BRIEF COMMUNICATION

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Cerium nitrate improves salt tolerance of wheat seedlings by regulating the antioxidant capacity of chloroplasts

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Abstract

The effects of $\text{Ce}(\text{NO}_3)_3$ on the antioxidant capacity in the chloroplasts of salt-stressed wheat (*Triticum aestivum* L.) seedlings were investigated. The results showed that salt stress (40, 80, 120, or 160 mM NaCl) significantly increased the activities of ascorbate peroxidase, glutathione reductase, superoxide dismutase, catalase, and glutathione peroxidase, the content of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), but significantly reduced the ratios of reduced ascorbate (AsA) to dehydroascorbate (DHA) and reduced glutathione to oxidized glutathione (GSH/GSSG), the content of chlorophylls (Chls) and carotenoids, net photosynthetic rate, plant height, plant dry mass and root length compared with the control. Compared with NaCl stress alone, application of $\text{NaCl} + \text{Ce}(\text{NO}_3)_3$ further enhanced the activities of above mentioned enzymes, increased the ratios of AsA/DHA and GSH/GSSG, the content of Chls and carotenoids, net photosynthetic rate, plant height, plant dry mass, and root length, and significantly reduced the content of MDA and H_2O_2 in chloroplasts. Meanwhile, $\text{Ce}(\text{NO}_3)_3$ alone also significantly enhanced the above mentioned indicators, but had no significant effect on the content of MDA and H_2O_2 compared with the control. Our results suggested that $\text{Ce}(\text{NO}_3)_3$ up-regulated the antioxidant capacity of chloroplast, which improved the wheat salt tolerance.

Additional key words: ascorbate peroxidase, carotenoids, catalase, chlorophylls, glutathione peroxidase, glutathione reductase, H_2O_2 , malondialdehyde, net photosynthetic rate, *Triticum aestivum*.

Salt stress is a major abiotic stress that seriously affects the growth and development of plants (Ryu and Cho 2015). One important feature of this stress is the induction of oxidative damage to plants by inducing the accumulation of reactive oxygen species (Guo *et al.* 2017). Chloroplast is an important cell compartment for its function in photosynthesis. However, chloroplast is highly vulnerable to oxidative stress. To protect itself against oxidative damage, chloroplast has a complex antioxidant system including antioxidant enzymes and non-enzymatic compounds. The antioxidant enzymes mainly include superoxide dismutase (SOD), catalase (CAT), ascorbate

peroxidase (APX), and glutathione reductase (GR). Non-enzymatic substances mainly include reduced ascorbate (AsA) and reduced glutathione (GSH).

Rare earth elements (REEs) have interesting effects on plant growth (Ouyang *et al.* 2003). It has been documented that cerium (Ce) is an important REE, which can protect plants against environmental stresses including excess of copper, cadmium, UV-B, and salinity (Mao *et al.* 2012, Wu *et al.* 2014, Liu *et al.* 2016, Rossi *et al.* 2016). It also plays important roles in fighting against oxidative damage induced by various stresses (Wu *et al.* 2014, Liu *et al.* 2016). Previous studies related with the role of Ce in

Submitted 6 September 2017, last revision 24 October 2018, accepted 22 November 2018.

Abbreviations: APX - ascorbate peroxidase; AsA - reduced ascorbate; Car - carotenoids; CAT - catalase; Chl - chlorophyll; DHA - dehydroascorbate; GPX - glutathione peroxidase; GR - glutathione reductase; GSH/GSSG - the ratio of reduced glutathione to oxidized glutathione; MDA - malondialdehyde; P_N - net photosynthetic rate; REES - rare earth elements; SOD - superoxide dismutase.

Acknowledgements: This study was funded by the National Natural Science Foundation of China (Grant No. 31701502), “Open project of Crop Science Characteristic Discipline of Henan Province”, “Important Science and Technology Specific Project of Henan Province (151100110700)”, and the starting fund for High-level Talents (Doctor) of Henan Institute of Science and Technology (2011010).

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fighting against oxidative damage have been focused on tissue levels. However, there is still little knowledge about its role at the chloroplast level, especially in wheat. Thus, it seems to be important to elucidate whether and how Ce regulates the antioxidant capacity in chloroplast in salt-stressed wheat. For this aim, we investigated the effects of $\text{Ce}(\text{NO}_3)_3$ on the activities of antioxidant enzymes and the content of antioxidants, MDA, and H_2O_2 , in chloroplasts as well as the content of chlorophylls (Chls) and carotenoids in the leaves, and net photosynthetic rate of salt-stressed wheat seedlings.

Seeds of wheat (*Triticum aestivum* L. cv. Bainong 207) were germinated in Petri dishes with filter paper moistened by distilled water and grown in a climate chamber under day/night temperatures of 25/15 °C, a photosynthetic active radiation of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a 10-h photoperiod. When the first leaf was fully expanded, the seedlings were transferred into plastic boxes filled with a half-strength Hoagland solution and kept their roots in darkness. The solution was exchanged every two days. When the third leaf was fully expanded, the seedlings of uniform height were selected for all the experiments. Concentrations of NaCl used were: 40, 80, 120, and 160 mM. Obvious wilting seedlings was observed only in those treated with 120 and 160 mM NaCl for 48 h, whereas there was no obvious wilting in seedlings treated with 40 and 80 mM NaCl. Therefore, we selected 80 mM NaCl as a suitable treatment concentration. The roots were placed in beakers containing 100 cm^3 of 80 mM NaCl for 48 h in a climate chamber under above mentioned conditions. To select a suitable concentration of Ce, the effects of different concentrations of $\text{Ce}(\text{NO}_3)_3$ on plant height, plant dry mass, and Chl content were measured, and then 10 μM $\text{Ce}(\text{NO}_3)_3$ was selected for the next experiments. In order to study the effect of $\text{Ce}(\text{NO}_3)_3$, plants were pre-treated with 10 μM $\text{Ce}(\text{NO}_3)_3$ for 12 h and then exposed to the salt stress or a half-strength Hoagland solution for 48 h. Control plants were treated with a half-strength Hoagland solution alone or with 10 μM $\text{Ce}(\text{NO}_3)_3$. All the solutions were prepared by dissolving corresponding chemicals in the half-strength Hoagland solution. After treatment for 48 h, the top fully expanded leaves were collected, frozen in liquid nitrogen, and then kept at -80 °C until analyses.

Intact chloroplasts were obtained from fresh wheat leaves by density-gradient centrifugation in *Percoll* gradients (Wang *et al.* 2009). Each fresh sample of leaves (50 g) was homogenized in a blender in 200 cm^3 of an ice-cold isolation buffer consisting of 330 mM mannitol, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM MgCl_2 , 2 mM sodium ascorbate, and 30 mM 3-(N-morpholino) propanesulfonic acid (Mops; pH 7.6). The homogenate was filtered through four layers of cheesecloth and centrifuged at 4 000 g for 30 s. The resulting crude chloroplast pellet was suspended in 5 cm^3 of a suspension medium (330 mM mannitol, 2 mM EDTA, and 50 mM Mops; pH 7.8). The chloroplast suspension (2 cm^3) was layered on 50 % (v/v) *Percoll* and

centrifuged at 5 000 g for 10 min. A band near the bottom containing intact chloroplasts was diluted five times with the suspension medium to remove *Percoll*. The suspension was then centrifuged at 12 000 g for 10 min. The pellet was re-suspended in the same medium and used for further assays. All operations were performed at 0 - 4 °C.

The intact chloroplast samples were diluted 10 fold with an ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1 % (m/v) 3-[(3-cholamidopropyl) dimethylammonium]-2-hydroxy-1-propanesulfonate (CHAPSO), and with the addition of 1 mM AsA in the case of APX and DHAR assays. Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium according to Giannopolitis and Ries (1977). One unit of SOD activity was defined as the amount of enzyme required to cause a 50 % inhibition of nitroblue tetrazolium 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 of GSH decreased per min. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) by monitoring a decrease in absorbance at 290 nm, and one unit of APX activity was defined as the oxidation of 1 μmol of AsA per min. Glutathione reductase (GR, EC 1.6.4.2) activity was monitored at 340 nm (Grace and Logan 1996). One unit of GR activity was defined as the reduction of 1 μmol of NADPH per min. Catalase (CAT, EC 1.11.1.6) activity was done according to Kato and Shimizu (1987). The specific activities of above mentioned enzymes were expressed as U mg^{-1} protein. Protein content was measured according to Bradford (1976) using bovine serum albumin as a standard.

For AsA and DHA assays, the intact chloroplast samples purified from 2 g of leaves were homogenized with 5 cm^3 of 5 % (m/v) ice-cold meta-phosphoric acid. The homogenates were centrifuged at 4 °C and 12 000 g for 20 min. Then, the supernatants were immediately used for assays. The AsA and DHA were measured according to Hodges *et al.* (1996), and GSSG and GSH were measured according to Griffith (1980).

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content according to the thiobarbituric acid reaction (Heath and Packer 1968). Hydrogen peroxide content was determined by measuring the absorption of titanium-hydroperoxide (Brennan and Frenkel 1977). The amount of H_2O_2 was calculated from a standard H_2O_2 curve.

The top fully expanded leaves were collected and immediately used to measure the content of Chl and Car according to Lichtenthaler and Wellburn (1983). Net photosynthetic rate (P_N) was determined by a photosynthesis measuring system (*LiCor-600*, Lincoln, USA) from 10:00 to 12:00.

All dry samples of each treatment were ground and mixed thoroughly. To analyze the content of Ce in roots and shoots, a fine powder (0.5 g) from roots and shoots

was digested in a mixture (7 cm³ of concentrated HNO₃ and 1 cm³ of concentrated HClO₄) at 170 °C by the method of Dai *et al.* (2013). Subsequently, the concentrations of Ce in extracts were determined by flame atomic absorbance spectrometry (*Hitachi 180-80*, Tokyo, Japan). A standard curve was prepared by using a series of diluted solutions of a commercially available standard.

After treatment of 7 d, plant height and root length were measured by a ruler. Fresh mass of seedlings was recorded, and then the seedlings were oven dried at 80 °C for 72 h, and dry mass determined.

Results presented are means of six replications. The means were compared by one-way analysis of variance and the Duncan multiple range test at a 5 % level of probability. To select a suitable Ce(NO₃)₃ concentration, the effects of different Ce(NO₃)₃ concentrations on plant height, dry mass, and Chl content under the salt stress were investigated. Among 5, 10, 30, and 60 µM Ce(NO₃)₃, 10 µM Ce(NO₃)₃ significantly increased all these parameters under the salt stress (Table 1). These results suggest that 10 µM Ce(NO₃)₃ was a suitable concentration for this study.

Table 1. Effects of different concentrations of Ce(NO₃)₃ on plant height, plant dry mass, Chl and Ce content in roots and shoots. The plants were treated as follows: half-strength Hoagland solution (control); 80 mM NaCl (NaCl), and 80 mM NaCl with different Ce(NO₃)₃ concentrations. The plants were pre-treated with Ce(NO₃)₃ for 12 h and then exposed to NaCl for 48 h for Ce content or 7 d for plant height, plant dry mass, and Chl content. Means ± SDs, *n* = 6, different letters indicate significant differences at *P* < 0.05.

Treatment	Plant height [cm]	Plant dry mass [mg plant ⁻¹]	Chl content [mg g ⁻¹ (f.m.)]	Ce content in roots [mg kg ⁻¹ (d.m.)]	Ce content in shoots [mg kg ⁻¹ (d.m.)]
Control	16.9 ± 1.70b	110.0 ± 10.7b	2.10 ± 0.23bc	0.5 ± 0.00h	0.2 ± 0.00f
NaCl	13.4 ± 1.25d	86.5 ± 7.15d	1.62 ± 0.14de	0.5 ± 0.00h	0.2 ± 0.00f
5 µM Ce(NO ₃) ₃	17.8 ± 1.63b	116.6 ± 10.3b	2.21 ± 0.19b	8.2 ± 0.69f	1.4 ± 0.12e
5 µM Ce(NO ₃) ₃ + NaCl	14.1 ± 1.60cd	91.0 ± 9.11cd	1.74 ± 0.22d	6.0 ± 0.52g	1.0 ± 0.11e
10 µM Ce(NO ₃) ₃	19.6 ± 1.77a	128.4 ± 11.1a	2.42 ± 0.22a	18.5 ± 1.82d	3.8 ± 0.41c
10 µM Ce(NO ₃) ₃ + NaCl	15.3 ± 1.64c	99.7 ± 8.09c	1.92 ± 0.26c	15.0 ± 1.73e	2.2 ± 0.26d
30 µM Ce(NO ₃) ₃	17.0 ± 1.53b	113.0 ± 9.60b	2.15 ± 0.18b	28.7 ± 2.22b	7.0 ± 0.60b
30 µM Ce(NO ₃) ₃ + NaCl	12.1 ± 1.37de	81.1 ± 7.13d	1.60 ± 0.17de	23.5 ± 1.93c	4.6 ± 0.41c
60 µM Ce(NO ₃) ₃	15.3 ± 1.62c	100.7 ± 9.90c	1.95 ± 0.19c	34.9 ± 3.06a	9.8 ± 0.79a
60 µM Ce(NO ₃) ₃ + NaCl	11.0 ± 1.35e	72.4 ± 6.17e	1.43 ± 0.13e	28.3 ± 2.30b	6.3 ± 0.70b

Salt stress significantly increased the activities of APX, GR, SOD, CAT, and GPX in chloroplasts compared with the control (Table 2). The Ce(NO₃)₃ pre-treatment significantly increased the activities of above enzymes under the salt stress compared with salt stress alone. After 48 h of treatment, the activities of APX, GR, SOD, CAT, and GPX increased by 53.3, 54.5, 30.7, 38.9, and 55.6 %, respectively. Meanwhile, Ce(NO₃)₃ alone also significantly increased the activities of above enzymes compared with the control. These results suggest that Ce(NO₃)₃ up-regulated the reactive oxygen species scavenging in the chloroplasts of salt-stressed wheat seedlings by increasing the activities of APX, GR, SOD, CAT, and GPX.

The salt stress significantly decreased the ratios of AsA/DHA and GSH/GSSG in chloroplasts compared with the control (Table 2). The Ce(NO₃)₃ significantly increased the ratios of AsA/DHA and GSH/GSSG under the salt stress compared to the salt stress alone. After 48 h of treatment, the ratios of AsA/DHA and GSH/GSSG increased by 21.4 and 26.7 %, respectively. Meanwhile, Ce(NO₃)₃ alone also significantly increased the ratios of AsA/DHA and GSH/GSSG compared with the control. The above results suggest that Ce(NO₃)₃ could regulate the redox state of chloroplasts.

The salt stress significantly increased the content of MDA and H₂O₂, and decreased P_N and the content of Chls and Car compared to the control (Table 2). The Ce(NO₃)₃ significantly decreased the content of MDA and H₂O₂, increased P_N and the content of Chl and Car under the salt stress compared to the salt stress alone. After 48 h of treatment, the content of MDA and H₂O₂ decreased by 37.0 and 35.7 %, respectively, and P_N and the content of Chls and Car increased by 21.7, 17.7, and 38.6 %, respectively. Meanwhile, Ce(NO₃)₃ alone also significantly decreased the content of MDA and H₂O₂ and increased P_N and the content of Chls and Car compared to the control. These results suggest that Ce(NO₃)₃ had an important role for acquisition of salt tolerance in the chloroplasts of wheat seedlings.

To further investigate whether Ce(NO₃)₃ has an important role for salt tolerance of wheat seedlings, the effects of Ce(NO₃)₃ on plant height, plant dry mass, and root length under the salt stress were studied. The results show that the salt stress significantly decreased these indicators compared to the control (Table 2). The Ce(NO₃)₃ significantly increased these indicators under the salt stress compared to the salt stress alone. Meanwhile, Ce(NO₃)₃ alone also significantly increased these indicators compared to the control. These results further

suggest that $\text{Ce}(\text{NO}_3)_3$ had an important role in improving salt tolerance of wheat seedlings.

As can be expected, $\text{Ce}(\text{NO}_3)_3$ application significantly increased Ce content in both roots and shoots compared with the control or salt stress alone (Table 1). Compared with the control, $\text{Ce}(\text{NO}_3)_3$ alone increased Ce content in roots and shoots 36.9- and 18- fold, respectively.

Compared with the salt stress alone, the salt stress plus $\text{Ce}(\text{NO}_3)_3$ also significantly increased Ce content in both roots and shoots. The salt stress plus $\text{Ce}(\text{NO}_3)_3$ increased Ce content in roots and shoots 29- and 10-fold, respectively. These results indicate that $\text{Ce}(\text{NO}_3)_3$ can increase the accumulation of Ce in wheat seedlings.

Table 2. Effects of 80 mM NaCl and 10 μM $\text{Ce}(\text{NO}_3)_3$ on the content of chlorophylls (Chls) and carotenoids (Car), net photosynthetic rate (P_N), plant height, root length, plant dry mass, the specific activities of ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX), the ratios of ascorbate to dehydroascorbate (AsA/DHA) and glutathione to oxidized glutathione (GSH/GSSG), and the content of malondialdehyde (MDA) and H_2O_2 . The plants grown in a half-strength Hoagland solution (control) were pre-treated with 10 μM $\text{Ce}(\text{NO}_3)_3$ for 12 h and then exposed to NaCl for 48 h. Means $\pm$ SDs, $n = 6$, different letters indicate significant differences at $P < 0.05$.

Parameters	Control	$\text{Ce}(\text{NO}_3)_3$	NaCl	$\text{Ce}(\text{NO}_3)_3$ + NaCl
Chls [mg g^{-1} (f.m.)]	$2.11 \pm 0.25\text{b}$	$2.35 \pm 0.28\text{a}$	$1.58 \pm 0.13\text{d}$	$1.86 \pm 0.23\text{c}$
Car [mg g^{-1} (f.m.)]	$0.74 \pm 0.08\text{b}$	$0.92 \pm 0.10\text{a}$	$0.44 \pm 0.05\text{c}$	$0.61 \pm 0.08\text{b}$
P_N [$\mu\text{mol}(\text{CO}_2) \text{m}^{-2} \text{s}^{-1}$]	$6.12 \pm 0.75\text{b}$	$7.25 \pm 0.77\text{a}$	$4.20 \pm 0.44\text{d}$	$5.11 \pm 0.48\text{c}$
Plant height [cm]	$17.10 \pm 1.20\text{b}$	$19.60 \pm 1.25\text{a}$	$14.00 \pm 1.11\text{d}$	$15.50 \pm 1.20\text{c}$
Root length [cm]	$13.30 \pm 1.24\text{b}$	$15.70 \pm 1.92\text{a}$	$10.00 \pm 1.07\text{d}$	$11.50 \pm 1.50\text{c}$
Plant dry mass [mg plant^{-1}]	$108.30 \pm 9.90\text{b}$	$122.40 \pm 11.3\text{a}$	$85.00 \pm 7.70\text{d}$	$97.40 \pm 3.90\text{c}$
APX [U mg^{-1} (protein)]	$0.88 \pm 0.07\text{c}$	$1.39 \pm 0.10\text{b}$	$1.50 \pm 0.10\text{b}$	$2.29 \pm 0.18\text{a}$
GR [U mg^{-1} (protein)]	$0.76 \pm 0.05\text{c}$	$1.00 \pm 0.08\text{b}$	$1.11 \pm 0.10\text{b}$	$1.71 \pm 0.16\text{a}$
CAT [U mg^{-1} (protein)]	$1.25 \pm 0.11\text{c}$	$1.70 \pm 0.15\text{b}$	$1.82 \pm 0.19\text{b}$	$2.52 \pm 0.29\text{a}$
SOD [U mg^{-1} (protein)]	$1.70 \pm 0.14\text{c}$	$2.25 \pm 0.18\text{b}$	$2.62 \pm 0.23\text{b}$	$3.42 \pm 0.31\text{a}$
GPX [U mg^{-1} (protein)]	$0.50 \pm 0.04\text{c}$	$0.77 \pm 0.06\text{b}$	$0.89 \pm 0.09\text{b}$	$1.38 \pm 0.13\text{a}$
AsA/DHA	$19.20 \pm 0.17\text{b}$	$21.80 \pm 0.19\text{a}$	$14.10 \pm 0.16\text{d}$	$17.10 \pm 0.16\text{c}$
GSH/GSSG	$21.60 \pm 0.19\text{b}$	$23.90 \pm 0.25\text{a}$	$15.20 \pm 0.16\text{d}$	$19.02 \pm 0.17\text{c}$
MDA [nmol g^{-1} (f.m.)]	$3.81 \pm 0.33\text{c}$	$2.93 \pm 0.23\text{d}$	$10.01 \pm 0.92\text{a}$	$6.30 \pm 0.54\text{b}$
H_2O_2 [$\mu\text{mol g}^{-1}$ (f.m.)]	$0.35 \pm 0.03\text{c}$	$0.22 \pm 0.02\text{d}$	$1.38 \pm 0.16\text{a}$	$0.89 \pm 0.10\text{b}$

Many studies have proved that salt stress could induce oxidative damage in plants (Fatehi *et al.* 2012, Sorkheh *et al.* 2012). In our study, an enhanced lipid peroxidation, as indicated by the increased content of MDA and H_2O_2 , was observed in the chloroplasts of wheat seedlings in response to the salt stress. This result suggests that the salt stress induced oxidative stress to the chloroplasts of wheat seedlings. To cope with the oxidative stress, the antioxidant capacity in the chloroplasts of wheat seedlings was up-regulated.

It is increasing evidence that Ce can relieve oxidative damage in plants exposed to a variety of stresses including salt and heavy metal stresses (Liu *et al.* 2016, Rossi *et al.* 2016). It has also been documented that Ce could protect *Brassica napus* and maize against salt stress (Qu *et al.* 2013, Rossi *et al.* 2016). Other studies also showed that Ce could increase the AsA/DHA and GSH/GSSG ratios (Hou *et al.* 2018, Zheng and Guo 2018). However, little is known about effect of Ce on antioxidative responses in chloroplasts under salt stress. Our results show that $\text{Ce}(\text{NO}_3)_3$ could increase the activities of SOD, GPX, APX, GR, CAT and the ratios of AsA/DHA and GSH/GSSG in the chloroplast of wheat seedlings, which indicates that $\text{Ce}(\text{NO}_3)_3$ could improve tolerance

mechanisms to salt by enhancing antioxidant enzymes and the regulation of redox potential in chloroplasts. Compared with the results of other studies, our findings suggest that $\text{Ce}(\text{NO}_3)_3$ had the same effects on the antioxidant capacity of plants at both tissue and cellular levels.

There is a close relationship between the content of photosynthetic pigments and the antioxidant capacity in chloroplasts because oxidative damage can lead to the degradation of photosynthetic pigments. Besides, Car are one important type of antioxidants. Thus, a high content of photosynthetic pigments mean a high antioxidant capacity. In our study, $\text{Ce}(\text{NO}_3)_3$ significantly increased the content of Chls and Car, which resulted in an increase in net photosynthetic rate. Besides, we found that the uptake of Ce by roots and shoots depended on its concentration. From the present data, we also found that Ce could be accumulated in plant tissues, especially in roots.

Some studies showed that a low concentration of Ce has positive effects on plants, but a high concentration of Ce has negative effects (Xu *et al.* 2016). In our study, the low concentration of $\text{Ce}(\text{NO}_3)_3$ could markedly increase wheat plant height and plant dry mass under the salt stress compared with the salt stress alone similarly as was reported by (Xu *et al.* 2016) in onion.

In conclusion, our results indicate that $\text{Ce}(\text{NO}_3)_3$ protected the chloroplast of wheat seedlings against oxidative stress and improved P_N and plant growth under

the salt stress. These results provide new knowledge to the antioxidant mechanism regulated by Ce in wheat chloroplasts under salt stress.

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