Hydrogen peroxide acts as a signal molecule in CO_2 laser pretreatment-induced osmotic tolerance in wheat seedlings

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ABSTRACT

The objective of this study was to test whether hydrogen peroxide (H_2O_2) is involved in laser pretreatment-induced water tolerance in wheat seedlings due to its nature as a second messenger in stress responses. The results showed that 3 min laser pretreatment could enhance water tolerance in wheat seedlings by decreasing the concentration of malondialdehyde (MDA), the production rate of superoxide radical (O_2^-), and increasing the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) and the concentration of glutathione (GSH), and having a positive physiological effect on the growth of osmotic stress seedlings. But the promotive effect of laser pretreatment-induced water tolerance in wheat seedling was effectively reversed by addition of 2 mmol AsA (ascorbic acid) or 5 μmol DPI (diphenyle iodonium), but exogenous 100 U/mL CAT could not reversed laser pretreatment-induced protective effect on wheat seedlings under osmotic stress. The results suggest that H_2O_2 metabolism was involved as signal in the processes of laser-induced water acclimation and laser-induced protective effect was shown to be likely related to NADPH oxidase-dependent H_2O_2 production.

Keywords: laser; osmotic stress; lipid peroxidation; antioxidative system

The laser has been widely used in the field of biology with the development of laser technology. Laser was used widely as pre-sowing seed treatments to increase seed germination and seedling growth (Qi et al. 2002). Previous studies indicated that suitable doses of laser irradiation improved plant metabolism, enzymatic activities and the concentration of chlorophyll (Qiu et al. 2010). Qiu et al. (2008) had preliminarily proved that suitable laser pretreatment of embryos enhanced water tolerance of wheat seedlings by decreasing the concentration of malondialdehyde (MDA), the production rate of superoxide radical (O_2^-) and increasing the activities of ascorbate peroxidase (APX) and peroxidase (POD). The long-term physiological effect of CO_2 laser treatment on UV-B or osmotic stress damaged plants was also observed (Qiu et al. 2002, 2008). However, the physiological mechanism of the effect of laser pretreatment on seed germination and seedling growth was unknown.

Drought is the biggest limiting factor for plant growth and crop production and recent global climate change made this situation more serious (Wang et al. 2003). One important consequence of abiotic stresses is an increase in the cellular levels of reactive oxygen species (ROS), which show toxicity to the metabolic functions after conversion to H_2O_2 (Sairam et al. 2004). There is compelling evidence about the biological activity of ROS with emphasis on the function of hydrogen peroxide (H_2O_2) as a signal molecule in plants (Hung et al. 2005). Chamnogpol et al. (1998) reported that H_2O_2 can act as an intermediate signal upstream of both ethylene and salicylic acid during plant stress responses, or can serve as a second messenger in signal transduction pathways, leading to stress acclimation. Available information suggest that H_2O_2 directly regulates the expression of numerous genes, some of which are involved in plant defense and antioxidants, cell rescue/defense proteins, and signaling proteins such as kinase,

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phosphatase, and transcription factors (Hung et al. 2005). Hence, H_2O_2 signaling is of potential significance to any program aimed at improving crop tolerance to environmental stresses.

Exogenous application of H_2O_2 increases chilling tolerance by enhancing the glutathione level of mung bean seedlings (Murphy et al. 2002). Azevedo Neto et al. (2005) reported that addition of H_2O_2 to the nutrient solution induces salt tolerance by enhanced activities of antioxidants and reduced peroxidation of membrane lipids in leaves and roots of maize as an acclimation response. In these studies, H_2O_2 can be viewed as a signal molecule in the regulation of plant growth and development, whereas suitable doses of laser irradiation also had a positive effect on plant growth. So we put forward a hypothesis that H_2O_2 metabolism was involved as signal in the processes of laser-induced water acclimation. The aim of this investigation was to examine the relationship of H_2O_2 and laser pretreatment in water tolerance, in order to better understand the physiological and biochemical mechanism of laser-induced water acclimation.

MATERIAL AND METHODS

Plant materials. The uniform seeds of wheat (*Triticum aestivum* L. cv. Mianyang 26, obtained from Yangling Breeding Center of National Wheat Engineering Research Center of China) were randomly divided into two groups: (1) without any treatment; (2) seeds exposed to CO_2 laser irradiation.

Laser treatment: seeds were sterilized for 3 min by 0.01% $HgCl_2$ and were washed for 30 min by flowing water. A CO_2 laser (Wavelength 10 600 nm, power density 20.1 mW/mm^2, beam diameter 5 mm) directly irradiated the embryo of wheat seed for 3 min. Three minute laser pre-treatment was chosen according to our previous work (Qiu et al. 2008). Ten replications of 40 pure seeds were used. One seed was pretreated only once by laser irradiation. The seeds were exposed to laser one by one. No laser irradiation and no osmotic stress were regarded as the control (CK). The CO_2 laser (Model No. MSHCO2-A-C800MM) was made in the Northwest University (Xi´an, China).

Untreated seeds and CO_2 laser-pretreated seeds were grown in Petri dish (diameter 18 cm), flushed daily with Hoagland solution, in a growth chamber under a 12 h photoperiod at 250 μmol/m^2/s provided by fluorescent lamps, 70% relative humidity and 25°C/18°C (day/night). When the seedlings were 12 days old (with two fully expanded leaves), they were treated with 10% (w/v) polyethylene glycol (PEG 6000) solution for 3 days. 10% (w/v) PEG6000 treatment was chosen according to our previous work (data not shown). On 3 days of osmotic stress, leaves and roots were sampled for various analyses. See Table 1 for details about experimental design.

Diphenylene iodonium (DPI) (NADPH oxidase inhibitor) was purchased from Sigma (USA). All above chemicals were added to Hoagland solution with or without PEG6000. All of the solutions were regenerated once a day.

Lipid peroxidation and production rate of O_2^-. MDA concentration was measured according to Qiu et al. (2008). The production rate of O_2^- was determined using the method of Elstner and Heupel (1976).

Antioxidant enzymes activities and antioxidant compounds concentration. Activity of superoxide

Table 1. The methods of CO_2 laser pretreatment, H_2O_2 treatment and osmotic stress

Group/treatment	CO_2 laser treatment (min)	Osmotic stress % (w/v)	CAT (U/mL)	AsA (mmol)	DPI (μmol)
Hoagland (the control, CK)	0	0	0	0	0
PEG 6000 (P)	0	10	0	0	0
Laser treatment + PEG 6000 (L + P)	3	10	0	0	0
Laser treatment + PEG 6000 + 100 U CAT (L + P + C)	3	10	100	0	0
Laser treatment + PEG 6000 + 2 mmol AsA (L + P + A)	3	10	0	2	0
Laser treatment + PEG 6000 + 5 μmol DPI (L + P + D)	3	10	0	0	5
Hoagland + 100 U CAT (CK + CAT)	0	0	100	0	0
Hoagland + 2 mmol AsA (CK + AsA)	0	0	0	2	0
Hoagland + 5 μmol DPI (CK + DPI)	0	0	0	0	5

dismutase (SOD) (EC1.15.1.1) was assayed according to Giannopolitis and Ries (1977). Catalase (CAT) activity (EC1.11.1.6) was determined using the method of Cakmak and Marschner (1992). Peroxidase (POD, EC 1.11.1.7) activity was determined as described by Zhang and Kirham (1994). The activity of APX (EC 1.11.1.1) was assayed according to Nakano and Asada (1981). GR activity was determined as described by Foyer and Halliwell (1976). Glutathione content was determined according to Ellman (1959). Ascorbic acid (AsA) concentration was determined using the method of Tonamura (1978).

Growth parameter. Green parts in the plant and root were oven dried at 80°C until constant weight and weighed using electronic scale as dry weight (g). Plant height and root length were also measured.

Statistics. All of the data were subjected to ANOVA and are presented here as the mean ± SE of at least three independent experiments.

RESULTS AND DISCUSSION

Osmotic stress induces the accumulation of ROS and causes oxidative damage to the plant (Anjum et al. 2011). We demonstrate that the lesser effects of 10% (w/v) PEG6000 on lipid peroxidation in laser-pretreated wheat seedlings. As showed in Figure 1, osmotic stress (P) induced toxic effects on plant growth and development. However, laser-pretreated wheat seedlings under osmotic stress (L + P) can counteract oxidative damage and have protective effect against stressful conditions. It was assumed that plant undergo significant morphological and metabolic changes in response to drought. Plant height, root length, dry weight and root dry weight increased significantly by the interaction (Figure 1), which was concomitant with decreased production rate of O_2^- and the concentration of MDA (Figure 2). The same phenomena was found in the laser-pretreated wheat seedlings exposed to UV-B radiation or drought stress, which also has reverse detrimental effects (Qi et al. 2002). To determine whether H_2O_2 is involved in MDA concentration and the production rate of O_2^- decreasing in wheat seedlings induced by laser pretreatment, 12-day-old seedlings pretreated with 3 min laser radiation under osmotic stress were treated with 100 U/mL CAT, 2 mmol AsA or 5 μmol DPI.

Here, exogenous CAT was used to determine the effect of laser pretreatment on wheat seedlings under osmotic stress. CAT, a membrane impermeable scavenger of H_2O_2, can degrade extracellular H_2O_2 to molecular oxygen and water (Hideo et al. 2003).

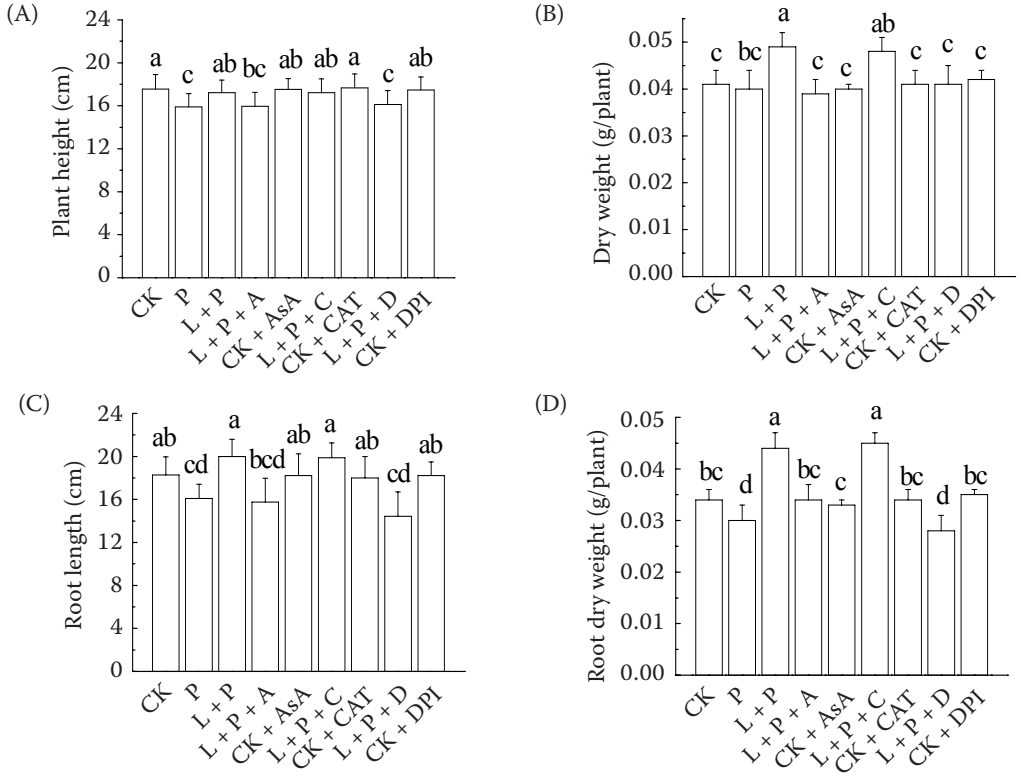

Figure 1. Effect of CO_2 laser treatment on growth and development in wheat seedlings under osmotic stress for 3 days. Bars are means ± standard error of 18 determinations. Means with different letters above bars were significantly different at 0.05 level according to the Duncan's multiple range test

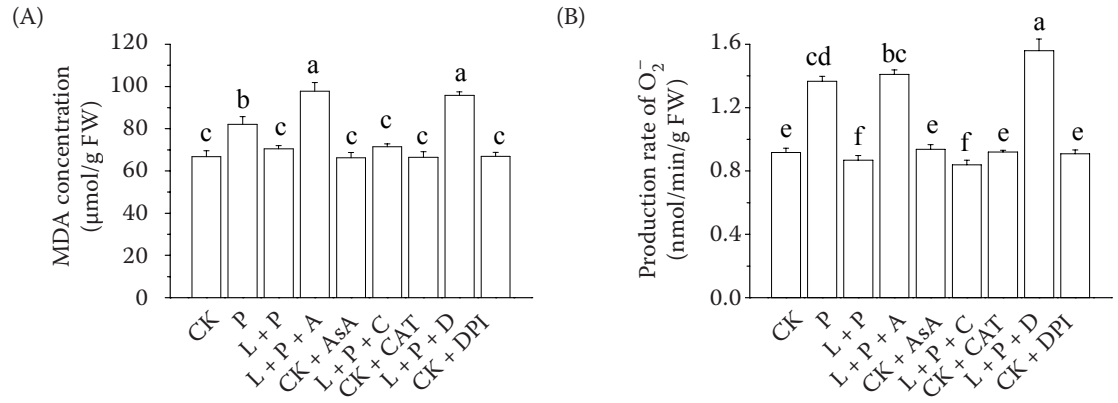

Figure 2. Effect of CO_2 laser treatment on MDA concentration (A) and the production rate of O_2^- (B) in wheat seedlings under osmotic stress for 3 days. See notes to Figure 1. Bars are means ± standard error of 6 determinations

Exogenous application of H_2O_2 was able to induce salt tolerance by enhanced activities of antioxidants and reduced peroxidation of membrane lipids in leaves and roots of maize and this effect could be eliminated by exogenous CAT (Azevedo Neto et al. 2005). In the experiment, exogenous 100 U/mL CAT could not reverse laser pretreatment-induced MDA concentration and the production rate of O_2^- decreasing in wheat seedlings under osmotic stress (Figure 2). As CAT is not a cell-permeable

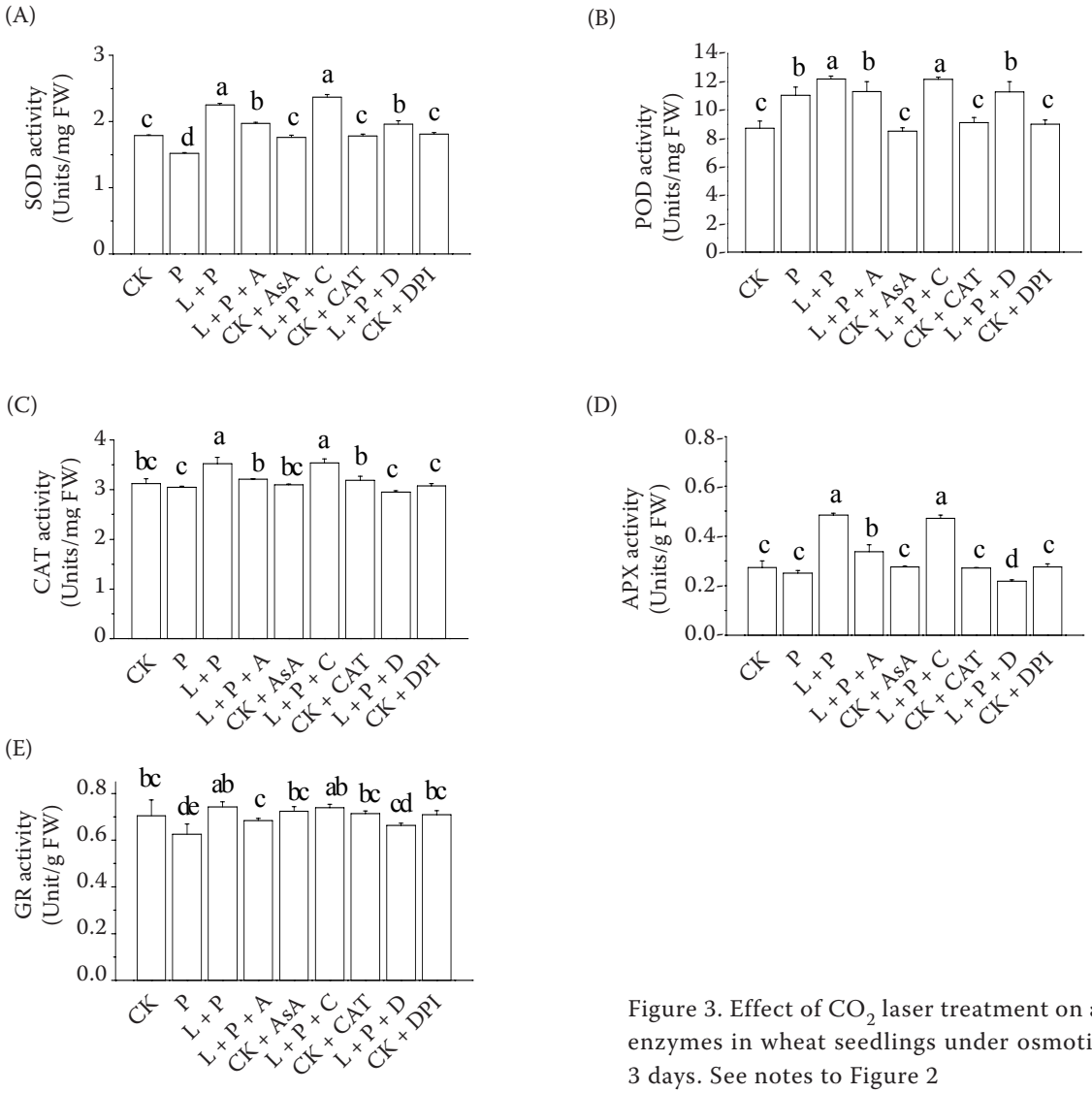

Figure 3. Effect of CO_2 laser treatment on antioxidant enzymes in wheat seedlings under osmotic stress for 3 days. See notes to Figure 2

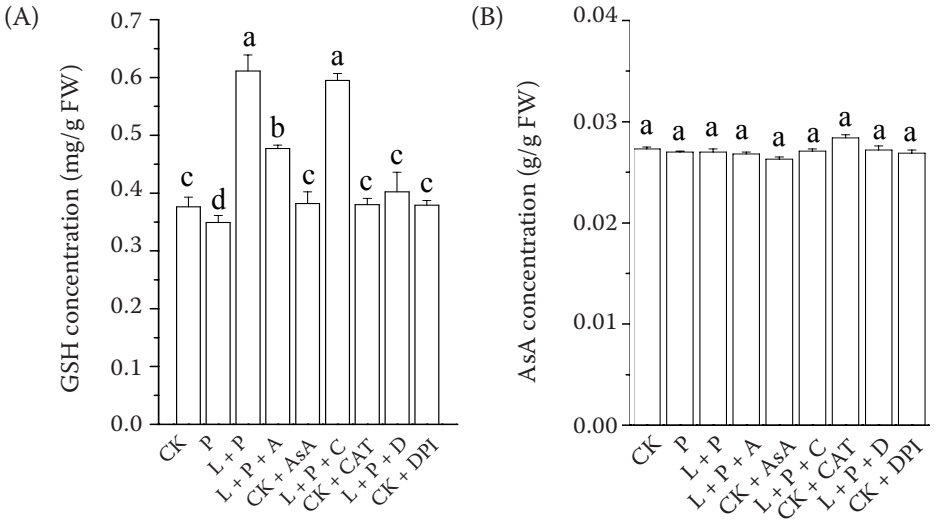

Figure 4. Effect of CO_2 laser treatment on GSH (A) and AsA (B) concentration of wheat seedlings under osmotic stress for 3 days. See notes to Figure 2

molecule, the exogenous CAT was not able to remove the endogenous H_2O_2 in laser pretreated seedlings, it could not eliminate the effect of laser pretreatment and no effect of laser pretreatment-induced water tolerance was observed. This result was similar to Li et al. (2009).

Also AsA is an H_2O_2 scavenger and one of the most important reducing substrates for H_2O_2 removal in cells (Noctor and Foyer 1998). Our experiments showed that 2 mmol AsA could effectively reversed laser pretreatment-induced MDA concentration and the production rate of O_2^- decreasing in wheat seedlings (Figure 2). The results suggest that laser pretreatment-induced protective effect on lipid peroxidation under osmotic stress was related to a pathway involving H_2O_2.

In plant cells, H_2O_2 can be generated by specific enzymes such as plasma membrane-localized NADPH oxidases. NADPH oxidases are the main enzymes that produce H_2O_2 in cells (Foreman et al. 2003). DPI is a specific inhibitor of NADPH oxidases and strongly inhibits the NADPH oxidases production of H_2O_2 in plant cells (Desikan et al. 1998). Li et al. (2009) detected that 10 μmol DPI treatment for 24 h markedly inhibited adventitious root formation of mung bean seedlings. Our experiments showed that 5 μmol DPI could effectively reverse laser pretreatment-induced MDA concentration and the production rate of O_2^- decreasing in wheat seedlings (Figure 2). The results imply that the inhibitory effect of DPI on laser pretreatment-induced MDA concentration and the production rate of O_2^- decreasing in wheat seedlings were due to the DPI inhibition of NADPH oxidase production of H_2O_2. The endogenous H_2O_2 in cells may be a signal molecule in laser pretreatment-induced MDA concentration and the production rate of O_2^- decreasing in wheat seedlings.

Several studies have produced evidence for a signaling role for H_2O_2 (Hung et al. 2005). The addition of H_2O_2 to plant tissues or its experimental generation was demonstrated to act as a signal in the induction of gene expression of the enzymes CAT, APX, POD, and GR (Van et al. 2001). Our results indicated clearly that prior CO_2 laser irradiation of embryos of plants could raise SOD, POD, CAT, APX, and GR activities and increase the concentration of GSH (Figures 3 and 4), which negatively correlated with decreasing production rate of O_2^- and the concentration of MDA (Figure 2). Because of higher activity of SOD, POD, CAT, APX, and GR and higher concentrations of GSH in laser-pretreated seedlings, free radical production would be eliminated quickly. As a result, they prevented lipid peroxidation and MDA production, and thus the plant cells were protected from osmotic stress damage. It is unknown, however, whether H_2O_2 is involved in laser-induced antioxidant enzymes and antioxidant compounds in wheat seedlings under osmotic stress. Therefore, the effect of H_2O_2 on SOD, POD, CAT, APX, and GR activities and concentrations of AsA and GSH in wheat seedling under osmotic stress were determined, although convincing data were obtained by the H_2O_2-induced increase in plant tolerance or resistance against salt or osmotic stress (Azevedo Neto et al. 2005). As shown in Figures 3 and 4, the promotive effect of the laser pretreatment was suppressed in the presence of the H_2O_2 scavenger (AsA) or NADPH oxidase inhibitor (DPI) indicating that laser pretreatment-

induced protective effect might be partly due to the intracellular production of H_2O_2.

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