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Exogenous p-hydroxybenzoic acid regulates antioxidant enzyme activity and mitigates heat stress of cucumber leaves

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ABSTRACT

To elucidate a physiological mechanism of heat stress mitigated by p-hydroxybenzoic acid (PHBA), seedlings of *Cucumis sativus* cv. Jinchun no. 4 were watered for 2 d with the Hoagland nutrient solution containing 0.5 mM PHBA, and then they were watered with the Hoagland nutrient solution only and were subjected to normal (25/18 °C) and elevated (42/38 °C) temperatures for 3 d. We investigated whether PHBA could protect cucumbers from heat stress and whether the protective effect was associated with regulation of antioxidant enzymes and lipid peroxidation. 0.5 mM PHBA was chosen since it improved growth inhibition and decreased levels of superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and malonaldehyde (MDA) under heat stress more than other concentrations of PHBA. At 2 d, supply of 0.5 mM PHBA elevated the activities of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11), catalase (EC 1.11.1.6), monodehydroascorbate reductase (EC 1.6.5.4), dehydroascorbate reductase (EC 1.8.5.1) and glutathione reductase (EC 1.6.4.2) in cucumber leaves. When the PHBA-pretreated seedlings were exposed to heat, the antioxidant enzyme activities were changed further and were higher than those in heat treatment alone, and this was consistent with the increased transcript levels of *Cu/Zn-SOD*, *Mn-SOD* and *guaiacol peroxidase* (*GPX*) genes and coincided with the enhanced contents of reduced glutathione. Heat increased the levels of $O_2^{\bullet -}$, H_2O_2 and MDA and made 62.5% of leaf edges being dried up. Compared to heat treatment, the combination of 0.5 mM PHBA and heat decreased the levels of $O_2^{\bullet -}$, H_2O_2 and MDA, while it resulted in the dried leaf edges to be 29.17%. We conclude that pretreatment with 0.5 mM PHBA enhances antioxidant enzyme activities under heat stress; thus, it decreases lipid peroxidation to some extent and enhances heat tolerance of cucumber seedlings.

1. Introduction

Heat stress is a serious threat to crop production in many areas of the world (Hall, 2001). Lots of researchers have thereby focused on mechanisms of the stress (Wahid et al., 2007), of which heat causes oxidative stress in plants and leads to accumulation of reactive oxygen species (ROS) including superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (Almeselmani et al., 2006). The overproduction of ROS does harm to cell membranes (Xu et al., 2006), induces lipid peroxidation and even results in cell death (Molassiotis et al., 2006). To protect cells from the ROS damage, plants evolve antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), glutathione peroxidase (GSH-Px), ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) (Mittler, 2002) and the non-enzymatic antioxidants including the reduced glutathione (GSH) (Kumar and Knowles, 1993).

Phenolic compounds are ubiquitous constituents of higher plants (Manach et al., 2004). As an acclimation mechanism of plants against thermal stress, endogenous phenolic compounds are accumulated in tomato and watermelon (Rivero et al., 2001). On the other hand, when supplying exogenous phenolic compounds such as salicylic acid, cinnamic acid and caffeic acid, the resistance of plants to stress environments is increased (Krishna and Surinder, 2003; Shi et al., 2006; Li et al., 2011). p-Hydroxybenzoic acid (PHBA; 4-hydroxybenzoic acid) is one of the phenolic compounds and comes from the CoA ester of p-hydroxycinnamic acid through an oxidation-like mechanism (Loscher and Heide, 1994). According to Viitanen et al. (2004), chorismate pyruvate-lyase catalyzes the direct conversion of chorismate to PHBA and pyruvate. It has been shown that PHBA is unable to stimulate an alternative respiratory pathway in tobacco and thus does not induce heat production (van der Straeten et al., 1995). However, PHBA at 0.5 mM enhances drought tolerance of winter wheat and elevates freezing tolerance of spring wheat, and there is correlation between these stress resistance and antioxidant enzymes (Horváth et al.,

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2007). We hypothesize that maybe there is another physiological mechanism of PHBA and that PHBA at a certain concentration can modulate antioxidant enzymes and thereby enhance heat tolerance of plants. Up to now, there is no report that PHBA pretreatment alleviates the damage of heat stress to plants.

Cucumber is sensitive to heat (Zhang et al., 2012). In this study, its seedlings were pretreated with 0.5 mM PHBA for 2 d and then were subjected to heat stress (42/38 °C, day/night) for 3 d. Our aim was to examine whether PHBA application could protect plants from heat stress and whether the protective effect was in relation to regulation of antioxidant enzyme activities and lipid peroxidation. Our work may help to explore a physiological mechanism of heat stress abated by PHBA and to enlarge the application potential of PHBA in alleviating abiotic stresses.

2. Material and methods

2.1. Plant materials and treatments

Cucumber (*Cucumis sativus* cv. Jinchun no. 4) seeds were germinated on moist gauze at 25 °C for 2 d and were then planted into 10-cm plastic pots that were filled with sand. Cucumber seedlings were cultivated at 25 °C and were watered twice per day with the Hoagland nutrient solution, which contained 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 2 mM $MgSO_4$, 10 μM $MnSO_4$, 50 μM H_3BO_3, 0.7 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$ and 70 μM Fe-EDTA-Na_2. At the two-leaf stage when the second true leaves from below were fully expanded, cucumber seedlings were chosen for treatments in the preliminary or formal experiments.

In the preliminary experiments, 32 cucumber seedlings were divided into four groups (eight plants per group) and were separately watered for 2 d with the Hoagland nutrient solution containing different concentrations (0, 0.25, 0.5 and 0.75 mM) of PHBA. Then, the sand for planting cucumbers was rinsed with water for 6 times and with the Hoagland nutrient solution for 6 times. Subsequently, all seedlings were exposed to heat conditions (42/38 °C, day/night) for 3 d. To choose an optimum concentration of PHBA that mitigated heat stress better, indexes of plant growth were determined (Sun et al., 2012), while the second leaves in each group were collected for assaying physiological parameters. Three different sets of plants grown at different time were used for the preliminary experiments.

Based on the results of preliminary experiments, 56 cucumber seedlings at the two-leaf stage were divided into seven groups (eight plantlets per group) to study the effects of PHBA pretreatment on heat-stressed cucumbers in the formal experiments. Three groups of seedlings were watered with the Hoagland nutrient solution containing an optimum concentration (0.5 mM) of PHBA, and three groups of cucumbers were watered with the Hoagland nutrient solution only, while one group of plantlets was used to sample the second leaves and to investigate the physiological parameters of cucumber seedlings at the start of experiments. After 2 d, the sand for planting all seedlings was rinsed with water for 6 times and with the Hoagland nutrient solution for 6 times. Then, one group of PHBA-pretreated and one group of PHBA-untreated cucumber seedlings were used to sample the second leaves and to investigate the physiological parameters of cucumber seedlings at 2 d after the start of experiments. Meanwhile, two groups of PHBA-pretreated cucumbers were separately exposed to normal (25/18 °C, day/night) and high (42/38 °C) temperatures with 75% relative humidity of air and a photoperiod of 12 h light (300 μmol m^{-2} s^{-1})/12 h darkness, and they were named as "PHBA pretreatment" and "PHBA + heat treatment", respectively. Moreover, two groups of PHBA-untreated plants were separately subjected to two temperatures as indicated above and were designated "control" and "heat treatment", respectively. After 3 d of heat treatment, the second leaves from the above four groups were harvested to investigate the physiological parameters of cucumber seedlings at 5 d after the start of experiments. Three different sets of plants grown at different time were used for the formal experiments.

2.2. Determination of malondialdehyde content

Malondialdehyde (MDA) was extracted with 10% trichloroacetic acid, and its content was determined at 450, 532 and 600 nm following the procedures that were described by Dhindsa et al. (1981) and modified by Xu et al. (2008).

2.3. Measurement of H_2O_2 content

The content of H_2O_2 in leaves was determined according to the method of Bernt and Bergmeyer (1974) with some modifications. After ground with liquid nitrogen, 0.3 g of leaves was homogenized into 1 ml of 100 mM sodium phosphate buffer (pH 6.8) and was centrifuged at 4 °C and 18,000 × *g* for 20 min. Then, 0.5 ml of supernatant was mixed with 2.5 ml of peroxide reagent [consisting of 83 mM sodium phosphate (pH 7.0), 0.005% o-dianisidine and 1 mM peroxidase] and was incubated at 30 °C for 10 min. The reaction was stopped through adding 0.5 ml of 1 M perchloric acid. The absorbance was measured at 436 nm, and the content of H_2O_2 in leaves was calculated from a standard curve of H_2O_2 reagent.

2.4. Determination of the formation rate of $O_2^{\bullet -}$

The formation rate of $O_2^{\bullet -}$ in leaves was determined according to Elstner and Heupel (1976) with modifications. After ground with liquid nitrogen, cucumber leaves (0.2 g) were homogenized into 3 ml of 65 mM phosphate buffer (pH 7.8) and then were centrifuged at 4 °C and 5000 × *g* for 10 min. Subsequently, the supernatant (0.75 ml) was mixed with 0.675 ml of 65 mM phosphate buffer (pH 7.8) and 0.075 ml of 10 mM hydroxylamine chlorhydrate. When 0.375 ml of 17 mM sulfanilamide and 0.375 ml of 7 mM alpha-naphthylamine were added, the mixture was kept at 25 °C for 20 min and then was performed with 2.25 ml of aether. After centrifugation at 4 °C and 1500 × *g* for 15 min, the absorbance was measured at 530 nm, and the formation rate of $O_2^{\bullet -}$ was calculated from a standard curve of $NaNO_2$ reagent.

2.5. Extraction of antioxidant enzymes

When ground with liquid nitrogen, 0.2 g of leaves was suspended into 2 ml of ice-cold HEPES buffer (25 mM, pH 7.8) containing 0.2 mM EDTA and 2% PVP. The homogenate was centrifuged at 4 °C and 12,000 × *g* for 20 min, and the resulting supernatants were used to determine the activities of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), GPX (EC 1.11.1.7), DHAR (EC 1.8.5.1), MDHAR (EC 1.6.5.4) and GR (EC 1.6.4.2) (Ramiro et al., 2006). To extract GSH-Px (EC 1.11.1.9), 0.3 g of liquid-nitrogen-ground leaves was homogenized into 0.3 ml of HEPES buffer (25 mM, pH 7.8) that contained 0.2 mM EDTA and 2% PVP. The HEPES buffer (25 mM, pH 7.8), which contained 0.2 mM EDTA, 2% PVP and 2 mM ascorbate, was used for APX (EC 1.11.1.11) extraction.

2.6. Assay of antioxidant enzyme activities

The activity of SOD was determined at 560 nm following Hwang et al. (1999). One unit of SOD activity was defined as the amount of