

## DUAL ROLE OF ABSCISIC ACID ON ANTIOXIDATIVE DEFENSE IN GRASS PEA SEEDLING (*LATHYRUS SATIVUS L.*)

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### Abstract

The effects of exogenous abscisic acid (ABA) application on the antioxidant defenses were investigated in grass pea (*Lathyrus sativus L.*) seedlings. Four treatment combinations of ABA ( $1 \times 10^{-3}$  nM ABA) and PEG (10 % polyethylene glycol, PEG 6000) were designed to evaluate their short-term (48 h) effect: (1) Well-watered group (Control group1), (2) PEG treatment, (3) ABA treatment, and (4) PEG + ABA treatment. In addition, 2 other treatments were used to evaluate the long-term (15 d) effect of ABA: well-watered group (Control group2) and ABA treatment. Time-course analyses of ABA content, the production of malondialdehyde (MDA) and  $H_2O_2$ , and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and peroxidase (POD) in water-stressed leaves showed that a significant increase in ABA content preceded that of MDA and  $H_2O_2$  in long term experiment, which was followed by a substantial increase in the activities of four antioxidant enzymes. Under the short-term drought stress, ABA application promoted the activities of antioxidant enzymes, and reduced the accumulation of MDA and  $H_2O_2$  significantly. On the contrary, long term application of exogenous ABA increase the generation of MDA and  $H_2O_2$  significantly. It could be argued that during a successive application of exogenous ABA, ABA played a dual role by which the beneficial role in the initial stage shifted to a detrimental one under prolonged treatment in up-regulating protective defense strategies in plants.

### Introduction

Drought stress is a major limiting factor to crop production worldwide. Plants can respond and adapt to drought stress by altering a series of adaptive physiological and biochemical processes, such as regulating cellular metabolism and invoking various defense mechanisms (Lange *et al.*, 1981; Sachs & Ho, 1986; Bohnert & Jensen, 1996; Shen *et al.*, 2001; Xiong *et al.*, 2005; Xiong *et al.*, 2006abcd). It has been extensively believed that the plant hormone abscisic acid (ABA) plays a major role in plants adaptation to drought stress. In the last two decades, physiological aspects of ABA signal directed specifically at crop development have been topical research foci, both in the whole-plant level (Sachs & Ho, 1986; Zhang & Davies, 1989, 1990a, 1990b, 1991; Jia & Zhang, 1997; Xiong *et al.*, 2006d) and in the cellular level (Skriver & Mundy, 1990; Pla *et al.*, 1993; Straub *et al.*, 1994; Gosti *et al.*, 1995; Shen *et al.*, 2001; Rakwal & Komatsu, 2004).

One mode of ABA action may be related to its role in the oxidative stress in plant cells. It has been extensively documented that ABA can cause an increased generation of  $H_2O_2$  (Sakamoto *et al.*, 1995; Jiang & Zhang, 2001; Murata *et al.*, 2001; Zhang *et al.*, 2003), and induce the expression of antioxidant genes (Anderson *et al.*, 1994; Guan & Scandalios, 1998; Kamminaka *et al.*, 1999). In addition, ABA also increases the activities of antioxidant

enzymes such as SOD, CAT, APX, and GR in plant tissues (Bohnert & Jensen, 1996; Murata *et al.*, 2001; Jiang & Zhang, 2002; Shao *et al.*, 2005). Antioxidative systems provide protection against the toxic effects of activated oxygen species including scavenging H<sub>2</sub>O<sub>2</sub>, one of the three reactive oxygen species (ROS) *in vivo*.

The effects of ABA, antioxidant enzymes and ROS on the antistress regulation have been extensively studied till now. It is evident that plants are frequently suffering oxidative stress. ROS played a negative role in regulating the antioxidant system (Jiang & Zhang, 2001; Mittler, 2002; Garnczarska *et al.*, 2004), and positive role in signalling events that regulate ion channel activity and gene expression (Neil *et al.*, 2002; Foreman *et al.*, 2003). On the other hand, some studies found that ABA also promotes the seed development at the early stage of seed germination and prevents germination at the later stage (Koornneef *et al.*, 1984; Neil *et al.*, 2002). Unfortunately, most of studies only separately emphasized one aspect of ABA effects and data about the interaction between ABA, antioxidant enzymes and ROS are largely missing.

Actually, the majority of existing experiments only emphasized beneficial effects of ABA on plants, such as closing of stomata (Assmann & Armstrong, 1999) and improving freezing tolerance (Rinne *et al.*, 1998). Sharp *et al.*, (2000) proposed that ABA accumulation during water stress may often function to help maintain plant growth, rather than to inhibit growth as is commonly believed (Sharp *et al.*, 2000). It is also possible that ABA acts to inhibit shoot growth in drying soil under conditions where shoot water deficits do not occur, but this remains to be demonstrated. Whether ABA acts as an inhibitor or promoter of anti-drought defense in plants under drought stress, is important and of interest. Although water stress-induced ABA accumulation was revealed as early as in the 1960s (Wright & Hiron, 1969), little information has been available concerning ABA's overall roles in antistress adaptation, especially in antioxidant regulation.

Understanding the overall effects of ABA on biochemical detoxification strategies against oxidative stress is a key to manipulate the complicated adaptive mechanism for plants in a variable and continuous adverse environment. The objective of the present study was to investigate the dual effects of ABA on mediating plant antioxidant responses. Herein, the manipulation of activities of four antioxidant enzymes (POD, SOD, CAT and GR) and the levels of MDA and H<sub>2</sub>O<sub>2</sub> in short- and long-term ABA application may help to assess the comprehensive roles of ABA in grass pea (*Lathyrus sativus* L.) seedlings.

## Materials and Methods

**Plant material and growth conditions:** Grass pea (*Lathyrus sativus* L.) seeds were surface-sterilized with 0.2 % (w / v) HgCl<sub>2</sub> for 10 min., and rinsed with sterile running water, then were soaked in water and germinated in the dark at 4°C for 3-4 days. The seeds were grown in a Hoagland nutrient solution in a growth cabinet (Conviron PGV36 controlled environments, Asheville, North Carolina, USA) at 23 / 10°C with a photoperiod of 16 / 8 h light/dark, and watered daily. Fifteen days after germination, the plants were exposed to the following treatment combinations (see below in *Trial 1* and *2*), and then leaves were sampled for all investigations. The leaf samples collected were rinsed in distilled water, and then 10-gram leaf sample was harvested for each measurement.

**Exposure to PEG and exogenous ABA supplement:** *Trial 1:* A 48-h treatment was designed to evaluate the effect of ABA on antioxidant defense in short-term drought stress.

In the following combinations in the presence and absence of exogenous ABA or PEG, the leaves of 15-d-old grass pea seedlings were sprayed (the volume of the ABA solution sprayed each time was about 2 ml) using  $1 \times 10^{-3}$  nM ABA reagent, with four times spraying in each light period (each time at 4-h interval). For PEG treatment, the seedlings of grass pea were incubated in 10% PEG solution (PEG 6000, -0.7 MPa). Four experimental combinations were as follows: (1) well-watered group (Control group), (2) PEG treatment, (3) PEG + ABA treatment ( $1 \times 10^{-3}$  nM, the same below), and (4) ABA treatment. In PEG + ABA treatment, the pretreatment of PEG solution was performed one hour earlier than exogenous ABA application. All four groups were simultaneously grown. At the 0, 3, 12, 24, 36 and 48 hour after treatment initiation respectively, seedling leaves were collected in 5 replicates. All the above solutions were renewed every day (the same below).

*Trial 2:* A 15-d treatment was designed to assess the effect of ABA on antioxidant defense in long-term drought stress. The method of ABA treatment was the same to those in *Trial 1*, but the plants were grown for 15 days. Two treatment groups were as follows: (1) well-watered treatment, and (2) ABA treatment. The leaf samples were collected on the 0, 1, 3, 7, 10, and 15 day after the initiation of treatment. In the above two trials, the contents of ABA, MDA and H<sub>2</sub>O<sub>2</sub> were measured and the activities of CAT, POD, SOD and GR were monitored simultaneously.

**Determination of lipid peroxidation:** Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 1 g leaf fresh weight according to Madhava Rao & Sresty (2000). MDA is a product of lipid peroxidation by thiobarbituric acid reaction. The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>.

**Enzyme extraction and assay procedure:** A leaf sample (0.5 g each) was homogenized in a Waring Blender at 4°C. The grinding medium contained 0.05 M Potassium phosphate buffer (pH 7.8), 20 mmol / L  $\beta$ -mercaptoethanol, 1 mmol/L EDTA, and 0.1 mmol / L phenylmethanesulfonyl fluoride (PMSF) (Baker *et al.*, 1996). Polyvinylpyrrolidone (PVP) (0.2 mg / L) was added to the samples to scavenge leaf phenolics. Homogenates were centrifuged at 1700 g for 15 min at 4°C. The supernatant fractions were carried out at 0-4°C. All activities were determined at 25°C. We preferred to express all enzyme activities on a protein basis. Protein concentrations were measured according to the method of Bradford (1976), with BSA as a standard.

Superoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), using the method of Bearuchamp & Fridovich (1971). The 3 ml reaction mixture contained  $2.4 \times 10^{-6}$  mol / L riboflavin and 0.013 mol / L-methionine phosphate at pH 7.8. One unit of enzyme activity was the amount of enzyme bringing about 50% inhibition of the photochemical reduction of NBT (Dhindsa & Matowe, 1981).

Catalase activity was determined by measuring the decreasing rate in the absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm (Aebi, 1984). One unit was defined as the amount of enzyme catalyzing the decomposition of 1  $\mu$ mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> per minute calculated from the extinction coefficient for H<sub>2</sub>O<sub>2</sub> at 240 nm of 0.036 cm<sup>2</sup>  $\mu$ mol L<sup>-1</sup> (Luck, 1963).

Peroxidase activity was determined by using guaiacol as the substrate, with the method of Volk & Feierabend (1989). The molar extinction coefficient of tetraguaiacol ( $26.6 \text{ mM}^{-1}\text{cm}^{-1}$ ) was employed in the calculation of the enzyme concentration.

Glutathione reductase activity was determined in 0.5 mL of reaction mixture that contained  $0.1 \text{ mmol L}^{-1}$  Hepes buffer (pH 8.0),  $1.0 \text{ mmol L}^{-1}$  EDTA, and 50-100  $\mu\text{L}$  of enzyme extract. The reaction was initiated by the addition of  $1.0 \text{ mmol L}^{-1}$  oxidized glutathione (GSSG), and the rate of NADPH oxidation was monitored at 340 nm (Burke *et al.*, 1985). Glutathione reductase activity was expressed in nanomol / nmol of NADPH oxidized per minute per milligram of protein.

**Measurement of hydrogen peroxide:**  $\text{H}_2\text{O}_2$  was extracted and estimated following the method of MacNevin & Uron (1953) with slight modifications (Mondal & Choudhuri, 1981). Isolation was carried out with 5 g of leaf tissue in ice-cold acetone by the addition of 5% (w / v) titanyl sulfate and concentrated  $\text{NH}_4\text{OH}$  solution; the absorbance was taken at 415 nm against water blank. The  $\text{H}_2\text{O}_2$  content was calculated from a standard curve prepared in a similar way (Mukherjee & Choudhuri, 1983).

**Determination of ABA content:** Precisely weighed 500 mg sample was added to the 3 ml 80 % precooling methanol solution of  $4^\circ\text{C}$ . In the weak light, the mixture was homogenized under the ice-bath condition. Then, the thick liquid was transferred to the centrifuge tube for the centrifuge program, performed with 5000 turns per minute at  $4^\circ\text{C}$  for 10 min. Thereafter, the upper turbid liquid was separated carefully and 300  $\mu\text{l}$  were sampled. Under vacuum, methanol in it was cleared away. Thereafter, it was extracted for three times by 200  $\mu\text{l}$  ethylacetate and combined with ethylacetate phase (meanwhile, water phase would be abandoned). Once more, decompress treatment was used to get rid of ethylacetate from it. Furthermore, the obtained sample was dissolved with 300  $\mu\text{l}$  methanol, in the process of which the sample could be methesterified. The free-state ABA could be detected and its content could be recorded in the ABA ELISA (Enzyme Linked Immunosorbent Assay) box (purchased from Nanjing Agriculture University of China).

**Statistical analysis:** The results presented here were the mean of five replicates. Means in ABA content in seedling leaves of grass pea and all physiological parameters (four antioxidant enzymes, MDA and  $\text{H}_2\text{O}_2$ ) in either short-term (48 h) or long-term (15 d) treatment of exogenous ABA were compared by one-way analysis of variance and Student's unpaired *t*-tests at the 5% level of significance. Significant differences in all physiological parameters (four antioxidant enzymes, MDA and  $\text{H}_2\text{O}_2$ ) in short-term (48 h) treatment of exogenous ABA were discriminated using Student's unpaired *t*-test between control group and ABA treatment, and between PEG treatment and (PEG + ABA) treatment, respectively. The objectives of the two above analyses were to evaluate the differential role of exogenous ABA application in short- and long-term treatment respectively. Regression and correlation analyses were used to describe the relationships between ABA content, the activities of antioxidant enzymes and the generations of MDA and  $\text{H}_2\text{O}_2$ .

## Results

**The content of ABA in leaves under exogenous ABA application or PEG treatment:** Under the well-watered condition, the normal endogenous contents of ABA, MDA and  $\text{H}_2\text{O}_2$  in grass pea leaves were kept at the level of about  $1.88 \text{ nmol g}^{-1}$  DW,  $76.26 \text{ nmol g}^{-1}$  DW and

31.8 3  $\mu\text{mol g}^{-1}$  DW, and the normal endogenous activities of four antioxidant enzymes (SOD, CAT, POD and GR) can be seen in Table 1. The effects of drought stress on ABA content, the activities of antioxidant enzymes, and the production of MDA and  $\text{H}_2\text{O}_2$  are shown in Figures 1-3. Under the short-term (48 h) drought stress induced by PEG only, ABA content in the leaves increased rapidly during the initial 3 hours. At the third hour, it was 2.1-fold that of the control group. Soon afterwards, the increase rate started to slow down, reaching the highest level (4.46  $\text{nmol g}^{-1}$  DW, 2.12-fold that of the control group) at the 12<sup>th</sup> hour. With the lengthening of treatment time, ABA content started to decrease gradually (Fig. 1).

**Tables 1.** Normal endogenous level of relevant physiological parameters in seedling leaves of well-watered grass pea plant.

| Physiological Parameters                                    | Endogenous level<br>(mean $\pm$ SD) |
|---|-------------------------------------|
| CAT activity ( $\text{U mg}^{-1}$ protein)                  | 162.5 $\pm$ 13.6                    |
| SOD activity ( $\text{U mg}^{-1}$ protein)                  | 97.42 $\pm$ 8.43                    |
| POD activity ( $\text{U mg}^{-1}$ protein)                  | 175.17 $\pm$ 14.23                  |
| GR activity ( $\text{nmol GSSG mg}^{-1}$ protein)           | 171.33 $\pm$ 15.47                  |
| ABA content ( $\text{nmol g}^{-1}$ DW)                      | 1.88 $\pm$ 0.15                     |
| MDA content ( $\text{nmol g}^{-1}$ DW)                      | 76.26 $\pm$ 5.2                     |
| $\text{H}_2\text{O}_2$ content ( $\mu\text{mol g}^{-1}$ DW) | 31.83 $\pm$ 3.07                    |

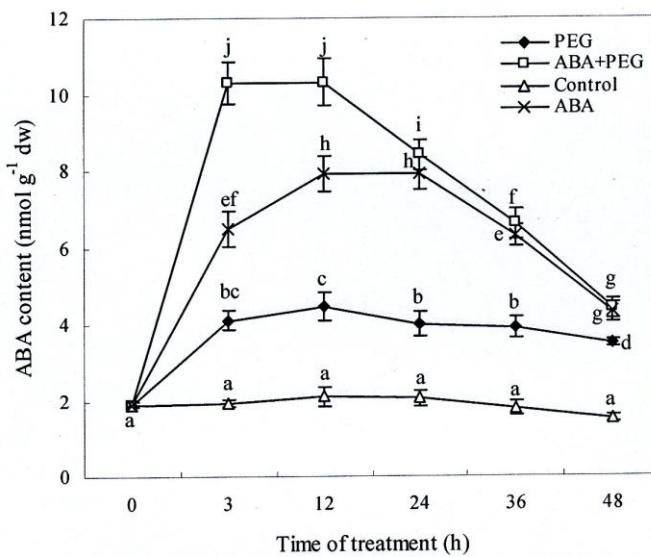


Fig. 1. Time-course of changes in ABA content in the leaves of grass pea seedlings under short-term (48 h) water stress initiated by PEG and exposed to the exogenous ABA solution.

Four treatments are designed as follows: (1) the well-watered (Control), (2) PEG treatment (exposed to 10 % PEG solution), (3) ABA treatment (exposed to  $1 \times 10^{-3}$  nM exogenous ABA) and (4) PEG + ABA treatment, respectively. Values are means  $\pm$  S.E. ( $n = 5$ ). Means denoted by the same letter did not significantly differ at  $p < 0.05$ .

Under the short-term treatment with exogenous ABA application only, the ABA content in the leaves rapidly increased during the initial 3 hours and then reached its maximum value (about 7.90 nmol g<sup>-1</sup> DW) during the 12<sup>th</sup> and 24<sup>th</sup> hour, 3.5 times that of the control group. After that, it slightly decreased from the 24<sup>th</sup> to 48<sup>th</sup> hour, but still remained at a relative high level (4.3 nmol g<sup>-1</sup> DW), significantly higher than that in the group of PEG treatment (Fig. 1).

Under the short-term treatment with both PEG and exogenous ABA, the ABA content in the leaves significantly increased by 5.3, 4.9, 4.1, 3.7 and 2.9-fold that of the control group at the 3<sup>rd</sup>, 12<sup>th</sup>, 24<sup>th</sup>, 36<sup>th</sup> and 48<sup>th</sup> hour respectively (Fig. 1). Each value of ABA content in leaves in this group was significantly higher than that of any other group at all the treatment times except for 48<sup>th</sup> hour. At the 48<sup>th</sup> hour, the ABA content in leaves in this group was close to that in ABA treatment group (without significant difference), but is still significantly higher than the other two groups (the control and PEG treatment). Therefore, the ranking of foliar ABA content was as follows: (PEG + ABA) group > ABA group > PEG group > control group. The common tendency in various treatment groups except for the control group was that ABA content rose rapidly at the beginning stage of treatment, then reached its peak values and finally fell down at the ending stage of treatment (Fig. 1).

**The positive effect of ABA on antioxidative defense:** In the well-watered group, the average activities of CAT, SOD and POD in the leaves remained about 162, 97 and 175 U mg<sup>-1</sup> protein, and the average activity of GR remained about 171 nmol GSSG mg<sup>-1</sup> protein, respectively (Table 1). In order to determine whether the increases in the production of MDA and H<sub>2</sub>O<sub>2</sub>, and the activities of antioxidant enzymes were related to the accumulation of ABA in the leaves of grass pea leaves exposed to drought water, the supplement experiments with PEG or exogenous ABA were performed. The comparative analysis on varying treatment combinations was used to test whether the negative effect of drought stress induced by PEG could be overcome by exogenously supplied ABA.

Experimental results showed that PEG pretreatment significantly inhibited the activities of four antioxidant enzymes, and significantly increased the generations of MDA and H<sub>2</sub>O<sub>2</sub> in leaves (Figs. 2-3). The activities of SOD, POD and CAT significantly decreased, and the activity of GR increased significantly within 24 h of PEG treatment. Since the 24<sup>th</sup> hour, they were maintained at the stable level (Fig. 2). On the other hand, the pretreatment of PEG led to a continuous increase in generations of MDA and H<sub>2</sub>O<sub>2</sub> within the 48 h of drought stress (Fig. 3), suggesting that drought stress induced by PEG caused the injury of membrane lipid peroxidation.

Based on statistical analysis between PEG treatment and PEG+ABA treatment, the positive effects of exogenous ABA application on the above physiological parameters were observed in the seedling leaves of grass pea. As exogenous ABA was added to the plants exposed to PEG, the increase in ABA content in the leaves was to mainly alleviate the inhibition of drought stress on CAT, also partly for other three antioxidant enzymes, and reduced the production of MDA and H<sub>2</sub>O<sub>2</sub> significantly. Compared with those in PEG treatment, the activity of CAT increased by 6.8, 11.6, 15.2, 15.6, 15.4%, the activity of GR increased by 3.29, 9.7, 17.4, 16.2, and 16.4%, the activity of SOD increased by 3.89, 8.2, 10.4, 6.25 and 8.3%, and the activity of POD increased by 8.3, 15.2, 14.58, 20 and 17.2% at the 3<sup>rd</sup>, 12<sup>th</sup>, 24<sup>th</sup>, 36<sup>th</sup>, and 48<sup>th</sup> hour after treatment respectively in PEG + ABA treatment (Fig. 2). At the same time, the generations of H<sub>2</sub>O<sub>2</sub> declined by 20, 21.9, 24.2, 26.5 and 26.6%; the generations of MDA declined by 6.57, 14.47, 21.95, 20.5 and 19.27% at the 3<sup>rd</sup>, 12<sup>th</sup>, 24<sup>th</sup>, 36<sup>th</sup> and 48<sup>th</sup> hour after treatment respectively in PEG + ABA treatment (Fig. 3).

The same results could be concluded through the comparison between the control group and

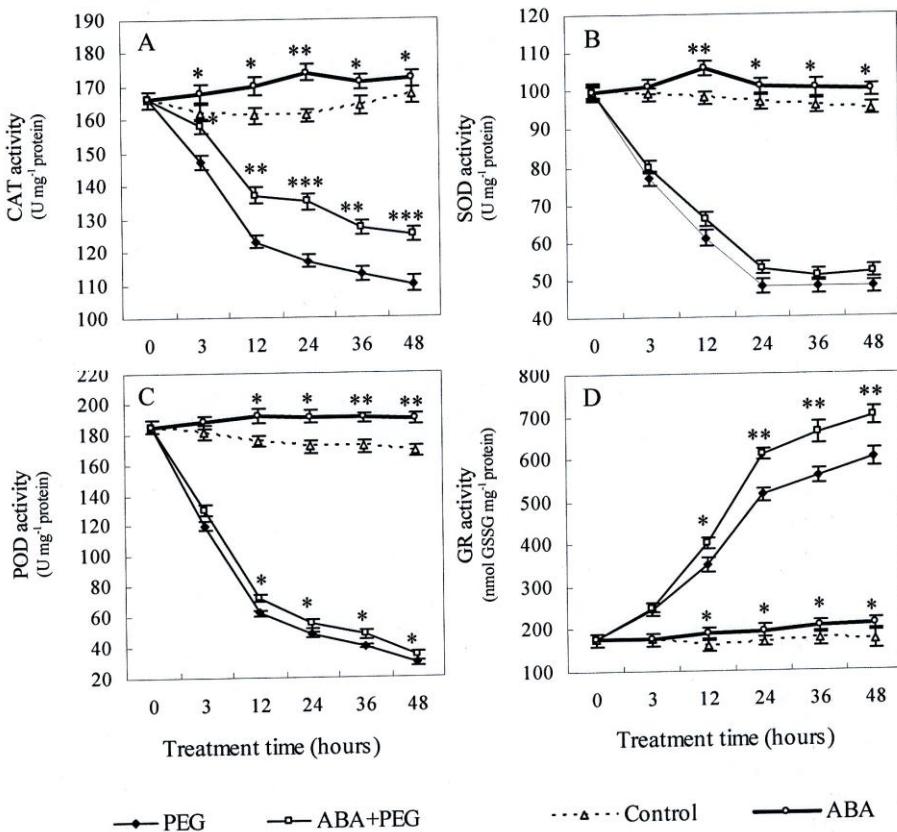


Fig. 2. The effects of short-term pretreatment with exogenous ABA on the activities of CAT (A), SOD (B), POD (C) and GR (D) in the leaves of grass pea seedlings exposed to the exogenous ABA solution.

Four treatments are designed as follows: (1) the well-watered (Control), (2) PEG treatment (exposed to 10 % PEG solution), (3) ABA treatment (exposed to  $1 \times 10^{-3}$  nM exogenous ABA) and (4) PEG + ABA treatment, respectively. The analyses of significant variance were performed between control group and ABA treatment, and between PEG treatment and PEG + ABA treatment, respectively. Comparison was made on the same treatment time. \*, \*\*, and \*\*\* indicated significant difference at  $P < 0.05$ , 0.01 and 0.001 respectively. Values are means  $\pm$  S.E. ( $n = 5$ ).

ABA treatment group. Under ABA treatment alone, exogenous ABA application generally increased the activities of four antioxidant enzymes and reduced the accumulation of MDA and  $H_2O_2$  significantly in the seedling leaves of grass pea (Fig. 3). During the whole 48-h treatment time, the extent of membrane lipid peroxidation in leaves tended to increase, judged by the MDA concentration as a typical criterion. The MDA concentration appeared to be positively correlated with the generation of  $H_2O_2$  in the seedling leaves of grass pea ( $r = 0.82**$   $P < 0.01$ ) (Fig. 3). Therefore, exogenous ABA application was able to reduce the

reaction of membrane lipid peroxidation caused by the accumulation of active oxygen species, suggesting that ABA played a positive role for plant to improve its antioxidant defense mechanism in plants.

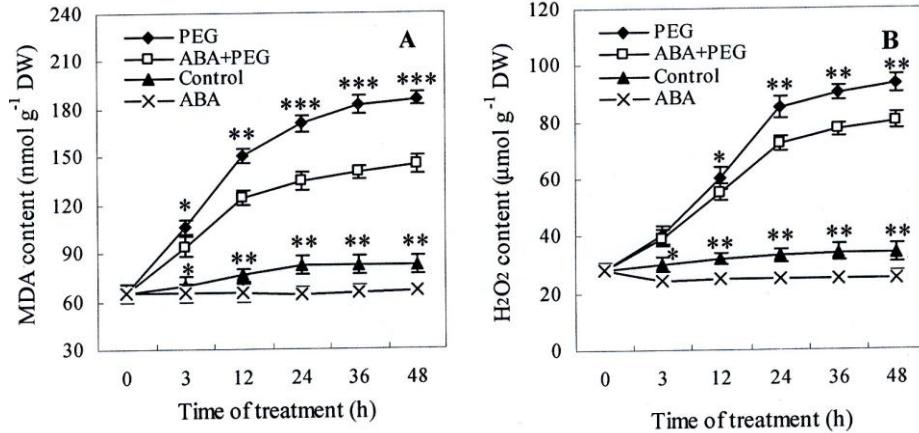


Fig. 3. The effects of short-term pretreatment with exogenous ABA on the production of MDA (A) and H<sub>2</sub>O<sub>2</sub> (B) in the leaves of grass pea seedlings exposed to the exogenous ABA solution.

Four treatments are designed as follows: (1) the well-watered (Control), (2) PEG treatment (exposed to 10 % PEG solution), (3) ABA treatment (exposed to  $1 \times 10^{-3}$  nM exogenous ABA) and (4) PEG + ABA treatment, respectively. The analyses of significant variance were performed between control group and ABA treatment, and between PEG treatment and PEG + ABA treatment, respectively. Comparison was made on the same treatment time. \*, \*\*, and \*\*\* indicated significant difference at  $P < 0.05$ , 0.01 and 0.001 respectively. Values are means  $\pm$  S.E. ( $n = 5$ ).

**The negative effect of ABA on antioxidative defense:** In order to determine the effect of long-term (15 d) exogenous ABA application on the activities of antioxidant enzymes and the accumulation of MDA and H<sub>2</sub>O<sub>2</sub>, the plants of grass pea were treated with  $1 \times 10^{-3}$  nM exogenous ABA for 15 days. The treatment of exogenous ABA led to the steep increase of the ABA content in leaves, 3.2-fold against the control value on the first day after treatment (Fig. 4). This continuous increase was kept on for 3 days, and on the third day, there was another relatively slow increase until the seventh day. Afterwards, ABA content stayed at the highest level till the fifteenth day. The ABA contents in the leaves were 4.4, 4.5 and 4.6-fold that of the control group at the 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> day, respectively (Fig. 4).

Time-course of the activities in four antioxidant enzymes and the generations of MDA and H<sub>2</sub>O<sub>2</sub> under long-term exogenous ABA application could be seen from Figs. 5 and 6. From the 1<sup>st</sup> to the 3<sup>rd</sup> day after treatment, the activities of CAT, GR, POD and SOD started to rise gradually, 1.08 and 1.12-fold (for CAT), 1.15 and 1.23-fold (for GR), 1.1 and 1.11-fold (for POD), and 1.06 and 0.95-fold (for SOD) the control values on the 1<sup>st</sup> and 3<sup>rd</sup> day, respectively. Generally, within 3 days after treatment, the activities of four antioxidant enzymes had a significant increase, except the activity of SOD started to decrease on the 3<sup>rd</sup> day after treatment (Fig. 5). Under the short-term treatment, ABA application improved the activities of all the four antioxidant enzymes.

From the 7<sup>th</sup> day onwards, however, the activities of CAT, SOD, POD and GR were found to decline significantly. On the 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> day, the activity of CAT was 0.89, 0.81 and 0.73-fold, the activity of SOD was 0.85, 0.71 and 0.57-fold, the activity of POD

was 0.93, 0.75 and 0.61-fold, the activity GR was 0.96, 0.87 and 0.76-fold the control values, respectively (Fig. 5). With the lengthening of treatment time, ABA application inhibited the activities of four antioxidant enzymes.

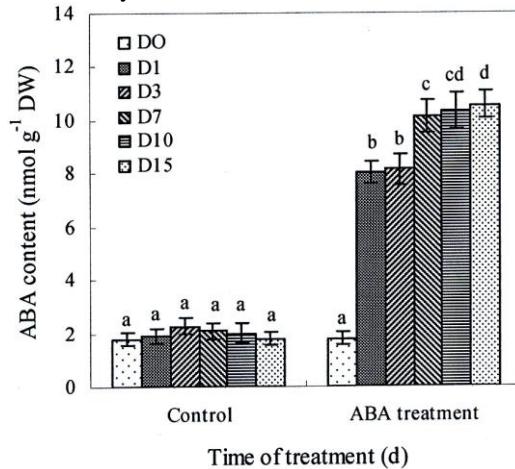


Fig. 4. Time-course of changes in ABA content in the leaves of grass pea seedlings exposed to the exogenous ABA solution. D0, D1, D3, D7, D10 and D15 represent the 0<sup>th</sup>, 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> day after treatments respectively (same to the below). Two treatments are designed to be the well-watered (Control) and ABA treatment (exposed to  $1 \times 10^{-3}$  nM exogenous ABA), respectively. Values are means  $\pm$  S.E. ( $n = 5$ ). Means denoted by the same letter did not significantly differ at  $P < 0.05$ .

The membrane lipid peroxidation appeared to be positively correlated with the contents of H<sub>2</sub>O<sub>2</sub> in the foliar cells of grass pea seedlings (Fig. 6). On the 1<sup>st</sup> and 3<sup>rd</sup> day after exogenous ABA application, the generations of MDA and H<sub>2</sub>O<sub>2</sub> tended to decrease. The production of MDA was 0.83, 0.81-fold the control values, and the generations of H<sub>2</sub>O<sub>2</sub> was 0.87, 0.84-fold the control values, respectively. But from the 7<sup>th</sup> day on, there were continuous increase in the accumulation of MDA and H<sub>2</sub>O<sub>2</sub>. On the 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> day, the concentrations of MDA were 1.2, 1.75 and 2.2-fold, and the generations of H<sub>2</sub>O<sub>2</sub> content were 1.2, 1.6 and 2.1-fold the control values respectively (Fig. 6). Therefore, short-term ABA application exhibited a beneficial role but long-term ABA application had a detrimental effect on the protective regulation mechanism of grass pea seedlings.

## Discussions

Although the scientific origins of abscisic acid (ABA) had been investigated early in the late 1940s, it was isolated and identified in the 1960s (Trouverie *et al.*, 2003). There is a strong evidence that the plant hormone abscisic acid (ABA) is a product of various environmental stresses, and considered to be an adaptation in response to these stresses (Zeevaart & Creelman, 1988). Additionally, ABA was to initiate a series of relevant reactions for plants in response to adversity stresses. Previously, a large number of efforts were made to emphasize its positive role in water stress-induced antioxidant defense against oxidative stress (Guan *et al.*, 2000; Jiang & Zhang, 2002). Jiang & Zhang (2002) found that pretreatment with an ABA biosynthesis inhibitor, tungstate, significantly suppressed the accumulation of ABA induced by water stress, reduced the increased generation of ROS, and

resulted in the up-regulation of antioxidant enzymes in the water-stressed *Zea mays* seedling leaves. These effects were completely prevented by the addition of ABA, which raised the internal ABA content (Guan *et al.*, 2000). However, the interrelationship between ABA, ROS and antioxidant defenses in drought stress signal transduction cascades is still not clear. The effects have been examined systematically of drought stress on ABA content, the generation of  $H_2O_2$ , and the activities of four antioxidant enzymes including SOD, CAT, POD and GR in grass pea leaves and the interrelationship among them under a PEG-induced drought stress. Our research indicated that the significant increase in ABA content preceded the significant increase of  $H_2O_2$  and MDA generation, and a marked decrease in the activities of CAT, SOD and POD and a marked increase in the activity of GR followed by the increase of  $H_2O_2$  and MDA in grass pea leaves under PEG-induced drought stress (Figs. 1-3).

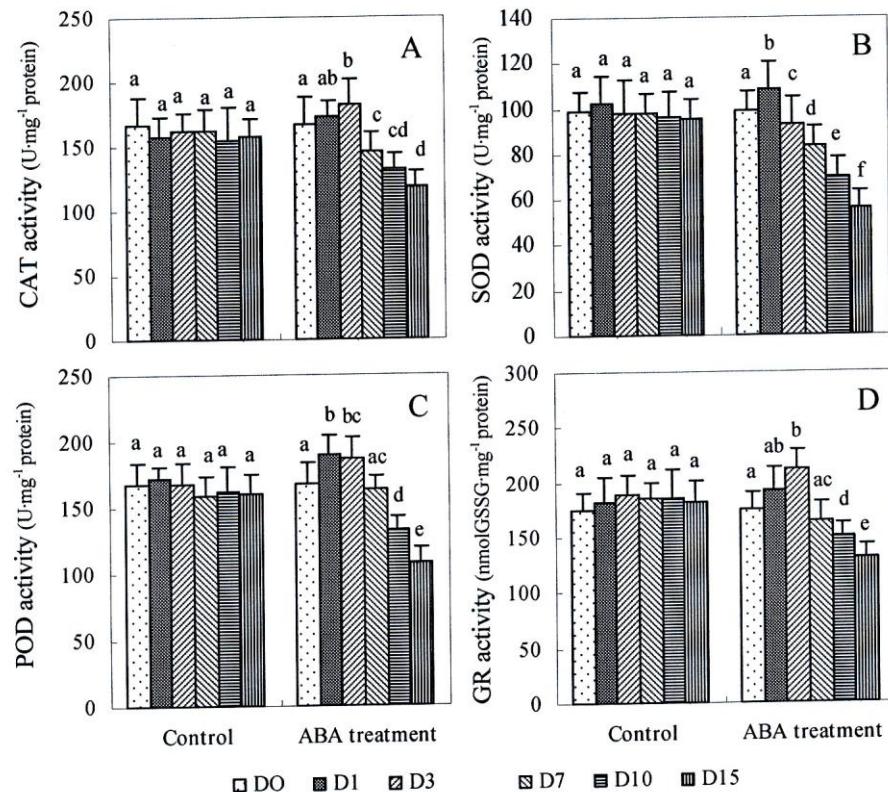


Fig. 5. The effects of long-term pretreatment with exogenous ABA on the activities of CAT (A), SOD (B), POD (C) and GR (D) in the leaves of grass pea seedlings exposed to the exogenous ABA solution. D0, D1, D3, D7, D10 and D15 represent the 0<sup>th</sup>, 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> day after treatments respectively. Two treatments are designed to be the well-watered (Control) and ABA treatment (exposed to  $1 \times 10^{-3}$  nM exogenous ABA), respectively. Values are means  $\pm$  S.E. ( $n = 5$ ). Means denoted by the same letter did not significantly differ at  $P < 0.05$ .

The increase in generations of  $H_2O_2$  and MDA was accompanied by the decrease of the activities in SOD, POD and CAT in drought-stressed leaves. This decrease was fully

prevented by the addition of exogenous ABA, which raised the internal ABA content (Figs. 1-2). Although an increase of the activity in GR followed the increase of the accumulation in  $H_2O_2$  and MDA, the GR activity had a further increase after the addition of ABA in drought-stressed leaves. PEG-induced drought stress resulted in a significant up-regulation in the activities of antioxidant enzymes, which was suppressed by the increase in internal ABA content. This result can be supported by the relevant data in ABA treatment. In this study, the leaf-spraying treatment of exogenous ABA in leaves was to increase the ABA concentration in the leaves. Under non-stressed conditions, the generation of H and MDA were found to decrease massively and the activities of four antioxidant enzymes were to significantly increase with respect to the control values (Figs. 2-3).

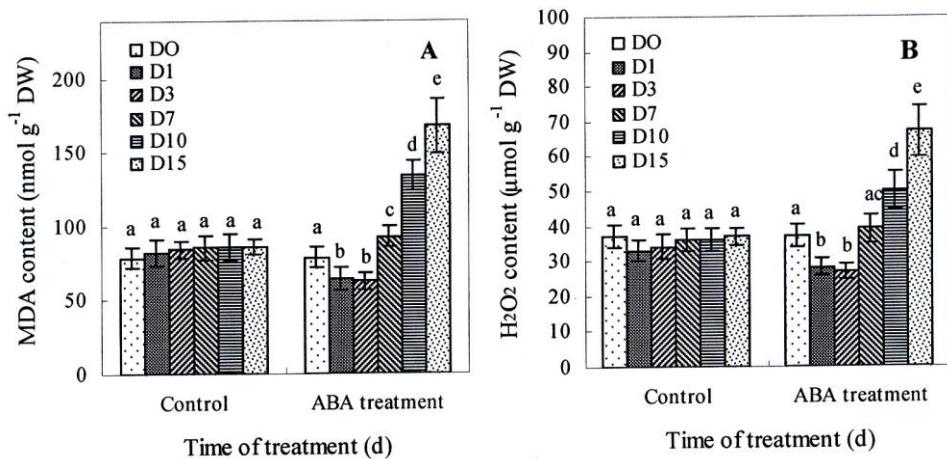


Fig. 6. The effects of long-term pretreatment with exogenous ABA on the production of MDA (A) and  $H_2O_2$  (B) in the leaves of grass pea seedlings exposed to the exogenous ABA solution. D0, D1, D3, D7, D10 and D15 represent the 0<sup>th</sup>, 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup>, 10<sup>th</sup> and 15<sup>th</sup> day after treatments respectively. Two treatments are designed to be the well-watered (Control) and ABA treatment (exposed to  $1 \times 10^{-3}$  nM exogenous ABA), respectively. Values are means  $\pm$  S.E. ( $n = 5$ ). Means denoted by the same letter did not significantly differ at  $P < 0.05$ .

Plant cell membranes are often the initial site of drought damage, and membrane leakage has often been used as a measure of plant damage (Wang, 1981; Rajasekaran & Blake, 1999; Borsos-Matovina & Blake, 2001; Cho & Seo, 2005), and that is often indicated by the increased MDA. The thiobarbituric acid reagent substances (TBARS) were thought to be the most popular index to measure the level of membrane lipid peroxidation. More specifically, most understanding concerning it was that MDA content (TBARS was largely focused on it), which would be the major index to judge the extent of lipid peroxidation (Gossett *et al.*, 1994; Cho & Seo, 2005). It has long been distinct that the *in vivo* free radical in plants was to accumulate and trigger a series of physiological or biochemical reactions including membrane lipid peroxidation. In this case, plant injuries would occur eventually. Herein, the antioxidant system was thought to play a vital role when plants were injured under water stress.  $H_2O_2$ , one of free radicals, causes lipid peroxidation and the resulting increase in the permeability of cell membranes can induce senescence (Droillard *et al.*, 1987; Jiang & Zhang, 2002; Cho & Seo, 2005). Recent researches suggested that ABA was likely

to inhibit the expression of some genes (Shriver & Mundy, 1990; Creelman & Mason, 1990; Colorado *et al.*, 1994). For example, the expression of some drought-induced genes depended on the increase of ABA level and the expression of late embryogenesis abundant-protein (LEA) gene was closely related to the elevated ABA concentration. LEA was considered to act as a dehydration protectant. The extent of membrane injuries can be alleviated by the increase in LEA synthesis induced by ABA. It was, by regulating *In vivo* active oxygen metabolism especially by regulating the change of H<sub>2</sub>O<sub>2</sub> generation, for ABA to inhibit the membrane lipid peroxidation and then to release the injury caused by water stress. Therefore, the short-term supplement of exogenous ABA in PEG-treated grass pea seedlings increased the capacity of protective adaptation mechanism, suggesting that ABA played a contributory and active role in improving the grass pea' adaptation to drought stress.

It has long been known that CAT mostly existed in the peroxisome. Its major function was to clear away all H<sub>2</sub>O<sub>2</sub> produced in the cytoplasm. Moreover, the H<sub>2</sub>O<sub>2</sub> in the chloroplast could be mainly eliminated in the Foyer-Halliwell Cycle, in which GR and APX were considered to be two critical enzymes (Asada, 1992). In the exogenous ABA treatment, the increase in activities of CAT and GR was to eliminate the H<sub>2</sub>O<sub>2</sub> in the cytoplasm as well as the chloroplast. Therefore, the amount of H<sub>2</sub>O<sub>2</sub> would drop down and then the membrane lipid peroxidation would be restrained. In this case, the plant injury caused by water stress was effectively to be released. So, it could be assumed that ABA was beneficial to the crop' adaptation to the short-term (48h) drought stress.

If the grass pea seedlings were treated with the exogenous ABA for a long time leaf-spraying, the results showed that at the front stage of treatment (0 ~ 3 days) the ABA content in the blade rose rapidly and significantly, and the activities of CAT and GR were to increase slightly (Figs. 4-5), whereas the amount of MDA and H<sub>2</sub>O<sub>2</sub> somewhat decreased (Fig. 6). It suggested that after grass pea leaves absorbed exogenous ABA, ABA would promote the activities of the antioxidant enzymes to reduce the amount of active oxygen (H<sub>2</sub>O<sub>2</sub>). That led to lower the extent of membrane lipid peroxidation and thus, in turn, lessen the plant injury caused by the outer stressful surroundings. On the other hand, in later period of treatment (3 ~ 15 days), the foliar ABA level was still maintained relatively high, and correspondingly the activities of CAT, SOD, POD and GR were to drop down wholly (Figs. 4-5). More importantly, at that moment, MDA and H<sub>2</sub>O<sub>2</sub> started to accumulate quickly and significantly (Fig. 6), showing that the peroxidative reaction in membrane lipid had become stronger and the plant injury would be aggravated more seriously. Herein, the long-term ABA application could lead to the plant senesce. It could also be concluded that ABA turned to its adverse aspects of protecting plant against suffering from *In vitro* stresses *i.e.*, long-term water stress still stimulated the synthesis of ABA but ABA in this condition was harmful to plant adaptation to drought stress.

In conclusion, at the initial stage of drought stress (short-term stress), plants were subjected to mild water stress. Under mild water stress, water-stress-induced ABA accumulation triggers the increased generation of reactive oxygen species (ROS), which may involve a trans-plasma membrane NAD(P)H oxidase (Jiang & Zhang, 2002) and, in turn, leads to the induction of the antioxidant defense system in plants (Guan *et al.*, 2000). Our data suggested that at the initial stage of stress, the content of H<sub>2</sub>O<sub>2</sub> was highly correlated with the activities of all four antioxidant enzymes (SOD, CAT, POD and GR). Therefore, ABA's positive role was performed in the pathway in which H<sub>2</sub>O<sub>2</sub> accumulation triggered the increased activities of four antioxidant enzymes. In the prolonged drought conditions, plants were subjected to severe water stress. Under severe water stress, internal ABA in

leaves would accumulate excessively. In this case, the content of H<sub>2</sub>O<sub>2</sub> was negatively correlated with the activities of all four antioxidant enzymes. So, the amount of H<sub>2</sub>O<sub>2</sub> surpassed a certain threshold, significantly higher than that of under mild stress. So, excessive accumulation of H<sub>2</sub>O<sub>2</sub> started to inhibit the activities of four antioxidant enzymes and in turn, triggered the negative effect of ABA.

ABA had its own dual characteristics *i.e.*, it was beneficial in adaptation to the short-term draught stress and it turned to be harmful in long-term drought stress. Considering the complexity of ABA and H<sub>2</sub>O<sub>2</sub> action, although another possibility in the induction of the antioxidant defense system in response to drought stress cannot be ruled out, our results had confirmed the ABA's dual roles in a possible pathway in plant response to short-term and long-term water stress.

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