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The role of brassinosteroids in the regulation of the plasma membrane H^+-ATPase and NADPH oxidase under cadmium stress

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ABSTRACT

The present research aim was to define the role of brassinosteroids (BRs) in plant adaptation to cadmium stress. We observed a stimulating effect of exogenous BR on the activity of two plasma membrane enzymes which play a key role in plants adaptation to cadmium stress, H^+-ATPase (EC 3.6.3.14) and NADPH oxidase (EC 1.6.3.1). Using anti-phosphothreonine antibody we showed that modification of PM H^+-ATPase activity under BR action could result from phosphorylation of the enzyme protein. Also the relative expression of genes encoding both PM H^+-ATPase and NADPH oxidase was affected by BR. To confirm the role of BR in the cadmium stimulating effect on activity of both studied plasma membrane enzymes, an assay in the presence of a BR biosynthesis inhibitor (propiconazole) was performed. Moreover, as a tool in our work we used commercially available plant mutants unable to BR biosynthesis or with dysfunctional BR signaling pathway, to further confirm participation of BR in plant adaptation to heavy metal stress. Presented results demonstrate some elements of the brassinosteroid-induced pathway activated under cadmium stress, wherein H^+-ATPase and NADPH oxidase are key factors.

1. Introduction

Cadmium (Cd) is a non-essential element, very toxic to plants, which disturbs many metabolic processes. It inhibits photosynthesis and respiration, disturbs the water balance and nutrient management, and alters enzyme activity [1]. Moreover, toxicity of Cd may be related with the oxidative burst as a result of reactive oxygen species (ROS) enhanced generation [2]. Overproduction of ROS causes disturbance of redox balance and oxidative damage of macromolecules in plant cells [3]. Oxidation of membrane fatty acids and proteins result in an increase in permeability of the plasma membrane (PM), membrane enzyme inactivation and membrane integrity disruption. To survive Cd stress, plants have evolved mechanisms to activate optimal responses and adapt to adverse environment conditions. The plant molecular response to Cd stress is mediated through immobilization of Cd by means of the cell wall and synthesis of chelators such as phytochelatins, metallothioneins and organic acids [2]. Cd detoxification also involves vacuolar compartmentalization and synthesis of heat shock proteins (HSPs) [4]. On the other hand several phytohormones regulate the plant responses to various stresses [5], but very few studies focus on hormone crosstalk involved in the plant response to Cd. Villiers et al. [6] reported significant similarity between Cd and brassinosteroid (BR) induced responses at the gene expression level. BRs are steroids phytohormones fulfilling multiple functions in plant growth [7] and development [8], regulating various biological processes among others cell division and elongation, and synthesis of nucleic acid and protein [9]. Moreover, BRs are involved in plant responses abiotic stress such as salinity [10], drought stress [11] and temperature extremes [12], but the underlying BR mediated stress response mechanisms in plants are unclear.

The BR signaling cascade is very well known, and many elements of this pathway have been discovered [13]. Binding of BR to transmembrane receptor BR insensitive 1 (BRI1) resulted in receptor autophosphorylation and the following link of the co-receptor BRI1-associated receptor kinase 1 (BAK1). Consecutive transphosphorylation of the BRI1/BAK1 receptor kinase complex results in activation of downstream BR signaling processes, leading to the regulation of transcription factors, affecting target gene expression. On the other hand, few data elucidate the events in the cell surface within the plasma membrane directly after perception of the hormone by the plasma membrane receptor BR insensitive 1 (BRI1). Our understanding of BR signaling and the role of BR in plant metabolic processes derives from research

Abbreviations: 24EBL, 24-epibrassinolide; BAK1, BRI1-associated receptor kinase 1; BR, brassinosteroid; BRI1, transmembrane receptor brassinosteroid insensitive; Cd, cadmium; DAB, 3,3′-diaminobenzidine; DMSO, dimethylsulfoxide; DPI, diphenyleneiodonium; H_2O_2, hydrogen peroxide; HSPs, heat shock proteins; MS, Murashige and Skoog medium; $O_2^{\cdot -}$, superoxide radical anion; PCR, polymerase chain reaction; Pcz, propiconazole; PM, plasma membrane; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

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involving BR insensitive mutants. To date, there have been described many mutants with disruption of BR perception on the plasma membrane surface with mutation of the receptor (*bri1*) or co-receptor (*bak1*) and BR biosynthesis with mutation of enzymes involved in the BR synthesis pathway (*dwarf*). *Arabidopsis* plants are a helpful tool for BR study in view of commercial mutants availability and wide genome information. New reports on the molecular machinery of BR perception indicate that BR binding could trigger activation of the plasma membrane (PM) located H^+-ATPase by a yet unknown mechanism and modify the membrane potential [14]. Our previous study [15–19] showed that PM H^+-ATPase and NADPH oxidase, which modified its activity, are the key enzymes in adaptation of plants to various stress conditions including Cd stress. Now we focus on the role of BR in PM H^+-ATPase and NADPH oxidase activities under Cd stress. The first living structure in the plant cell exposed to Cd is the plasma membrane. Cd damaging the plasma membrane structure causes disorder of its function and loss of essential substances. The repair process in plant cells exposed to Cd ions requires generation of an electrochemical proton gradient through the plasma membrane which allows maintenance of secondary transport, ionic balance, eliminating toxic ions and refilling nutrients. The plasma membrane proton pump (PM H^+-ATPase) is the only pump which can generate an electrochemical gradient. The PM proton pump is an enzyme that catalyzes one-way proton transport across the PM using ATP as an energy source and plays a key role in regulation of ion homeostasis in the cytosol. The PM proton pump is encoded by a multigene family [20]. Moreover, environmental stress could be a factor which activate PM H^+-ATPase genes. Besides genetic regulation of the PM proton pump, there is also possible regulation of this enzyme activity by rapid changes in post-translational level as a result of reversible phosphorylation [21].

Hydrogen peroxide (H_2O_2) as a second messenger could be an important molecule that mediates in signaling network triggering during the plant response to stress stimuli. Some authors have indicated change of gene expression of plasma membrane PM H^+-ATPase under abiotic stresses. In a previous study [22] the stimulating effect of H_2O_2 on the expression level of a gene encoding the PM proton pump was reported. In our previous study [15] we found that PM proton pump activity is correlated with NADPH oxidase activity. PM NADPH oxidase is the main source of the superoxide radical anion ($O_2^{\cdot -}$) in the apoplast, and as an unstable molecule it is converted into H_2O_2 which transfers across membranes passively or through water channels [23]. NADPH oxidase activity can be modulated at the gene expression level. In cucumber nine genes encoding NADPH oxidase *CsRbohA-J* were investigated [15]. Moreover, rapid generation of ROS, which can serve as protective agents, is a part of the plant adaptation to stress [24,25]. In our previous study [15] we found evidence for an important role of ROS in the plant response to Cd. Our results showed that PM NADPH oxidase could be a part of plant response to Cd stress by affecting the activity of PM H^+-ATPase, whereas Xia et al. [26] reported that BR application enhanced tolerance to various stresses preceded by increased NADPH oxidase activity. The aim of present investigates was to demonstrate the role of BRs in the modification of the plasma membrane enzymes (H^+-ATPase and NADPH oxidase) in adaptation of plants to Cd stress conditions.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L. Visconsin) were germinated in darkness for 48 h at 25 °C and then transferred for 6 days to a medium containing: 1.7 mM KNO_3, 1.7 mM $Ca(NO_3)_2$, 0.33 mM KH_2PO_4, 0.33 mM $MgSO_4$, and the microelements 75 μM ferric citrate, 10 μM $MnSO_4$, 5 μM H_3BO_4, 1 μM $CuSO_4$, 0.01 μM $ZnSO_4$, and 0.05 μM Na_2MoO_4 (control conditions). Some of the plants were treated with 10 μM $CdCl_2$ for 3 days and then returned to control conditions for another 3 days (Cd, post-stressed plants). Some of the plants exposed to Cd were transferred to control conditions containing additionally Pcz (propiconazole). For the phytohormone (24-EBL – 24-epibrassinolide) and/or DPI (diphenyleneiodonium) treatment analysis cucumber seedlings after 5 days of growth on the control medium were transferred to the same medium containing additionally 24-EBL and/or DPI for 24 h. Plants were cultivated hydroponic at 25 °C by day and 22 °C by night under a 16 h photoperiod (light intensity: 180 μmol m^{-2} s^{-1}).

Seeds of *Arabidopsis thaliana* plants, ecotype Columbia (Col-0) and BR signaling: BRI-1 (N503371) BAK1-1 (N6125) or synthesis: DWARF-4 (N520761) mutants were gained from the Nottingham Arabidopsis Stock Centre (NASC) University of Nottingham. The seeds were sterilized with 3% sodium hypochlorite and sown on top of cut, dark Eppendorf tubes filled with 0.5 × MS medium pH 5.7 with 0.8% agar. After stratification at 4 °C for 3 days the tubes were transferred to a plant growth chamber to a nutrient medium containing: 22.3 μM EDTA, 0.5 mM $MgSO_4$, 31.3 μM NaOH, 5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.13 mM $NH_4H_2PO_4$, 29.3 μM NH_4NO_3 and the microelements 22.4 mM ferric citrate, 9.68 H_3BO_3, 2.03 mM $MnCl_2$, 0.314 mM $ZnSO_4$, 0.21 mM $CuSO_4$, 0.139 mM MoO_3, 0.0859 mM $Co(NO_3)_2$ to maximize the yield of vegetative tissue. Plants were grown hydroponically under an 8 h photoperiod while vegetative tissue growth is favored, preventing flowering (light intensity: 180 μmol m^{-2} s^{-1}) at 22 °C by day and 20 °C by night. Six-week old plants were transferred for 6 days to low concentrated medium in which Cd is well uptake and in which the metal does not precipitate, containing: 0.5 mM $Ca(NO_3)_2$, 0.13 mM $NH_4H_2PO_4$, 2.5 mM KNO_3, 1.25 mM K_2SO_4, 0.5 mM $CaSO_4$, 0.5 mM $MgSO_4$, 22.3 μM EDTA, 31.3 μM NaOH, 29.3 μM NH_4NO_3 and the microelements 22.4 mM ferric citrate, 9.68 H_3BO_3, 2.03 mM $MnCl_2$, 0.314 mM $ZnSO_4$, 0.21 mM $CuSO_4$, 0.139 mM MoO_3, 0.0859 mM $Co(NO_3)_2$ (control conditions). Some of the plants were treated with 10 μM $CdCl_2$ for 3 days and then returned to control conditions for another 3 days (Cd, post-stressed plants). 50 mg of root tissue was ground in liquid nitrogen to isolate total RNA.

2.2. Chemicals

The BR: 24-EBL (24-epibrassinolide) was dissolved in 96% ethanol to achieve a stock solution of 10 mM and diluted in nutrient medium to a final concentration range of 1 nM–100 μM in 24 h. The stock solution was stored at −20 °C. The inhibitor of NADPH oxidase DPI was dissolved in DMSO (dimethylsulfoxide) and then added to the nutrient medium to the final concentration of 20 μM 24 h before the isolation of PM. The inhibitor of BRs synthesis Pcz was dissolved in 96% ethanol and then supplied to the nutrient medium to the final concentration 1 μM for 3 days.

2.3. Isolation of PMs

According to Larsson's method [27] modified by Kłobus plasma membrane vesicles from cucumber roots were isolated using a two-phase system [28]. The plant samples were homogenized in an extraction buffer containing 25 mM BTP-MES pH 7.5, 5 mM DTT, 330 mM sorbitol, 5 mM EDTA, 0.5 mM PMSF, 0.2% BSA, and 5 mM KCl. The homogenate was filtered and centrifuged at 18 000*g* for 10 min. Microsomal vesicles were pelleted from the supernatant by centrifugation at 80 000*g* for 30 min 4 °C. The pellet subsequently was suspended in a buffer containing 5 mM BTP-MES pH 7.5, 5 mM KCl, 330 mM sorbitol, 0.1 mM EDTA and the PM was isolated by loading the microsomal fraction into a two phase system containing 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 330 mM sorbitol, 5 mM KCl, and 5 mM BTP-MES (pH 7.5) and centrifuged at 500*g* for 5 min. The PM fraction accumulated in the upper phase was diluted in buffer containing 5 mM BTP-MES pH 7.5, 5 mM KCl, 330 mM sorbitol, 0.1 mM EDTA and centrifuged for 30 min at 80 000*g*. The obtained PM fraction from cucumber roots was resuspended in the same buffer and used for further analysis or quickly frozen in liquid N_2 and stored at −80 °C.