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Reverse function of ROS-induced CBL10 during salt and drought stress responses

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ABSTRACT

Cellular levels of Ca^{2+} and reactive oxygen species (ROS) are maintained at low levels in the cytosol but fluctuate greatly when acting as second messengers to decode environmental and developmental signals. Phytohormones are primary signals leading to various changes in ROS or Ca^{2+} signaling during synergistic and antagonistic cross-talk. In this study, we found that brassinosteroids (BRs), hormones involved in diverse plant developmental processes, promote ROS production. To identify downstream signaling components of ROS during BR-mediated plant development, we searched for genes whose expression remained unchanged by ROS only in BR- signaling mutants and found *calcineurin B-like (CBL) 10*, which encodes a CBL should be changed to CBL10. protein that senses calcium. ROS-induced *CBL10* expression was nullified and endogenous *CBL10* expression in the shoot was low in the BR-signaling mutant. Using a *cbl10* mutant and a transgenic plant overexpressing *CBL10*, we showed that BR sensitivity during hypocotyl growth decreased in the *cbl10* mutant under salt stress, providing an additional mechanism for positive regulation of salt stress by CBL10. We also demonstrated that CBL10 negatively affects tolerance to drought and is not mediated by abscisic acid-induced signaling. Our results suggest that Ca^{2+} signaling through CBL10 differently affects the response to abiotic stresses, partly by regulating BR sensitivity of plant tissues.

1. Introduction

Brassinosteroids (BRs) are involved in a variety of plant developmental processes, such as cell expansion and division, vascular differentiation, leaf architecture, pollen tube elongation, and fruit development [1]. In addition to these important roles in life-long development, BRs also regulate skotomorphogenesis in response to light and resistance to a wide variety of abiotic and biotic stresses [2,3]. BRs confer thermo-tolerance through synthesis of heat-shock proteins [4] and induce high resistance to bacterial and fungal pathogens in tobacco and rice [5]. However, how BRs exert positive effects on stress tolerance is not understood.

Exquisite synergistic and antagonistic cross-talk between phytohormones is decoded by second messengers such as Ca^{2+}, inositol triphosphate, and reactive oxygen species (ROS), which carry the signals further downstream to effectors that exert specific functions, depending on the original primary signals. Increases in cytosolic calcium and ROS production levels are rapid responses to pathogenic attacks in plants and animals [6,7]. Various plant hormones, such as abscisic acid (ABA) and BRs induce ROS production by affecting expression of *Atrboh* genes encoding NADPH oxidases, which regulates stress tolerance or stomatal closure as well as the accumulation of other hormones [8,9]. Ca^{2+} is also involved in various processes, including pollen tube growth and root hair elongation [10,11], as well as responses to light, plant hormones, and environmental stresses [12,13]. In addition, ABA-induced ROS production activates Ca^{2+} signaling in guard cells resulting in stomata closure [14,15] and Ca^{2+}-induced cellular ROS accumulation in root hair [16], suggesting mutual relationship between ROS and Ca^{2+}. Therefore, cellular levels of Ca^{2+} and ROS, which apparently fluctuate greatly due to many environmental and developmental signals, are tightly regulated to be maintained in low levels in the cytosol through the intracellular and extracellular compartmentalization or specific scavenging mechanisms [13,17].

Changes in cellular Ca^{2+} are perceived by Ca^{2+} binding proteins, such as calmodulins (CaMs) and calcineurin B-like proteins (CBLs). As these calcium sensors do not have intrinsic activities, their conformational changes upon Ca^{2+} binding transmit the Ca^{2+} signal to target proteins [18], whereas calcium-dependent protein kinases and calcium and calmodulin-dependent protein kinases are directly activated by Ca^{2+} binding [19].

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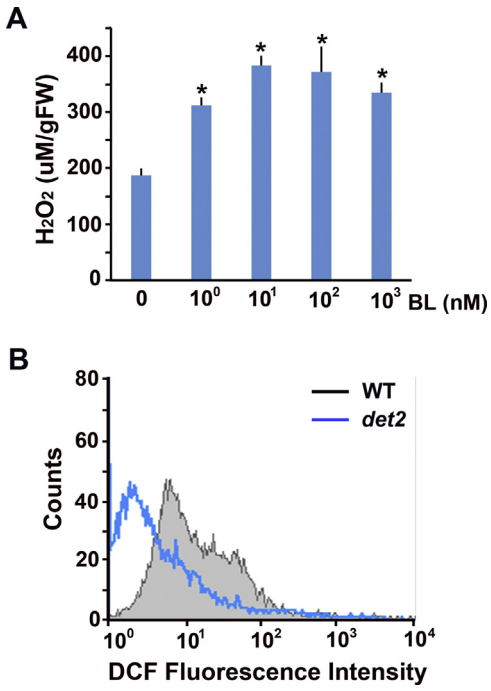

Fig. 1. Brassinosteroids (BRs) promote reactive oxygen species (ROS) production. (A) H_2O_2 contents were quantitatively measured in response to different concentrations of brassinolide (BL). Ten-day-old seedlings were treated with the indicated concentrations of BL for 1 h. Bar denotes standard error. Analysis of variance is used to compare seedling responses to treatments with or without BL ($^*P \leq 0.001$). (B) ROS production was measured in *det2* and wild type protoplast after a 15-min exposure to BL. Fluorescence from 30,000 protoplasts was counted each time. All experiments were repeated five times.

CBLs have sequences similar to that of yeast calcineurin, which is a protein phosphatase involved in salt tolerance [20]. In *Arabidopsis*, there are 10 members of CBLs composed of four EF-hand motifs to which Ca^{2+} binds [18,21]. Expression of CBLs is differentially regulated primarily by environmental changes, such as cold, drought, and salinity, and by stress-induced plant hormones, such as ABA [22]. Therefore, their physiological functions are well-correlated with abiotic stress responses. Lack of *CBL1* or its closest homolog *CBL9* causes hypersensitivity to osmotic stress [23,24]. Overexpression of *CBL1* results in enhanced tolerance to salt and drought stress in *Arabidopsis*. However, the same transgenic plants are less resistant to freezing [23]. The *cbl9* mutant shows hypersensitivity to ABA during seed germination and early seedling development, whereas ABA sensitivity of *cbl1* mutant is not altered [24]. CBL4/salt-overly sensitive 3 (SOS3) and CBL10 sense the increase in Ca^{2+} induced by salt [25,26].

In this study, we examined the role of BR in ROS production and identified *CBL10* as a gene whose expression is induced by ROS in wild type plants but not in the BR-signaling mutant. Using *cbl10* T-DNA insertional mutant and transgenic plants overexpressing *CBL10*, we demonstrated the role of CBL10 under salt and drought.

2. Materials and methods

2.1. Plant materials and growth conditions

We used *Arabidopsis thaliana* Columbia-0 (Col-0) as the wild type in all experiments. BR-biosynthetic mutant *det2* and BR signaling mutant *bri1-9* seeds used in this study were in Col-0 background. The *cbl10* T-DNA insertional knock-out mutant seeds (Salk_056042) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). To generate transgenic plants overexpressing *CBL10*, wild type cDNA was PCR-amplified with *CBL10 ORF-F* and *CBL10 ORF-R* primers containing BamHI and SalI restriction sites, respectively. The amplified fragments were cleaned, and cut with BamHI and SalI, and ligated to the pBluescript vector cut with using the same restriction enzyme set. The plasmid construct (*pBS-CBL10 ORF*) from transformed colonies was confirmed to have no mis-incorporated nucleotides by sequencing. To prepare the binary construct for plant transformation, CBL10 open reading frame was re-amplified with CBL10 ORF-F3 and CBL10 GW-R using *pBS-CBL10 ORF* as the template. The amplified fragment was cloned into pGW103 vector using the gateway system. The resulting *pGW103-CBL10OE* construct was transformed into wild type Col-0 using the *Agrobacterium*-mediated floral dipping method. Transformants were selected using the herbiside Basta.

For the growth of plants on plates, seeds were sterilized with 75% ethanol containing 0.05% Tween-20 for 15 min and washed twice with 95% ethanol. The dried seeds were germinated in 1/2 Murashige and Skoog (MS) medium (Duchefa, Haarlem, Netherlands) containing 0.8% phytoagar. For growth of plants in soil, seeds were sown directly onto Sunshine #5 soil. All plants were grown at 22 °C under long-day conditions (16 h light/8 h dark) under 82 μmol m^{-2} s^{-1} light intensity (Delta OHM, Caselle di Selvazzano, Italy).

2.2. Measuring ROS production

To measure the H_2O_2 level, we used an Amplex Red Peroxidase/H_2O_2 Assay Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol with some modifications as described by Kim et al. [27]. After treating 10-day-old seedlings with 1 μM brassinolide (BL) for 1 h, crude extracts were obtained from the seedlings in extraction buffer (50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM EDTA, and a protease inhibitor cocktail; Roche Diagnostics, Manheim, Germany), and reaction mixtures containing horseradish peroxidase (0.2 U/mL) were prepared according to the manufacturer's protocol. After incubation for 30 min at room temperature, absorbance was measured at a wavelength of 571 nm using a spectrophotometer (DU730; Beckman Coulter, Inc., Fullerton, CA, USA). To measure ROS levels by a flow cytometry, protoplasts were isolated from the wild type and *det2* mutants that were grown for 4 weeks according to the procedure described by Yoo et al. [28] and incubated with 5 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Millipore, Bedford, MA, USA) for 5 min. Fluorescence intensity was measured using a FACScan flow cytometer (Beckton Dickinson, San Jose, CA, USA) with excitation and emission set to 488 nm and 530 nm, respectively.

2.3. Determination of chlorophyll contents

Chlorophyll was extracted overnight from each sample in 5 mL 100% ethanol at room temperature. Absorbance of cooled solution containing chlorophyll was measured at 663 nm and 645 nm using a spectrophotometer (DU730; Beckman Coulter, Inc., USA) and calculated by Arnon's equation [29].

2.4. Assessment of tolerance to drought treatment

Drought condition was applied according to the description of Fu et al. [30]. Each seed was sown in soil pots saturated with the same amount of water and grown for 3 weeks under the conditions described in Section 2.1. During this period, humidity was maintained constant by controlled irrigation using a portable hygrometer. After a 3-week growth period, irrigation was interrupted for 9–12 days and resumed. The number of plants that resumed growth was observed 7 days after re-watering commenced.