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Effect of 24-*epi*brassinolide on *Brassica napus* alternative respiratory pathway, guard cells movements and phospholipid signaling under salt stress

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ABSTRACT

Using *Brassica napus* roots we observed statistically significant increase in alternative respiratory pathway in response to exogenous 24-*epi*brassinolide (EBL) under optimal conditions and salinity. Also we observed activation of phospholipid signaling under the same conditions in response to EBL by measuring levels of lipid second messengers – diacylglycerol (DAG) and phosphatidic acid (PA). We found that brassinosteroids cause closure of stomata in isolated leaf disks while inhibitors of alternative oxidase cancelled these effects. This study demonstrates that BRs activate total respiration rate, alternative respiratory pathway, production of PA and DAG, stimulate stomata closure and growth under optimal conditions and salinity. Also, specific inhibitor of brassinosteroids biosynthesis decreased alternative respiratory pathway and production of lipid messengers in rape plants.

1. Introduction

BRs are steroid class of phytohormones involved in regulation of cell proliferation and differentiation [1–3], cell adaptation and induction of plant tolerance to a range of abiotic [4–6] and biotic [7–9] stresses that are often accompanied by reactive oxygen species (ROS) production and oxidative stress.

Alternative oxidase (AOX) as a key component of the alternative respiratory pathway is involved in adaptation to a range of environmental stresses, namely salinity [10], drought [11], metals [12–15], pathogens [16,17]. AOX activation decreases ROS level in mitochondria and protect mitochondrial respiratory chain from self-inhibition and oxidative stress development [18]. Despite a series of experimental work of the last 2–3 years, there are many open questions concerning the role of the BRs in the AOX regulation under stress conditions [19–21]. One of the important function of the AOX is the control of ROS and nitric oxide (NO) levels to protect plant cells against possible oxidative stress [18,22].

BRs are believed to be involved in regulation of ROS cytotoxic effects in plant cells via induction of different antioxidant systems [23–26]. At the same time, BRs induce ROS-dependent signaling via activation of NADPH oxidase and production of ROS and NO molecules as second messengers [27–30]. These data point at possible interdependence between BRs and AOX in the process of ROS production. Activation of ROS signaling by BRs has a great impact on guard cells functioning as one of the key component in maintaining water status under stress conditions, particularly salinity [31].

We have recently found that EBL is involved in regulation of alternative respiratory pathway affecting AOX1A isoform in *Arabidopsis thaliana* green leaves under salinity conditions [19,20]. Moreover, radioisotope and bioinformatics approach revealed possible protein molecular targets – mitochondrial

Abbreviations: AOX, alternative oxidase; BRs, brassinosteroids; BRZ, brassinazole; COX, cytochrome oxidase; DAG, diacylglycerol; DGK, diacylglycerol kinase; DW, dry weight; EBL, 24-*epi*brassinolide; mETC, mitochondrial electron transport chain; NBD, nitrobenzoxadiazole; NEM, *N*-ethylmaleimide; NO, nitric oxide; PA, phosphatidic acid; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; TLC, thin-layer chromatography.

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chaperones - which can be involved in BR-induced stimulation of cell respiration and mitochondria adaptation [32]. Taking into consideration the results of our previous work, the following conclusion can be drawn: BRs are capable to induce alternative respiratory pathway and activate molecular targets involved in adaptation of respiratory chain. At this moment, role of BRs in activation of alternative respiratory pathway and regulation of stress adaptation is actively studied in plants [21].

In present work we investigated the role of BRs (24-*epi*brassinolide, EBL) in alternative respiratory pathway activity, formation of phosphatidic acid (PA) and guard cell movements under salinity as a part of adaptation mechanism which aimed at reduction of ROS cytotoxic effects in mitochondria and induction of ROS signaling.

2. Materials and methods

2.1. Plant materials and growth conditions

Brassica napus breed "Magnat" plants have been used in this investigation. Prior the experiments plants have been grown for 3 days on 1/3 strength Hoagland-Arnon medium. On the 3-rd day plants were transferred for 24 h to plots with growth medium supplemented with 100 mM NaCl or/and EBL (final concentration - 10^{-7} M) or brassinazole (final concentration - 10^{-6} M). Growth conditions were 22 ± 2 °C with a 16-h photoperiod (photosynthetic photon flux density of 300 µmol photons m^{-2} s^{-1} generated with Fluora lamps, Osram). Roots of the 4-th day old plants were measured for respiration intensity and production of lipid second messengers.

2.2. Chemicals

Brassinazole was obtained from TCI-Europe (Germany), EBL was synthesized in the Laboratory of Steroid Chemistry (Institute of Bioorganic Chemistry, NAS of Belarus), phosphatidylcholine-nitrobenzoxadiazole (phosphatidylcholine-NBD) was from Invitrogen (USA), Tris-HCl, potassium cyanide (KCN), salicylhydroxamic acid (SHAM) were obtained from Sigma-Aldrich.

2.3. Investigation of cell respiration activity

Cell respiration activity was measured by monitoring oxygen consumption by the leaves (50 mg) in a 1 ml of air-saturated 5 mM Tris-HCl buffer (pH 6,0) thermostated at 25 ± 0.2 °C. Oxygen consumption was recorded with polarograph Oxygraph (Hansatech Instruments, UK, England) and converted from voltage to oxygen concentration with Oxygraph Plus software (Hansatech Instruments, UK, England). To determine the rates of total respiration, cyanide-sensitive and alternative respiratory pathways inhibitory analysis was used. Potassium cyanide (KCN, 1 mM in final volume) and salicylhydroxamic acid (SHAM, 3 mM in final volume) were used as inhibitors of cytochrome oxidase (COX) and AOX respectively. Maximal activity of alternative respiratory pathway

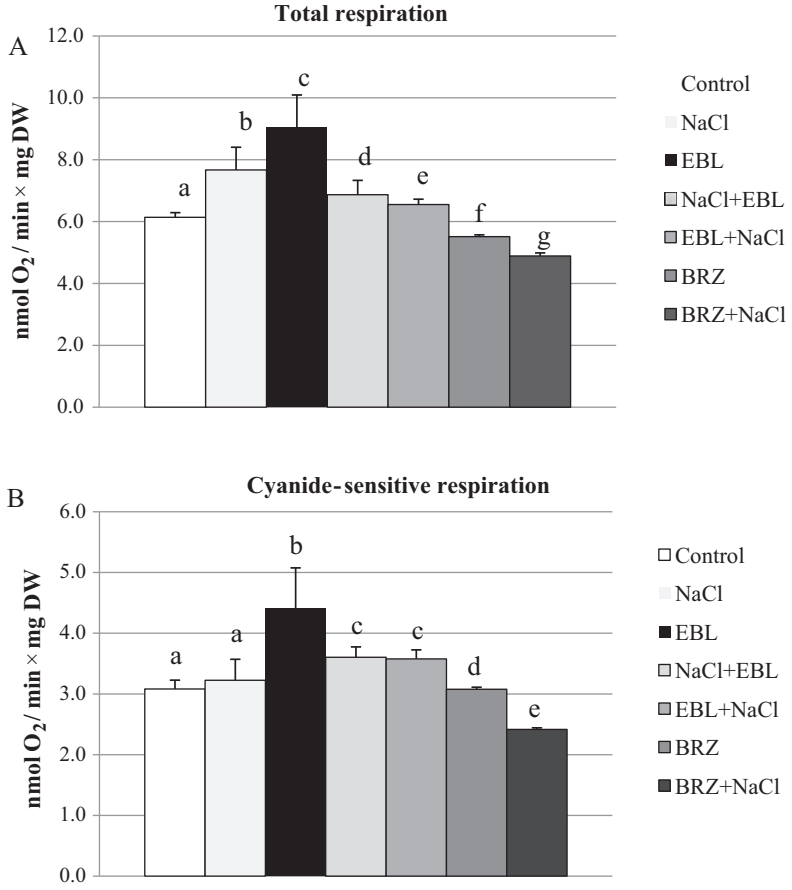

Fig. 1. Effect of BRs on *B. napus* respiration rate. A. Influence of EBL and BRZ on total respiration rate of *B. napus* roots in control conditions and under salinity 100 mM NaCl. B. Influence of EBL and BRZ on cyanide-sensitive respiration rate of *B. napus* roots in control conditions and under salinity 100 mM NaCl. Plants prior the analysis were grown for 3 days on 1/3 strength Hoagland-Arnon medium and then were transferred to experimental medium administrated with specific substances: 100 mM NaCl for 24 h; 10^{-7} M EBL for 24 h; 100 mM NaCl 24 h + 10^{-7} M EBL 24 h; 10^{-7} M EBL 24 h + 100 mM NaCl 24 h; 10^{-6} M BRZ 24 h; 10^{-6} M BRZ + 100 mM NaCl 24 h. Values followed by different letters are significantly differing at $P \leqslant 0.05$ according to LSD test. DW – dry weight, BRZ – brassinazole.