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BRIEF COMMUNICATION

Effects of lanthanum on the antioxidant capacity of chloroplasts and chlorophyll fluorescence parameters of maize seedlings under chromium stress

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Abstract

Effects of lanthanum chloride ($LaCl_3$) on chromium tolerance of maize were investigated at chloroplast level. The results showed that Cr stress significantly increased activities of ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, superoxide dismutase, and glutathione peroxidase, nonphotochemical quenching, malondialdehyde (MDA) and hydrogen peroxide contents, but significantly reduced the ratios of reduced ascorbate to dehydroascorbate and reduced glutathione to oxidized glutathione, the contents of chlorophyll and carotenoids, maximum photochemical efficiency of PSII, photochemical quenching, quantum efficiency of PSII photochemistry, net photosynthetic rate, plant height and biomass, compared with control. Compared to Cr stress alone, $LaCl_3$ significantly reduced MDA and H_2O_2 contents and increased other indicators under Cr stress. $LaCl_3$ alone also enhanced above indicators except MDA and H_2O_2, compared with control. Our results suggested that $LaCl_3$ improved the Cr tolerance of maize crops by upregulating the antioxidant capacity and the function of chloroplasts.

Additional key words: antioxidant system; chlorophyll fluorescence; chromium injury; lanthanum chloride; plastid; *Zea mays*.

Chromium stress is a heavy metal stress that inhibits the growth and development of plants (Ma *et al.* 2016). Cr stress usually induces the accumulation of reactive oxygen species (ROS), which induces peroxidative damage to plants (Mahmud *et al.* 2017). Chloroplast is an important cell compartment for its basic role in photosynthesis. However, chloroplast is highly vulnerable to peroxidative damage induced by stresses. Fortunately, chloroplast has developed an intricate antioxidant defense system to protect itself against the harmful effects of peroxidative damage, including antioxidative enzymes and nonenzymatic compounds. The antioxidant enzymes mainly include superoxide dismutase (SOD), glutathione peroxidase (GPX), and ascorbate peroxidase (APX), *etc*. Nonenzymatic substances mainly include reduced ascorbate (AsA) and reduced glutathione (GSH), *etc*.

Lanthanum (La) is an important rare earth element, which can promote root organogenesis (Guo *et al.* 2012), mediate secondary metabolism synthesis (Zhou *et al.* 2012), and promote nitrogen metabolism (Huang *et al.* 2013), *etc*. Increasing evidence has proven that La could relieve peroxidative damage in plants under many stresses, including salt and water stresses (Zhang *et al.* 2006, Xu *et al.* 2007, Liu *et al.* 2016). However, little is known about antioxidative responses of plants to La under Cr stress at the chloroplast level. Thus, it is interesting to elucidate whether and how La regulates the antioxidant capacity in chloroplasts under Cr stress.

In this study, we investigated the effects of La on the activities of antioxidant enzymes, the ratios of reduced ascorbate to dehydroascorbate (AsA/DHA) and reduced glutathione to oxidized glutathione (GSH/GSSG), the contents of malondialdehyde (MDA) and H_2O_2 in the chloroplasts and chlorophyll fluorescence parameters of maize under Cr stress. The aim of this study was to elucidate the role of La in regulating Cr tolerance of maize and provide the theoretical basis for its application in

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Abbreviations: APX – ascorbate peroxidase; AsA/DHA – the ratio of reduced ascorbate to dehydroascorbate; Car – carotenoids; Chl – chlorophyll; DAT – days of treatment; DHAR – dehydroascorbate reductase; F_v/F_m – maximum photochemical efficiency of PSII; GPX – glutathione peroxidase; GR – glutathione reductase; GSH/GSSG – the ratio of reduced glutathione to oxidized glutathione; MDA – malondialdehyde; MDHAR – monodehydroascorbate reductase; P_N – net photosynthetic rate; q_N – nonphotochemical quenching; q_P – photochemical quenching; ROS – reactive oxygen species; SOD – superoxide dismutase; Φ_{PSII} – effective quantum yield of PSII.
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promoting Cr tolerance of maize crops.

Seeds of maize (*Zea mays*, cv. Xindan 29) were germinated in Petri dishes with filter paper moistened by distilled water and grown in artificial climate chamber under a day/night temperature of 25/15°C, PAR of 500 μmol(photon) m^{-2} s^{-1}, and a 10-h photoperiod. When the first leaf was fully expanded, the seedlings were transferred into plastic boxes filled with half-strength Hoagland's solution and their roots were kept in dark. The half-strength Hoagland's solution was exchanged every two days. When the third leaf was fully expanded, the seedlings of uniform height were selected for all further experiments.

The suitable concentration of chromium chloride [80 mg($CrCl_3$) L^{-1}] was selected from following concentrations including 40, 80, 120, and 160 mg($CrCl_3$) L^{-1}. The obvious wilting phenomenon was observed in the seedlings treated by 120 and 160 mg($CrCl_3$) L^{-1} after 48 h, while there was no obvious wilting in the seedlings treated by 40 and 80 mg($CrCl_3$) L^{-1} after 48 h (Figure 1*A*). Therefore, we selected 80 mg($CrCl_3$) L^{-1} as the suitable treatment concentration. After placing in distilled water for 12 h, the roots were placed in beakers containing 100 ml of 80 mg($CrCl_3$) L^{-1} for 48 h at 25°C with a continuous light intensity of 500 μmol(photon) m^{-2} s^{-1}. Beakers were wrapped with aluminium foil to keep roots in dark. In order to select a suitable concentration of La, the effects of different concentrations of lanthanum chloride on the contents of MDA and H_2O_2 was tested, and then 30 μM $LaCl_3$ was selected for the further experiment (Table 1S, *supplement*). To study the effect of $LaCl_3$, plants were pretreated with 30 μM $LaCl_3$ for 12 h and then exposed to 80 mg($CrCl_3$)L^{-1} or half-strength Hoagland's solution for 48 h under above conditions. Control plants were treated with half-strength Hoagland's solution alone. After treatment of 48 h, the top full expanded leaves from different treatments were collected and frozen in liquid nitrogen, and then kept at –80°C until analyses.

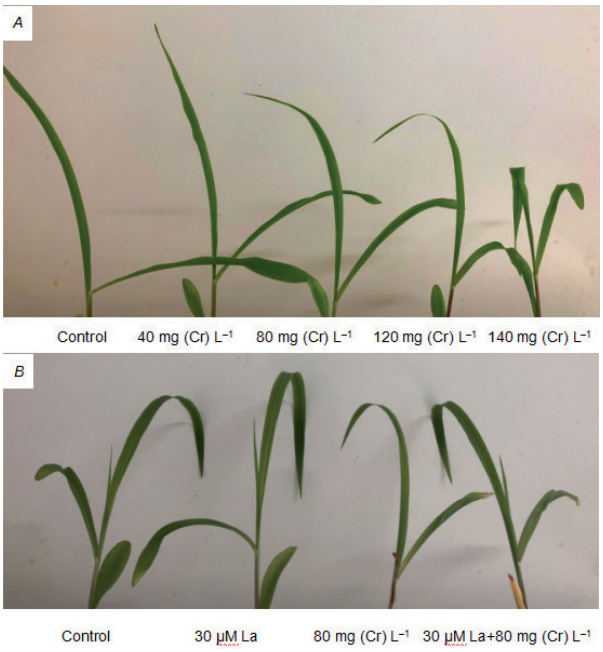

Fig. 1. Effects of different concentrations of Cr on the phenotype of maize seedlings under Cr stress after 48 h of treatment (*A*) and the suitable concentration of La on the phenotype of maize seedlings under Cr stress after 7 d of treatment (*B*). (*A*) The plants were treated as follows: Control – half-strength Hoagland's solution with $CrCl_3$ concentrations: 40, 80, 120, 160 mg(Cr) L^{-1}. The plants were exposed to Cr stress for 48 h. (*B*) The plants were treated as follows: Control – half-strength Hoagland's solution with $CrCl_3$ and $LaCl_3$: 30 μM La, 80 mg(Cr) L^{-1}, 30 μM La + 80 mg(Cr) L^{-1}. The plants were pretreated with $LaCl_3$ for 12 h, and then exposed to Cr stress for 7 d.

Intact chloroplasts were obtained from fresh maize leaves by density-gradient centrifugation in *Percoll* gradients according to the method of Wang *et al.* (2009). Each fresh sample of leaves (50 g) was homogenized in a blender with 200 ml of ice-cold isolation buffer consisting of 330 mM mannitol, 10 mM ethylenediamine tetraacetic acid (EDTA), 5 mM $MgCl_2$, 2 mM sodium ascorbate, and 30 mM 3-(N-morpholino) propanesulfonic acid (Mops) (pH 7.6). The homogenates were filtered through four layers of cheesecloth and centrifuged at 4,000 × *g* for 30 s. The resulting crude chloroplast pellets were suspended in 5 ml of suspension medium (330 mM mannitol, 2 mM EDTA, and 50 mM Mops pH 7.8). Chloroplast suspension (2 ml) was layered on 50 % *Percoll* and centrifugated at 5,000 × *g* for 10 min. The band near the bottom, containing intact chloroplasts, was diluted five times with the suspension medium to remove *Percoll*. The suspension was then centrifugated at 12,000 × *g* for 10 min. The pellets were resuspended in the same medium and used for further assays. All operations were performed at 0–4°C.

The intact chloroplast samples were diluted ten folds with ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1 % (w/v) CHAPSO, with the addition of 1 mM ASA for APX and DHAR assay. Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The absorbance was recorded at 560 nm (*TU-1810, Beijing Purkinje General Instrument Co.,Ltd.*, China), and one unit of SOD was defined as the amount of enzyme required to cause a 50 % inhibition of NBT reduction. Glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured according to He *et al.* (2006). One unit of GPX activity was defined as 1 μmol(GSH decreased) per min. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) by monitoring the decrease in absorbance at 290 nm for 1 min. One unit of enzyme was defined as the amount of APX catalyzing the oxidation of 1 μmol(ascorbate) per min. Glutathione reductase (GR, EC 1.6.4.2) activity was monitored at 340 nm for 3 min (Grace and Logan 1996). One unit of GR activity was defined as the reduction of 1 μmol(NADPH) per min. Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed according to Miyake and Asada (1992) at 340 nm for 3 min. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 μmol(NADH) per min. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured

according to Dalton *et al.* (1986) at 265 nm for 3 min. One unit of DHAR activity was defined as the amount of enzyme that produces 1 μmol(AsA) per min. The specific activities of above enzymes were expressed as Units mg^{-1}(protein). Protein concentration was measured by using bovine serum albumin as standard according to Bradford (1976).

The intact chloroplast samples purified from 2 g of leaves were homogenized with 5 ml of 5% ice-cold metaphosphoric acid. The homogenates were centrifuged at 4°C and 12,000 × *g* for 20 min. Then the resulting supernatants were immediately used for assay. AsA and dehydroascorbate (DHA) were measured according to Hodges *et al.* (1996). AsA/DHA was expressed as the ratio between the content of AsA and the content of DHA. Oxidized glutathione (GSSG) and GSH were measured according to Griffith (1980) GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. GSH content was then estimated from the difference between total glutathione and GSSG. A standard curve prepared by using GSH and GSSG was used in the calculation of the amounts of total glutathione, GSH, and GSSG. GSH/GSSG was expressed as the ratio between the content of GSH and the content of GSSG. Lipid peroxidation of chloroplasts was estimated by measuring MDA content according to the thiobarbituric acid (TBA) reaction (Heath and Packer 1968). H_2O_2 content was determined by measuring the absorption of titanium-hydroperoxide as described by Brennan and Frenkel (1977). The amount of H_2O_2 was calculated from the standardized H_2O_2 curve.

A *Yaxin-1161G* fluorometer (*Yaxin*, China) was used to measure the chlorophyll (Chl) fluorescence parameters from 10:00 to 12:00 h after 2 d of treatment (DAT). For dark adaptation, the leaves were covered for 30 min. Then Chl fluorescence parameters, such as maximum photochemical efficiency of PSII (F_v/F_m), photochemical quenching (q_P), nonphotochemical quenching (q_N), and quantum efficiency of PSII photochemistry (Φ_{PSII}), were measured by the fluorometer. Measurements were performed in a closed chamber under controlled conditions. Minimum fluorescence (F_0) was measured under a weak modulating radiation [0.5 μmol(photon) m^{-2} s^{-1}], and maximum fluorescence (F_m) was induced by a saturating pulse of radiation [2400 μmol(photon) m^{-2} s^{-1}]. The photosynthetic rate (P_N) was measured by photosynthesis system (*Licor-6400*, USA) at an irradiance of 500 μmol(photon) m^{-2} s^{-1} and a CO_2 concentration of 400 μmol mol^{-1} from 10:00 to 12:00 h after 2 DAT. After 7 DAT, plant height was measured by a ruler. Fresh masses of the seedlings of each treatment were recorded and then oven dried for 72 h at 80°C. Dry mass of the seedlings of each treatment were then recorded.

The experimental design was a randomized complete block design with six replications. The results presented were means of six replications. Means were compared by one-way analysis of variance (*ANOVA*) and *Duncan*'s multiple range test at the 5% level of significance.

Among different concentrations (10, 30, 50, and 100 μM), 30 μM $LaCl_3$ significantly decreased the contents of MDA and H_2O_2 in the chloroplast of maize seedlings under Cr stress (Table 1S, *supplement*), which suggested that 30 μM $LaCl_3$ was the suitable concentration for this study.

Compared with control, Cr stress significantly increased the activities of APX, GR, DHAR, MDHAR, SOD, and GPX in chloroplasts (Table 1). Pretreatment with $LaCl_3$ significantly increased the activities of above enzymes in chloroplasts under Cr stress, compared with Cr stress alone. After 48 h of treatment, $LaCl_3$ increased the activities of APX, GR, DHAR, MDHAR, SOD, and GPX by 34.7, 33.3, 29.9, 42.0, 19.9, and 31.4 %, respectively. Meanwhile, pretreatment with $LaCl_3$ alone also significantly increased the activities of above enzymes in chloroplasts, compared with control. These results suggested that $LaCl_3$ up-regulated the antioxidant metabolism in chloroplasts of maize by increasing the activities of above enzymes under Cr stress.

Compared with control, Cr stress significantly decreased the ratios of AsA/DHA and GSH/GSSG in chloroplasts (Table 1). Pretreatment with $LaCl_3$ significantly elevated the ratios of AsA/DHA and GSH/GSSG under Cr stress, compared to Cr stress alone. After 48 h of treatment, $LaCl_3$ increased the ratios of AsA/DHA and GSH/GSSG by 22.4 and 34.5%, respectively. Meanwhile, pretreatment with $LaCl_3$ alone also signifi-cantly elevated the ratios of AsA/DHA and GSH/GSSG, compared with control. Above results suggested that pretreatment with $LaCl_3$ regulated the redox state of the chloroplasts through AsA/DHA and GSH/GSSG under Cr stress.

Cr stress significantly declined the values of F_v/F_m, q_P, Φ_{PSII}, and P_N and increased q_N after 48 h of treatment, compared with control (Table 1). Compared to Cr stress alone, pretreatment with $LaCl_3$ followed by Cr stress significantly increased the values of F_v/F_m, q_P, q_N, Φ_{PSII}, and P_N. After 48 h of treatment, $LaCl_3$ increased F_v/F_m, q_P, q_N, Φ_{PSII}, and P_N by 23.1, 45.4, 20.9, 35.0, and 32.7%, respectively. These results suggested that $LaCl_3$ alleviated the negative effects of Cr stress on the photosynthetic apparatus of maize seedlings.

Cr stress significantly increased the contents of MDA and H_2O_2 in the chloroplasts, and decreased the contents of Chl and Car, plant height and plant biomass, compared with the control (Table 1). Pretreatment with $LaCl_3$ decreased the contents of MDA and H_2O_2 in the chloroplasts by 30.4 and 37.4 %, respectively. Pretreatment with $LaCl_3$ significantly increased the contents of Chl and Car, plant height and plant biomass by 17.0, 40.0, 11.5, and 13.6%, respectively. Meanwhile, pretreatment with $LaCl_3$ alone also significantly decreased the contents of MDA and H_2O_2 in the chloroplasts, and increased the contents of Chl and Car, plant height, and plant biomass, compared with the control. Above results were also proved by the phenotype of different treatments at 7 DAT (Fig. 1*B*). These results suggested that pretreatment with $LaCl_3$ has an important role in the acquisition of Cr tolerance of maize crops.

Many studies have proved that Cr stress induced oxidative damage in plants (Ma *et al.* 2016, Mahmud *et al.* 2017). In our study, an enhanced level of lipid peroxidation, as indicated by the contents of MDA and H_2O_2, was

Table 1. Effects of La on the activities of ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), superoxide dismutase (SOD), and glutathione peroxidase (GPX), the ratios of reduced ascorbate to dehydroascorbate (AsA/DHA) and reduced glutathione to oxidized glutathione (GSH/GSSG), and the contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in chloroplasts, the chlorophyll fluorescence parameters, plant height and biomass, and the contents of chlorophyll (Chl) and carotenoids (Car) of maize seedlings under Cr stress. The plants were treated as follows: Control – half-strength Hoagland's solution; La – 30 μM $LaCl_3$; Cr – 80 mg($CrCl_3$) L^{-1}; La + Cr – 30 μM $LaCl_3$ + 80 mg($CrCl_3$) L^{-1}. The plants were pre-treated with $LaCl_3$ for 12 h, and then exposed to Cr stress for 7 d. Plant height and biomass were measured after 7 d of treatment, other parameters were all measured after 48 h of treatment. Values represent mean ± standard deviations (SD), different *small letters* stand for significant difference between different treatments at $P<0.05$.

Parameter	Control	La	Cr	La + Cr
APX [U mg^{-1}(protein)]	1.17 ± 0.13^{d}	1.45 ± 0.16^{c}	1.93 ± 0.20^{b}	2.60 ± 0.24^{a}
GR [U mg^{-1}(protein)]	0.83 ± 0.09^{d}	1.15 ± 0.13^{c}	1.50 ± 0.14^{b}	2.00 ± 0.25^{a}
DHAR [U mg^{-1}(protein)]	0.75 ± 0.08^{d}	1.10 ± 0.10^{c}	1.44 ± 0.15^{b}	1.87 ± 0.19^{a}
MDHAR [U mg^{-1}(protein)]	0.52 ± 0.05^{d}	0.70 ± 0.08^{c}	1.00 ± 0.11^{b}	1.42 ± 0.15^{a}
SOD [U mg^{-1}(protein)]	1.38 ± 0.11^{c}	1.70 ± 0.16^{b}	2.11 ± 0.22^{b}	2.53 ± 0.27^{a}
GPX [U mg^{-1}(protein)]	0.25 ± 0.03^{c}	0.32 ± 0.04^{b}	0.35 ± 0.04^{b}	0.46 ± 0.06^{a}
AsA/DHA	20.20 ± 1.84^{b}	23.17 ± 2.11^{a}	14.63 ± 1.58^{d}	17.90 ± 1.93^{c}
GSH/GSSG	21.45 ± 2.37^{b}	24.15 ± 2.15^{a}	13.26 ± 1.24^{d}	17.84 ± 1.88^{c}
MDA[nmol g^{-1}(FM)]	4.33 ± 0.50^{c}	3.95 ± 0.48^{c}	13.11 ± 1.44^{a}	9.12 ± 1.03^{b}
H_2O_2 [μmol g^{-1}(FM)]	0.40 ± 0.05^{c}	0.35 ± 0.06^{c}	1.95 ± 0.22^{a}	1.22 ± 0.14^{b}
F_v/F_m	0.75 ± 0.09^{b}	0.85 ± 0.11^{a}	0.52 ± 0.07^{d}	0.64 ± 0.08^{c}
q_P	0.42 ± 0.05^{b}	0.53 ± 0.07^{a}	0.22 ± 0.03^{d}	0.32 ± 0.04^{c}
q_N	0.25 ± 0.04^{d}	0.33 ± 0.04^{c}	0.43 ± 0.05^{b}	0.52 ± 0.05^{a}
Φ_{PSII}	0.35 ± 0.04^{b}	0.44 ± 0.05^{a}	0.20 ± 0.03^{d}	0.27 ± 0.04^{c}
P_N [μmol m^{-2} s^{-1}]	6.52 ± 0.73^{b}	7.50 ± 0.88^{a}	3.85 ± 0.51^{d}	5.11 ± 0.58^{c}
Car [mg g^{-1} (FM)]	0.66 ± 0.07^{b}	0.78 ± 0.09^{a}	0.35 ± 0.04^{d}	0.49 ± 0.05^{c}
Chl [mg g^{-1} (FM)]	1.86 ± 0.21^{b}	2.06 ± 0.22^{a}	1.35 ± 0.13^{d}	1.58 ± 0.17^{c}
Plant height (cm)	17.00 ± 1.82^{b}	18.90 ± 2.00^{a}	13.00 ± 1.46^{d}	14.50 ± 1.30^{c}
Plant biomass [g(FM) $plant^{-1}$]	1.14 ± 0.13^{b}	1.28 ± 0.14^{a}	0.88 ± 0.10^{d}	1.00 ± 0.12^{c}

observed in the chloroplasts of maize under Cr stress. This result suggested that Cr stress induced oxidative stress to the chloroplasts. To cope with Cr stress, the antioxidant capacity in the chloroplast of maize was up-regulated indicated by the activities of antioxidant enzymes APX, GR, DHAR, MDHAR, SOD, and GPX.

La is an important rare earth element. Increasing evidence has proven that La can relieve oxidative damage in plants exposed to a variety of stresses, including salt and osmotic stresses (Zhang *et al.* 2006, Xu *et al.* 2007). But, the antioxidative responses of chloroplast in plants to La under Cr stress are still unclear. In the present study, our results showed that La could up-regulate the activities of SOD, GPX, APX, GR, DHAR, and MDHAR and increase the ratios of AsA/DHA and GSH/GSSG in chloroplasts, which, in turn, improved the antioxidant capacity of chloroplasts. Meanwhile, our results showed that La could increase the values of F_v/F_m, q_P, q_N, and Φ_{PSII} under Cr stress, which, in turn, improved the net photosynthetic rate. Above findings indicated that La could improve the function of photosynthetic apparatus in maize seedlings by increasing the antioxidant capacity in chloroplasts under Cr stress.

The redox state of plant cell is closely related to the ratios of AsA/DHA and GSH/GSSG. The ratios of AsA/DHA and GSH/GSSG can be controlled by enzymes of a recycling pathway in plants, including APX, GR, DHAR and MDHAR. In present study, we found that Cr stress increased the activities of APX, GR, DHAR, and MDHAR in the chloroplasts of maize. However, our results showed that Cr stress decreased the ratio of AsA/DHA, which was due to the oxidative stress induced by Cr stress. It has been reported that pretreatment with $LaCl_3$ increased the activities of APX and GR in *Saussurea involucrate* Kar. et Kir. under salt stress (Xu *et al.* 2007). In present study, we also found that pretreatment with $LaCl_3$ increased the activities of APX and GR in the chloroplasts of maize, which was consistent with previous study concerning salt tolerance. Besides, we also showed that $LaCl_3$ increased the activities of DHAR and MDHAR in the chloroplasts under Cr stress. Above results of our study suggested that pretreatment with $LaCl_3$ could increase the ratios of AsA/DHA and GSH/GSSG through the recycling pathway of AsA and GSH under Cr stress.

In our previous study, we found that $LaCl_3$ could improve the cadmium tolerance of maize seedlings by enhancing the biosynthetic and recycling metabolism of ascorbate and glutathione (Dai *et al.* 2017). In this study, we also found that $LaCl_3$ could improve the Cr tolerance of maize seedlings by enhancing the recycling metabolism of ascorbate and glutathione through enzymes APX, GR, DHAR, and MDHAR in the ascorbate-glutathione cycle

at the chloroplast level. However, whether $LaCl_3$ regulates the biosynthetic metabolism of ascorbate and glutathione is still unknown. Therefore, it will be interesting to investigate the role of $LaCl_3$ in regulating the biosynthetic metabolism of ascorbate and glutathione in maize under Cr stress. Besides, we found that $LaCl_3$ could improve the biomass of maize seedlings under both Cd and Cr stresses, which indicated that $LaCl_3$ can be used a regulator in the Cd or Cr resistance cultivation of maize crops.

In conclusion, our results suggested that $LaCl_3$ upregulated the antioxidant capacity in the chloroplasts of maize seedlings by increasing the activities of antioxidant enzymes and the ratios of AsA/DHA and GSH/GSSG, which protected the function of photosynthetic apparatus against Cr stress. These results provide new knowledge for the role of La in regulating the antioxidant mechanism in chloroplast of plants in response to Cr stress.

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