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Abscisic acid is involved in brassinosteroids-induced chilling tolerance in the suspension cultured cells from *Chorispora bungeana*

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ABSTRACT

The objective of this study was to investigate whether abscisic acid (ABA), a second messenger in chilling stress responses, is involved in brassinosteroids (BRs)-induced chilling tolerance in suspension cultured cells from *Chorispora bungeana*. The suspension cells were treated with 24-epibrassinolide (EBR), ABA, ABA biosynthesis inhibitor fluridone (Flu) and EBR in combination with Flu. Their effects on chilling tolerance, reactive oxygen species (ROS) levels and antioxidant defense system were analyzed. The results showed that EBR treatment markedly alleviated the decrease of cell viability and the increases of ion leakage and lipid peroxidation induced by chilling stress, suggesting that application of EBR could improve the chilling tolerance of *C. bungeana* suspension cultures. In addition, similar results were observed when exogenous ABA was applied. Treatment with Flu alone and in combination with EBR significantly suppressed cell viability and increased ion leakage and lipid peroxidation under low temperature conditions, indicating that the inhibition of ABA biosynthesis could decrease the chilling tolerance of *C. bungeana* suspension cultures and the EBR-enhanced chilling tolerance. Further analyses showed that EBR and ABA enhanced antioxidant defense and slowed down the accumulation of ROS caused by chilling. However, Flu application differentially blocked these protective effects of EBR. Moreover, EBR was able to mimic the effect of ABA by markedly increasing ABA content in the suspension cells under chilling conditions, whereas the EBR-induced ABA accumulation was inhibited by the addition of Flu. Taken together, these results demonstrate that EBR may confer chilling tolerance to *C. bungeana* suspension cultured cells by enhancing the antioxidant defense system, which is partially mediated by ABA, resulting in preventing the overproduction of ROS to alleviate oxidative injury induced by chilling.

Introduction

Low temperature is one of the most serious plant growth stresses in the world, and has become a major limitation to plant growth, development, survival and crop productivity (Yu et al., 2003). Exposure to low temperature induces the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide radical (O_2^-) and hydroxyl radical (•OH), which could cause oxidative stress at cellular level. Oxidative stress may be a significant factor in association with chilling-induced injury (Prasad et al., 1994; Fadzillah et al., 1996). To scavenge these toxic species and alleviate oxidative injury, plants have evolved an efficient antioxidant defense system consisting of several antioxidative enzymes, such as ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2), peroxidase (POD, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1), and some nonenzymatic antioxidants, including lipid-soluble antioxidants (α-tocopherol and β-carotene), and water-soluble reductants (ascorbate and glutathione) (Bowler et al., 1992; Zhou et al., 2005). Usually, the production of ROS is increased with the concomitant increases in antioxidant enzyme activities and antioxidant contents, suggesting that antioxidant defense system may have a general role in the acquisition of tolerance in plants under stress conditions such as chilling (Núñez et al., 2003).

Brassinosteroids (BRs) are a family of over 40 naturally occurring plant steroid hormones that are found in a wide range of organisms, from lower to higher plants (Ogweno et al., 2008; Bajguz and Hayat, 2009). They play critical roles in a variety of physiological responses in plants, including stem elongation, pollen tube growth, leaf bending and epinasty, root growth inhibition, ethylene biosynthesis,

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; AsA, ascorbic acid; BRs, brassinosteroids; CAT, catalase; EBR, 24-epibrassinolide; Flu, fluridone; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; OD, optical density; •OH, hydroxyl radical; O_2^-, superoxide radical; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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proton pump activation, vascular differentiation, nucleic acid and protein synthesis and photosynthesis. It has been proposed that the changes induced by BRs are mediated through the repression and/or depression of specific genes (Hasan et al., 2008). Furthermore, BRs have the ability to protect plants from various biotic and abiotic stresses, such as cold stress, water deficit, salt injury, pathogen infection, oxidative damage, thermal stress and metal stress (Bajguz and Hayat, 2009; Liu et al., 2009). BRs have been shown to have protective effects on eggplant, cucumber, maize and rice seedlings under chilling stress (Mandava, 1988; He et al., 1991; Hotta et al., 1998). In previous research, treatment with 24-epibrassinolide (EBR) resulted in the increase in chilling tolerance of rice seedlings through inducing effects on membrane stability and osmoregulation (Wang and Zeng, 1993). However, studies on the protective mechanisms of BRs in plants under chilling conditions are still limited.

In higher plants, abscisic acid (ABA) can act as a signal molecule in the responses to various abiotic stresses. ABA content is increased under environmental stress such as drought (Wang and Huang, 2003; DaCosta and Huang, 2007), heat (Teplova et al., 2000) and low temperature (Janowiak et al., 2002, 2003), which regulates gene expression and the relatively physiological and biochemical reactions. Recently, a role of ABA in the chilling tolerance of maize seedlings has been demonstrated (Janowiak et al., 2002, 2003). Previous studies showed that the increase in endogenous ABA content was closely related to the chilling tolerance of maize seedlings. In addition, application of exogenous ABA increased the chilling tolerance in maize, wheat, *Brassica napus*, chickpea and *Stylosanthes guianensis* (Zhou et al., 2005; Kumar et al., 2008). BRs-induced heat resistance has been demonstrated to be associated with the accumulation of ABA (Kurepin et al., 2008; Bajguz, 2009). However, it is not known whether BRs-induced chilling tolerance is mediated through ABA accumulation. Thus, we also investigated the role of ABA in the BRs-induced chilling tolerance. In this paper, the suspension cultured cells from *Chorispora bungeana* were treated with the ABA biosynthesis inhibitor fluridone (Flu) to examine the effects of BRs on chilling resistance, ROS levels and the antioxidant defense system under the inhibition of ABA biosynthesis, and to investigate whether ABA is involved in BRs-induced chilling resistance and BRs-mediated ROS levels as well as antioxidant defense in *C. bungeana* suspension cultured cells under chilling stress.

Materials and methods

Plant material

A wild species of *Chorispora bungeana* Fisch. and C.A. Mey. was obtained as described by Fu et al. (2006). The leaves of *C. bungeana* without serrate margins were selected for callus induction. The sterile leaves were cut into 10 mm segments, with margins finely cut off, and cultured on Murashige and Skoog (MS) basal medium containing 11.5 μM gibberellic acid (GA_3), 0.9 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 1.1 μM α-naphthalene acetic acid (NAA) under 25 °C with a 16-h photoperiod (irradiance of 80 $\mu mol\ m^{-2}\ s^{-1}$). The medium was refreshed every 2 weeks. Suspension cultured cells of *C. bungeana* were cultivated in liquid MS basal medium containing 0.9 μM 2,4-D, 1.1 μM NAA, 0.9 μM 6-benzylaminopurine (6-BA), and 0.9 μM kinetin (KT) on a rotary shaker (120 rpm). The liquid medium was also refreshed at weekly intervals. After five or more subcultures, the synchronous steady cultures were collected and used in the following experiments.

Chemicals and experimental treatments

To investigate the effect of BRs on chilling tolerance in *C. bungeana* suspension cultured cells, the 7-day-old stock cultures were transferred into liquid MS medium with EBR (Sigma, USA) of different concentrations or with sterilized water. Subsequently, the suspension cultures were incubated in the growth chamber set at 25 °C, 4 °C or 0 °C for 3 d and under continuous shaking at 120 rpm. In order to elaborate the influence of exogenously applied EBR on the ABA content, the suspension cells were treated with or without 0.1 μM EBR and harvested at different times after exposure to 25 °C, 4 °C or 0 °C for 1, 2, 3, 4 or 5 d.

Fluridone (Flu) was used as the ABA biosynthesis inhibitor. To investigate the role of ABA in the EBR-induced chilling tolerance, the 7-day-old stock cultures were immediately transferred into the different liquid MS mediums containing the following chemicals: (1) sterilized water; (2) 0.1 μM EBR; (3) 75 μM ABA (±enantiomer mixture; Sigma, USA); (4) 0.1 μM EBR + 50 μM Flu (Sigma, USA); and (5) 50 μM Flu. Subsequently, all the treated suspension cultures in same medium were divided into three groups. One was cultured under 25 °C and the other two were exposed to 4 °C or 0 °C for 3 d. Control cells were treated with sterilized water and exposed to a temperature of 25 °C. Following treatment, the suspension cultured cells were harvested and washed with sterilized distilled water to remove medium residues. The fresh cells were used for relative cell viability and ion leakage assays immediately and the others were frozen under liquid N_2, and then kept at −80 °C for determinations of other physiological parameters.

Relative cell viability assay

Chilling injury was determined by assessing viability of suspension cultured cells, which was measured by the 2,3,5-triphenyltetrazolium chloride (TTC) reduction test (Guo et al., 2006). Fresh cells (0.2 g) were incubated in 2.5 mL of 100 mM sodium phosphate buffer (pH 7.0) and 2.5 mL of 0.4% TTC in the dark at 25 °C for 13 h. The TTC solution was removed and the cells were washed with 5 mL of distilled water. The reduced TTC was extracted with 5 mL of 95% ethanol at 60 °C for 30 min and the absorbance was read at 485 nm. TTC reduction was expressed as the ratio of the extracted red formazan concentration per gram of treated cells to that of untreated cells.

Measurement of ion leakage

Ion leakage was determined according to Zhao et al. (2008) with some modifications. The suspension cultured cells (0.2 g) were placed with 16 mL of deionized water at 25 °C for 2 h. After incubation, the conductivity in the bathing solution was determined (C1). Then, the samples were boiled for 30 min, and the conductivity was read again in the bathing solution (C2). Relative ion leakage was expressed as a percentage of the total conductivity after boiling (Relative ion leakage % = C1/C2 × 100).

Analysis of lipid peroxidation

Malondialdehyde (MDA), an indicator of lipid peroxidation, was determined by the thiobarbituric acid (TBA) reaction. The samples were homogenized with a mortar and pestle in 5% trichloroacetic acid (TCA), and the homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was mixed with the equal volume of 5% TCA containing 0.67% TBA. The mixture was heated at 100 °C for 30 min and quickly cooled to room temperature. Absorbance was read at 450, 532, and 600 nm after centrifugation at 1000 × *g* for 10 min again. MDA content was calculated using the following formula: MDA (μM) = $[6.45(OD_{532} - OD_{600}) - 0.56OD_{450}]$ (Guo et al., 2006).