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Changes in Hydrogen Peroxide Content and Antioxidant Enzymes in Abscisic Acid-induced Antioxidant Defense in Leaves of Bean Seedlings

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Abstract: The plant hormone abscisic acid (ABA), as a stress signal, plays important roles in the regulation of plant responses to environmental stresses. The relationship among abscisic acid (ABA) accumulation, the generation of Reactive Oxygen Species (ROS) and the activities of several antioxidant enzymes such as Catalase (CAT), Ascorbate Peroxidase (APX) and Glutathione Reductase (GR) was investigated in leaves of detached bean (*Phaseolus vulgaris* L.) plants exposed to 10,100 and 1000 μM ABA [Detached leaves of bean were cut into leaf segments and floated in solutions containing 0, 10, 100 and 1,000 μM ABA, at 25°C and then used for assay of antioxidant enzymes]. Treatment with 10 and 100 μM ABA significantly increased the levels of O_2^- and H_2O_2, followed by an increase in activities of CAT, APX and GR in leaves of bean seedlings. Treatment with 1,000 μM ABA led to a more abundant generation of $O_2^-$and H_2O_2 .The activities of these antioxidative enzymes were reduced when compared with the treatment of 100 μM ABA. These results indicate that treatment with low concentrations of ABA (10 to 100 μM) induced an antioxidative defense response against oxidative damage, but a high concentration of ABA (1,000 μM) induced an excessive generation of ROS and led to an oxidative damage in plant cells.

Key words: Abscisic acid, reactive oxygen species, antioxidant defense system, oxidative damage, oxidative stress, *Phaseolus vulgaris*

INTRODUCTION

The phytohormone abscisic acid (ABA) regulates many important aspects of plant growth and development and its main function is to regulate plant adaptive responses to various adverse environmental conditions (Saleh, 2007; Cha-Um *et al*., 2007; Hou *et al*., 2010; Fatima and Bano, 1999; Ayub *et al*., 2000; Sivaci *et al*., 2008; Anjum *et al*., 1999; Kumari, 2010). How the ABA signal is transduced into a physiological or biochemical response has been an interesting research subject in the recent years.

Increasing evidence indicates that one mode of ABA action may be related to its role in the oxidative stress in plant cells. It has been documented that ABA can cause an increased generation of O_2^-(Jiang and Zhang, 2001; Laloi *et al*., 2004; Hu *et al*., 2005) and H_2O_2 (Guan *et al*., 2000; Pei *et al*., 2000; Jiang and Zhang, 2001) and CAT (Guan *et al*., 2000). Meanwhile, ABA also increases the activities of antioxidant enzymes such as SOD, CAT, APX, GR and MDA in plant tissues (Bellaire *et al*., 2000; Jiang and Zhang, 2001; Hu *et al*., 2005; Fryer *et al*., 2003; Park *et al*., 2004; Khorshidi and Nojavan, 2006).

Although, it has been shown that ABA can result in an oxidative stress, an enhancement in the capacity of oxidative stress tolerance may imply that plants need to mobilize the whole antioxidant defense systems including enzymatic and non-enzymatic constituents to resist oxidative damage in stressed plant tissues, rather than a few enzymes or metabolites. Meanwhile, since ABA causes oxidative stress in plants, it is possible that an oxidative damage might take place in plants exposed to the treatment of high concentrations of ABA. Different concentrations of ABA from 1 to 1,000 μM have been used to different plant tissues and have been shown to be able to induce gene expression and protein synthesis involved in antioxidative defences (Bellaire *et al*., 2000; Guan *et al*., 2000). In addition to H_2O_2, it is not clear whether other Reactive Oxygen Species (ROS), such as $O_2^-$and ·OH, are involved in oxidative stress in plants caused by ABA (Jiang and Zhang, 2001).

In this study, the effects of different concentrations of ABA on the levels of ROS including O_2^- and H_2O_2, the activities of antioxidant enzymes such as CAT, APX and GR were systematically studied in details. The objective of the present study was to find out a possible

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relationship between ABA, ROS, antioxidative defences and oxidative damage in bean plants.

MATERIALS AND METHODS

Plant material: The experiment was conducted in Faculty of Science, Mashad Branch, Islamic Azad University, Iran (2010).

The seeds of bean were supplied by the Agricultural Research Center of Khorasan, Iran. The plant Seeds were surface-sterilized with 5% NaHClO for 10 min and germinated at 28°C for 1 day. The seedlings were grown in trays of sand in a greenhouse at a temperature of 25-30°C and photoperiod of 14/10 h (day night) and watered daily. When the second leaf was fully expanded, they were collected and used for investigations.

Abscisic acid treatments: Detached leaves were cut into leaf segments (3 cm in length), rinsed with distilled water and then floated in solutions containing 0, 10, 100 and 1,000 µM ABA, at 25°C. Leaf segments were vacuum-infiltrated with these solutions before the incubation and immediately frozen in liquid N_2 and then stored at -80°C for further analysis.

Assays of antioxidant enzyme activities: Frozen leaf segments (0.5 g) were crushed into fine powder in a mortar and pestle under liquid N_2. Soluble proteins were extracted by homogenizing the powder in 10 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of 1 mM ASC in the case of APX assay. The homogenate was centrifuged at 15,000x g for 20 min at 4°C and the supernatant was used for the following enzyme assays. CAT (EC1.11.1.6) activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min (Aebi, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH7.0), 10 mM H_2O_2 and 200 µL of enzyme extract in a 3 mL volume.

GR (EC1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min in 1 mL of an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG and 200 µmL of enzyme extract. The reaction was initiated by adding NADPH. Corrections were made for the background absorbance at 340 nm, without NADPH (Shaedle and Bassham, 1977).

APX (EC1.11.1.11) activity was determined by following the decrease in A_{290} (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min in 1 mL of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ASC, 0.1 mM H_2O_2 and 200 µL of enzyme extract. The reaction was started by adding enzyme extract. Correction was done for the low, non-enzymatic oxidation of ASC by H_2O_2 (Nakano and Asada, 1981).

Determination of superoxide radical and hydrogen peroxide: O_2^- was measured as described by Erich and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of O_2^-, with some modifications. One g of frozen leaf segments was homogenized with 3 mL of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5,000x g for 10 min. The incubation mixture contained 0.9 mL of 65 mM phosphate buffer (pH 7.8), 0.1 mL of 10 mM hydroxylamine hydrochloride and 1 mL of the supernatant. After incubation at 25°C for 20 min, 17 mM sulfanilamide and 7 mM-naphthylamine were added to the incubation mixture. After reaction at 25°C for 20 min, ethyl ether in the same volume was added and centrifuged at 1,500x g for 5 min. The absorbance in the aqueous solution was read at 530 nm. A standard curve with NO_2^- was used to calculate the production rate of O_2^- from the chemical reaction of O_2^- and hydroxylamine.

The content of H_2O_2 was measured by monitoring the A_{415} of the titanium-peroxide complex following the method described by Brennan and Frenkel (1977). Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2.

RESULTS

Effects of different concentrations of ABA on levels of O_2^- and H_2O_2 in leaves of bean seedlings were investigated. Treatment with 10, 100 and 1,000 µM ABA caused a significant increase in levels of O_2^- (Fig. 1) and H_2O_2 (Fig. 2) compared with the control. At 12 h, O_2^- levels were 0.7, 0.8 and 1.4 (µmol g^{-1} dry wt. min) respectively, for the three concentrations of ABA used, that increased by 23, 40 and 63% in comparison with the control. H_2O_2 levels were 0.8, 1 and 1.5 (µmol g^{-1} fwt.) that increased by 39, 51 and 66% to the control.

Figure 3-5 show the effects of different concentrations of ABA on several antioxidative enzymes such as CAT (Fig. 3), the enzyme mainly responsible for eliminating H_2O_2 in the peroxisomes and APX (Fig. 4) and GR (Fig. 5), the two key enzymes of the Halliwell-Asada pathway for the removal of H_2O_2 in the chloroplasts. Treatment with 10 and 100 µM ABA caused a continuous increase in the activities of the three antioxidative enzymes . The leaves treated with 10 and 100 µM ABA enhanced the activities of CAT by 1 and

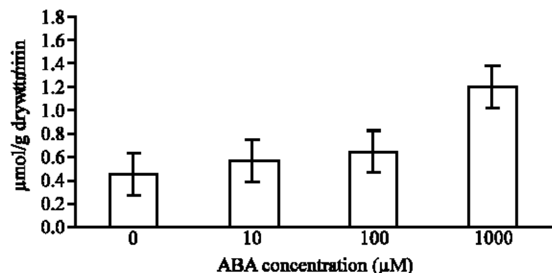

Fig. 1: Effects of ABA at different concentrations on levels of O_2^- in leaves of bean seedlings. Values are the means of three different experiments. Error bars represent SE with n = 3

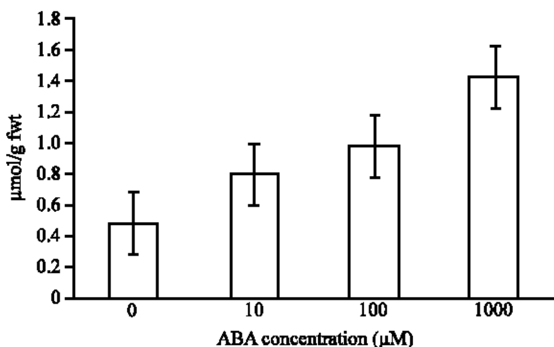

Fig. 2: Effects of ABA at different concentrations on levels of H_2O_2 in leaves of bean seedlings. Values are the means of three different experiments. Error bars represent SE with n = 3

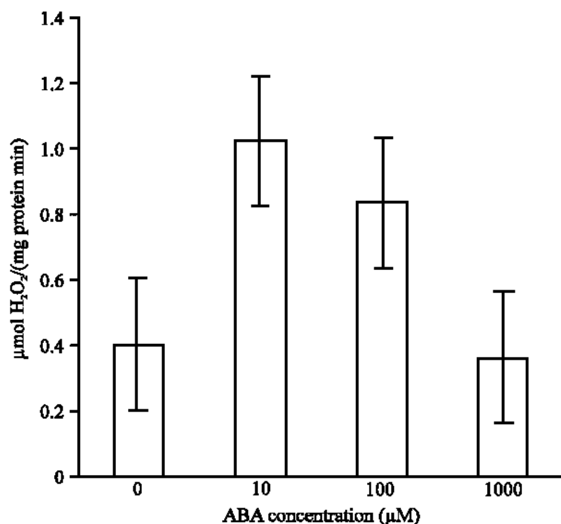

Fig. 3: Effects of ABA at different concentrations on activities of, CAT in leaves of bean seedlings. Values are the means of three different experiments. Error bars represent SE with n =3

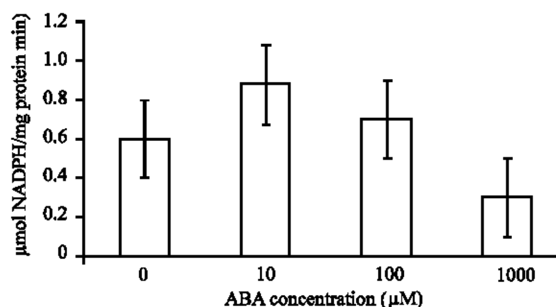

Fig. 4: Effects of ABA at different concentrations on activities of GR in leaves of bean seedlings. Values are the means of three different experiments. Error bars represent SE with n = 3

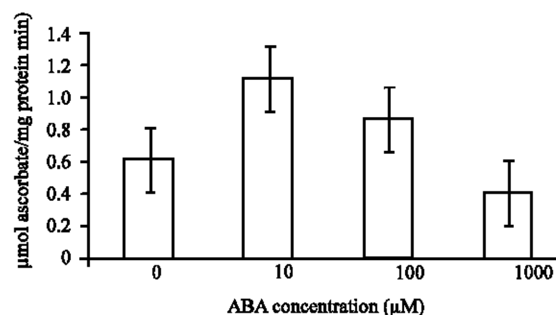

Fig. 5: Effects of ABA at different concentrations on activities of APX in leaves of bean seedlings. Values are the means of three different experiments. Error bars represent SE with n = 3

0.8 μmol H_2O_2/(mg protein min) i.e, 60 and 52%, respectively; when compared with the control (Fig. 3); GR by 0.9 and 0.7 μmol NADPH/(mg protein min) i.e, 35 and 16% (Fig. 4); APX by 1.15 and 0.9 μmol ascorbate/(mg protein min) i.e, 45 and 29% (Fig. 5). Activities of these antioxidative enzymes reduced in leaves treated with 1000 μM ABA in comparison with the control; CAT by 0.35 (10%), GR by 0.3 (23%) and APX by 0.4 (33%).

DISCUSSION

It has been shown that ABA can cause an increased production of H_2O_2, induce the expression of some antioxidant genes and enhance the activities of antioxidative enzymes such as SOD, CAT, GPX, APX and GR in plants (Bellaire *et al*., 2000; Guan *et al*., 2000). These suggest that ABA can result in an oxidative stress in plants. Our results not only have shown that ABA induced an increase in the content of H_2O_2 and the activities of CAT, APX and GR in leaves of bean seedlings but also further indicated that treatment of

ABA led to an increase in the level of O_2^-. These results further support that ABA results in an oxidative stress in plants.

ROS are generally believed as the by-products of oxygen metabolism and can have adverse effects such as lipid peroxidation, denaturation of proteins and mutation of DNA, all considered as cellular oxidative damage. Abundant evidence, however, has shown that ROS, especially H_2O_2 and O_2^-, are involved in cellular signaling process as secondary messengers. These induce a number of genes and proteins involved in stress defences, including CAT, GPX, GP, GR, GST, APX, SOD and pathogenesis-related (PR) protein (Bellaire *et al*., 2000; Guan *et al*., 2000). Although, ABA and ROS are thought to act as secondary messengers for the induction of antioxidant defences in stressed plants, the relationship between ABA and ROS in stress signal transduction cascades is still unclear. One possible model is that ABA-mediated metabolic changes might lead to an increase in endogenous ROS levels, which in turn induce antioxidative gene expression (Guan *et al*., 2000). Present results showed that a significant increase in levels of O_2^- and H_2O_2 occurred under ABA treatment and the increase in activities of antioxidative enzymes took place; meanwhile, the enhancement in the activities of these antioxidative enzymes was closely related to the levels of ROS induced by different concentrations of ABA in leaves of bean seedlings. These findings support the hypothesis mentioned above and suggests further that the generation of ABA-induced ROS may trigger the response of whole antioxidative defense systems against oxidative stress.

Although, ROS may act as secondary messengers to regulate gene expression and protein biosynthesis involved in the stress defences, these may only occur under subtoxic condition. ROS at higher concentrations are still cytotoxic. Our data showed that 10 to 100 μM ABA treatment significantly induced the increase in the levels of O_2^- and H_2O_2 and enhanced the capacity of protective systems in leaves of maize seedlings. Such an enhancement in the antioxidant defences is sufficient to scavenge these increased ROS. Treatment with 1,000 μM ABA led to a more abundant generation of O_2^- and H_2O_2. The capacity of antioxidative defense systems was not maintained at a higher level when compared with the treatment of 100 μM ABA. A marked oxidative damage occurred in leaves of bean seedlings. These suggest that the superoptimal concentration of 1,000 μM ABA may have induced a severe oxidative stress that cannot be controlled by the enhanced antioxidative defense systems.

There is only limited information about the mechanism with which ABA leads to the generation of ROS in plant cells. One speculation is that ABA-induced stomatal closing can cause the reduction in the availability of CO_2 for photosynthesis, which may lead to the generation of ROS from the misdirecting of electrons in the photosystems (Bowler *et al*., 1992). However, in guard cells of *Arabidopsis*, ABA-induced H_2O_2 production and the H_2O_2-activated Ca^{2+} channels are important mechanisms for ABA-induced stomatal closing (Pei *et al*., 2000). Diphenyleneiodonium chloride (DPI), a well-known inhibitor of trans-plasma membrane NAD (P)H oxidase (O_2^- synthase) (Orozco-Cardenas *et al*., 2001), blocked the production of ROS and partially inhibited ABA-induced stomatal closing. These results indicate that an oxidative burst occurred prior to the stomatal closing in the guard cells exposed to ABA treatment and suggest that a trans-plasma membrane NAD(P)H oxidase (O_2^- synthase) might be involved in the ABA signaling.

CONCLUSION

In conclusion, treatment with lower concentrations (10 to 100 μM) of ABA led to a relatively slight increase in the levels of O_2^- and H_2O_2 and induced the capacity of whole antioxidant defense systems against oxidative stress. Although, treatment with higher concentration of ABA (1,000 μM) no maintained the capacity of antioxidant defense at a higher level, a marked oxidative damage occurred.

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