C. The pellets were gently resuspended in 100 μL of Galbraith buffer and mixed with 300 μL of 1% (m/V) low melting point agarose (LMP) at 40°C. Onto each slide, 100 μL of such mixture was added and covered with a coverslip, followed by cooling at 4°C. Next, the coverslip-removed slides were placed in a horizontal tank containing freshly prepared cold electrophoresis buffer (1 mmol/L Na_2EDTA and 300 mmol/L NaOH, pH > 13.0). The slides were incubated for 20 min to allow DNA unwinding prior to electrophoresis at 0.72 V/cm for 25 min at 4°C. The slides were incubated in 400 mmol/L Tris buffer (pH 7.5) for 10 min, stained with 30 μL of 1‰ (m/V) ethidium bromide for 6 min, and then dipped in ice-cold water to remove the excess dye and covered with a coverslip. Three slides were prepared for each container, and 25 randomly chosen nuclei were photographed under fluorescence microscope in each slide (magnified 100 times). Comet length, tail length and tail moment of the nuclei were analyzed by CometScore image-analysis system to be as parameters of DNA damage. Similar trend were obtained in two independent experiments.

1.6 Determination of DPCs in root tip cells

DPCs formation was monitored based on fractionation of protein-bound and free DNA by sodium dodecyl sulfate/potassium (SDS/K^+) precipitation according to method described by Costa et al. (1996). Nuclei in root tips were isolated by gently slicing of root tips as described

as above. The pelleted nuclei were lysed in 0.5% (m/V) SDS solution. A 0.5 mL of 100 mmol/L KCl, 20 mmol/L Tris (pH 7.5) was added to each tube. After incubating for 10 min at 65°C, the tubes were placed on ice for 5 min, and then centrifuged at 6000 ×g for 5 min at 4°C. This washing step was repeated for more than two times, and supernatant was collected together by plastic tip pipette for quantification of free DNA contents in each treatment. The pellets were gently resuspended in 100 mmol/L of KCl, 20 mmol/L Tris-HCl (pH 7.5) and incubated again at 65°C for 10 min. Crosslinked DNA was released from the final K^+-SDS precipitates by treatment of 0.2 mg/mL proteinase K at 50°C for 3 hr. The tubes were placed on ice for 5 min and centrifuged at 12,000 ×g for 10 min at 4°C. The supernatant was collected to determine contents of crosslinked DNA. DNA standards were prepared at concentrations of 100, 200, 500, 1000, 2000 and 5000 ng/mL. The 2 mL of a standard DNA, free DNA or crosslinked DNA was mixed with 1 mL of 500 ng/mL of freshly prepared Hoechst 33258. The mixtures were placed in dark for 30 min. Fluorescence intensity was determined by excitation at 350 nm and emission at 450 nm using fluorescence spectrometry (Shimadzu RF-5301PC, 2000, Japan)

1.7 Statistical analyses

All the statistical analyses were performed using SPSS 13.0. The data were all presented as mean ± standard deviations of three replicates. Difference was considered to be significant at $p < 0.05$ (*) and highly significant at $p < 0.01$ (**) using one-way ANOVA by Dunnett's t-test. Representative photographs from each treatment were presented.

2 Results

2.1 Contents of La, Cu and K elements in roots changed in parallel with those in leaves

Contents of mineral nutrients in the leaves and roots were measured by ICP-OES. As shown in Table 1, contents of La, K and Cu in roots changed synchronously with those in leaves. The contents of La in the roots and leaves increased, while those of K decreased with increasing extraneous La. The contents of Cu in the roots and leaves slightly decreased at lower concentrations of La, and enhanced at higher ones.

2.2 Contents of Ca, Fe, Zn, Mg, Mn and P in roots changed inversely to those in leaves

Contents of Ca, Fe, Zn, Mg, Mn and P elements in roots changed inversely to those in leaves (Table 2). The contents of Ca, Fe and Zn in the roots were higher than those in the leaves, while the contents of Mg, Mn and P in the leaves were higher than those in the roots.

We also observed that the contents of Ca and Fe in the roots generally increased and those in the leaves decreased with increasing extraneous La (Table 2). The contents of Zn in the roots slightly increased at 0–2 mg/L of extraneous La and decreased at higher ones. The contents of Zn in the leaves changed inversely to those in the roots. The contents of Mg in the leaves decreased with the increasing extraneous La, while those in the roots increased with the extraneous La below 8 mg/L. The trend of Mn was in reverse with that of Mg. The contents of P in the roots slightly increased and then decreased with the increase of extraneous La at lower concentrations. However, the

Table 1 Contents of La, Cu and K in roots changed synchronously with those in leaves

Extraneous La (mg/L)	La content (μg/g dw)		K content (μg/g dw)		Cu content (μg/g dw)	
	Roots	Leaves	Roots	Leaves	Roots	Leaves
0	367 ± 46	7.2 ± 1.1	33970 ± 3042	24455 ± 2592	41.6 ± 2.6	19.0 ± 2.2
1	1082 ± 127*	11.9 ± 2.0	32905 ± 3613	22333 ± 2827	33.4 ± 3.3	17.9 ± 2.9
2	1193 ± 189**	14.8 ± 2.4**	30680 ± 2751	23040 ± 3001	29.8 ± 3.9*	19.2 ± 2.7
4	1964 ± 228**	16.0 ± 2.4**	28588 ± 3542	21428 ± 3410	36.2 ± 5.0	20.1 ± 3.0
8	2900 ± 411**	17.8 ± 2.9**	25136 ± 4121*	18901 ± 2284	48.3 ± 6.7	21.9 ± 3.3

Values are denoted as mean ± SD, $n = 3$, * $p < 0.05$, ** $p < 0.01$.

Table 2 Contents of Ca, Fe, Zn, Mg, Mn and P elements in roots and leaves

Extraneous La (mg/L)	Ca content (μg/g dw)		Fe content (μg/g dw)		Zn content (μg/g dw)	
	Roots	Leaves	Roots	Leaves	Roots	Leaves
0	2212 ± 236	1878 ± 140	570 ± 81	138 ± 15	175 ± 21	107 ± 10
1	2595 ± 260	1588 ± 218	666 ± 82	124 ± 16	203 ± 35	102 ± 12
2	2494 ± 185	1462 ± 201	635 ± 97	119 ± 16	217 ± 34	100 ± 17
4	2603 ± 295	1207 ± 133	807 ± 99*	110 ± 19	190 ± 26	104 ± 16
8	2810 ± 112	1050 ± 113	819 ± 114*	98 ± 18*	123 ± 23	113 ± 15
Extraneous La (mg/L)	Mg content (μg/g dw)		Mn content (μg/g dw)		P content (μg/g dw)	
	Roots	Leaves	Roots	Leaves	Roots	Leaves
0	793 ± 108	2880 ± 233	6.5 ± 0.5	16.7 ± 2.7	5339 ± 782	7850 ± 731
1	818 ± 119	2705 ± 328	5.4 ± 0.6	17.3 ± 2.4	5534 ± 831	6835 ± 815
2	936 ± 110	2624 ± 386	5.1 ± 0.9	17.9 ± 1.9	6077 ± 988	7469 ± 995
4	997 ± 107	2337 ± 333	2.8 ± 0.4**	18.4 ± 2.5	5673 ± 715	8093 ± 916
8	986 ± 161	1921 ± 328*	3.0 ± 0.5**	15.6 ± 2.5	5378 ± 746	8291 ± 1007

Values are denoted as mean ± SD, $n = 3$, * $p < 0.05$, ** $p < 0.01$.

contents of P in the leaves declined slightly at 1 mg/L of extraneous La and then enhanced at higher ones.

2.3 Changes in root lengths and shoot heights

Root lengths and shoot heights decreased with the increase of extraneous La. Significant reduction was observed at treatment of 8 mg/L of extraneous La ($p < 0.05$, $p < 0.01$) (Fig. 1).

2.4 DNA damage in cells of root tips and apical leaves

The nuclei isolated from root tips and apical leaves were performed by comet assay (Fig. 2). The tail lengths and tail moments of root tip cells enhanced with the increasing extraneous La from 0 to 2 mg/L, then tended to decrease, while those in the apical leaves peaked at 4 mg/L, followed by slight decline (Fig. 3).

2.5 DPCs in root tip cells

DPCs occurred in the root tip cells, which was detected by potassium-SDS precipitation method. The results showed that the proportion of DPCs increased with the extraneous La, which significantly enhanced compared with the control when the extraneous La was more than 4 mg/L (Fig. 4). In particular, the contents of DPCs were highly correlated with the root lengths ($r = –0.883$, $p < 0.05$) and shoot heights ($r = –0.923$, $p< 0.05$), respectively.

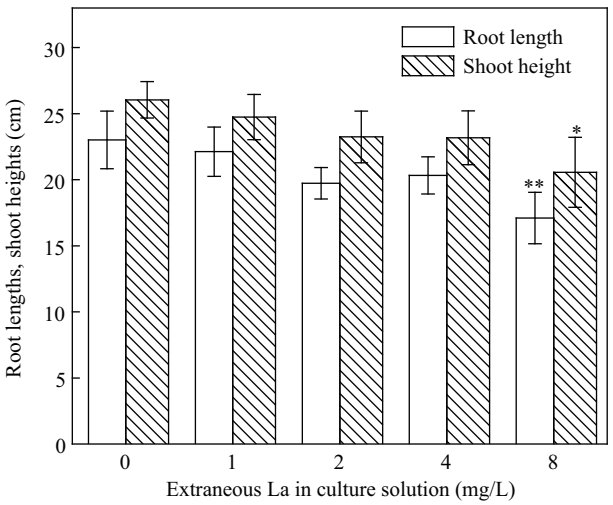

Fig. 1 Root lengths and shoot heights of *V. faba* seedlings cultured in extraneous La for 20 days. Values are denoted as mean ± SD, $n = 3$, * $p < 0.05$, ** $p < 0.01$.

3 Discussion

The applications of REEs in industrial, agricultural, forestry sectors and animal husbandry have accelerated the transport and accumulation of REEs in the environment (Wang et al., 2009). REEs were reported to exert hormetic effects (i.e., low-dose promotion and high-dose inhibition) on plant growth (Zeng et al., 2006; Wang et al., 2009; Wu et al., 2001; Ouyang et al., 2003; Kobayashi et al., 2007). However, their mechanisms remain fragmentary and need to be further investigated.

REEs can be accumulated in roots and aerial parts of plants following application of REEs (Wang et al., 2011; Xu et al., 2002). REE(s) were reported to disturb the distribution of mineral nutrients in plants (Wang et al., 2011; Hu et al., 2006). Whereas, low doses of La or Ce were also reported to have certain beneficial effects on uptake of nutrient elements such as Zn, Mn and Mo in corn seedlings (Qun et al., 1992). In particular, La^{3+} can bind to superficially located Ca^{2+} absorption sites in a less reversible manner than Ca^{2+}, and thus block Ca^{2+} channels and disturb the uptake of nutrient ions through Ca^{2+} channels (Hu et al., 2006). This may underlie the unbalance of these mineral nutrients in the roots and leaves due to La exposure in this experiment.

K, Ca, Mg, Fe, Cu, and Mn are essential nutrients for plant development. Therefore, absence or deficiency of such elements may lead to growth retardation or death. REEs could regulate plant growth by affecting the distribution of mineral elements in crop seedlings (Hu et al., 2004; Wang et al., 2008b, 2011). In the present study, the concentrations of extraneous La caused the unbalance of several nutrient elements and even their possible redistribution throughout the roots and leaves. For instance, the contents

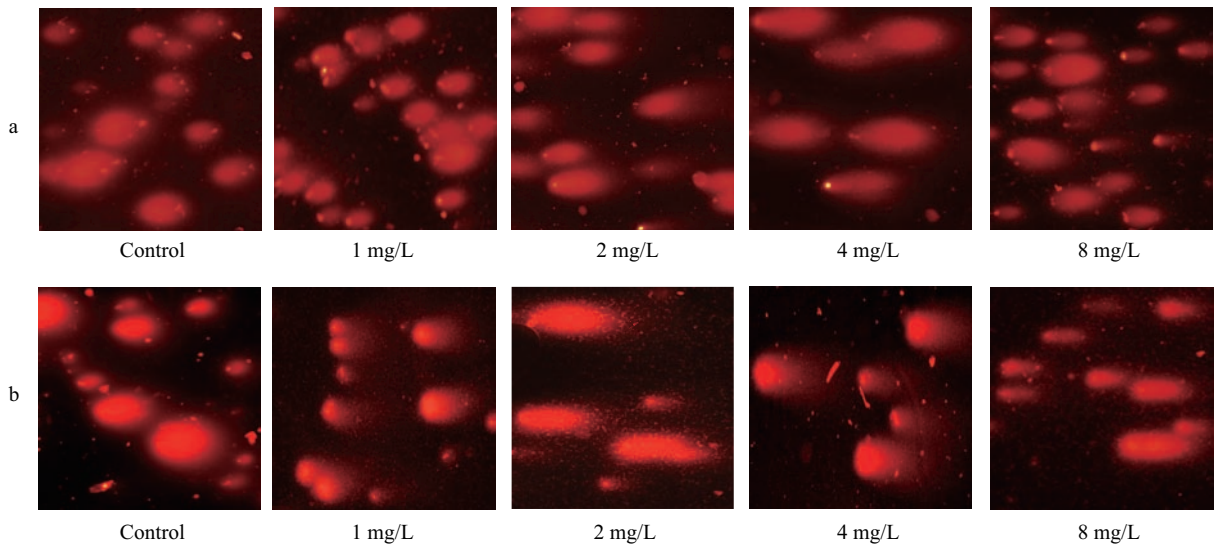

Fig. 2 Comet assay of nuclei in apical leaves (a) and root tips (b) of *V. faba* seedlings cultured in extraneous La for 20 days (magnified 100 times).

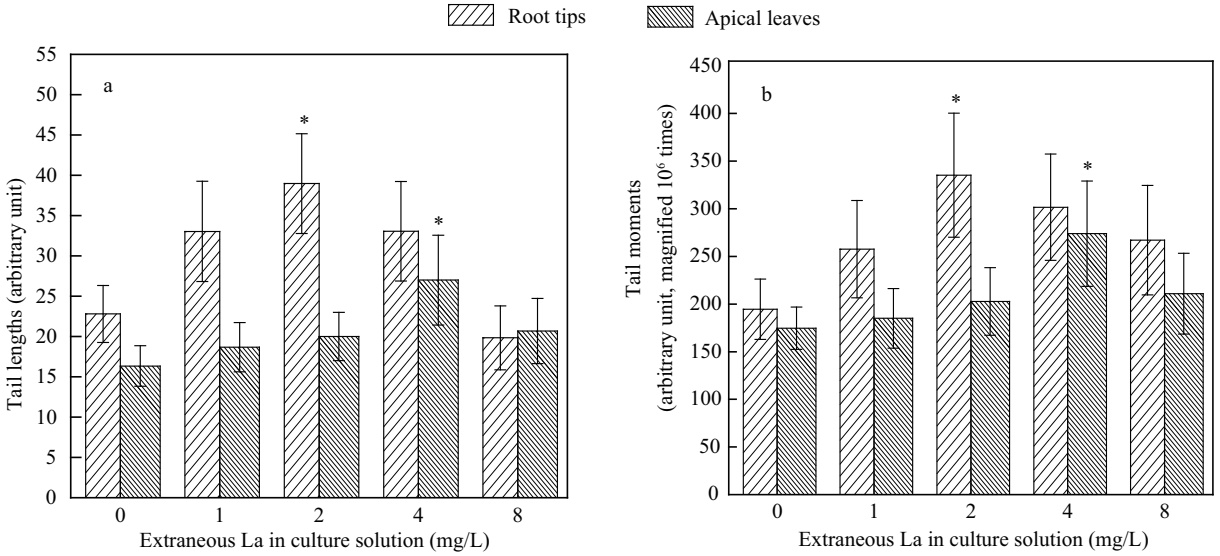

Fig. 3 Tail lengths (a) and tail moments (b) in comet assay of root tips and apical leaves of *V. faba* seedlings cultured in extraneous La for 20 days. Values are denoted as mean ± SD, $n = 3$, * $p < 0.05$.

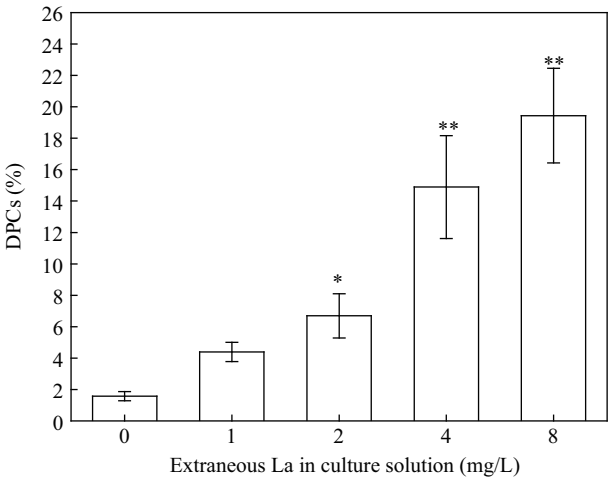

Fig. 4 DNA-protein crosslinks (DPCs) in root tip cells of *V. faba* seedlings cultured in extraneous La for 20 days. Values are denoted as mean ± SD, $n = 3$, * $p<0.05$, ** $p<0.01$.

of La, Cu or K elements in the roots generally altered in parallel with those in the leaves, while the contents of Ca, Fe, Zn, Mg, Mn or P in the roots altered inversely to those in the leaves, involving the differential alterations of shoot heights or root lengths of the seedlings (Fig. 1).

Our previous study demonstrated that concentrations of extraneous La led to disturbance of cell proliferation cycles in roots of *V. faba* L. seedlings (Wang et al., 2011). However, the related mechanisms are still not fully understood yet. Thus, DNA lesion and DNA-protein crosslinks were preferably investigated in this study.

SCGE is a sensitive method for detection of various DNA lesions in organisms. In the present study, the tail lengths and tail moments in root tip cells or apical leaves increased with the extraneous La at lower concentrations and tended to decline at higher concentrations (Fig. 3), indicating DNA lesion due to La^{3+} in the seedlings. In contrast to many different DNA lesions that lead to increased DNA migration in comet assay, crosslinks are the only known DNA modifications that cause an actual decreased DNA migration (Merk et al., 2000). We first tried to explore whether DPCs occurred in the nuclei of root tips or apical leaves according to method of Merk et al. (2000). After DNA unwinding in the comet assay, the slides were covered with 100 μL of 1 mg/mL proteinase K and incubated for 2 hr in a moist chamber at 37°C. The coverslips were removed and the slides were processed as described in SCGE protocol. However, the posttreatment of proteinase K in the slides could not prove it's reproducibility in the same treated group. We then turned to SDS/K^+ precipitation protocol to investigate DPCs in the root tips or apical leaves (Barker et al., 2005; Costa et al., 1996). This method can be used to measure the extent of DNA associated with protein after the protein is precipitated. The results showed that DPCs were induced and the proportion of DPCs increased with the increase of extraneous La^{3+} in the root tip cells after treatment by proteinase K (Fig. 4). This protocol was also conducted to investigate DPCs in the leaves. However, the fluorescence intensities of DNA were variously interfered by reserved chlorophyll, which was difficult to be cleared with general extraction methods. As a consequence, no reproducible results could be obtained. Then, we turned to a modified SCGE protocol, which was combined with proteinase K pretreatment of nuclei. Similarly, no satisfying results were achieved. Up to now, reports concerning DPCs detection in leaves have not been consulted. Therefore, DPCs detection method in leaves needs to be improved.

DPCs may retard the migration of DNA fragments, resulting in a reduced tail moment (Merk et al., 2000). Therefore, the elevated DPCs contents might underlie the reduced tail lengths and tail moments in the comet cells of root tips or apical leaves exposed to the increasing concentrations of La^{3+} (Fig. 3). DPCs also play a role as bulky helix-distorting adducts and physically block the progression of replication, transcription, DNA incision or repair (Barker et al., 2005). In our previous report, the cell cycle phases in root tips of *V. faba* seedlings were disturbed due to La^{3+} induction. The cell cycles were most probably

arrested at G1/S interphase by La^{3+} (Wang et al., 2011). DNA breaks usually occur rapidly and are easily repaired while DPCs develop slowly and can not be easily repaired (Merk et al., 2000). Therefore, the enhanced DPCs were inferred to be responsible for the inhibition and disturbance of cell proliferation cycles in the mitotic tissues of root tips. Root lengthening is generally related to apical meristem activity (Fusconi et al., 2006). Thus, the inhibited mitotic activities were inevitably involved in the growth of *V. faba* seedlings, which were reconfirmed by the highly negative correlation between the contents of DPCs in the roots and the root lengths ($r = –0.883$, $p < 0.05$) or shoot heights ($r = –0.923$, $p < 0.05$).

REEs exert low-dose promotion and high-dose inhibition on plant growth, a so-called hormetic effect or hormesis (Wu et al., 2001; Ouyang et al., 2003). Combined with our previous report and the present study, it may be concluded that the nutrient imbalance, DNA lesion and DNA-protein crosslinks contributed to the growth retardation of *V. faba* seedlings exposed to the concentrations of La^{3+}.

4 Conclusions

This study demonstrated that the concentrations of extraneous La led to redistribution and unbalance of mineral nutrients in roots and leaves of *V. faba* seedlings. The contents of La, Cu or K elements in the roots generally changed synchronously with those in the leaves, while Ca, Fe, Zn, Mg, Mn or P in the roots altered inversely to those in the leaves. DNA lesion and DNA-protein crosslink were also induced and enhanced with the increasing La. These results suggested that the mineral nutrient imbalance, DNA lesion and DNA-protein crosslink were involved in the growth retardation of the seedlings. This study may help to understand the mechanisms of REEs on plant growth.

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