Environmental and Experimental Botany 96 (2013) 52–58

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Preliminary studies on the involvement of glutathione metabolism and redox status against zinc toxicity in radish seedlings by 28-Homobrassinolide

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ARTICLE INFO

Article history:
Received 26 June 2013
Received in revised form 5 August 2013
Accepted 19 August 2013

Keywords:
28-Homobrassinolide
Glutathione metabolism
Cysteine
Phytochelatins
Redox status
Zinc stress

ABSTRACT

The effect of exogenous application of 28-Homobrassinolide (HBR) on radish (*Raphanus sativus* L.) seedlings under zinc (Zn^{2+}) stress on glutathione (GSH) production, consumption and changes in redox status was investigated. Zinc toxicity resulted in oxidative burst as evidenced by increased accumulation of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content. These stress indices were significantly decreased by HBR supplementation. Under Zn^{2+} stress, GSH pool was decreased, while the contribution of oxidized glutathione (GSSG) to total GSH increased (GSSH/GSH ratio), this translated into significant reduction of GSH redox homeostasis. In addition, an increase of phytochelatins (PCs) was observed. In radish seedlings under Zn^{2+} stress, the activities of gamma-glutamylcysteine synthetase (γ-ECS), glutathione synthetase (GS), glutathione peroxidase (GPX), glutathione-S-transferase (GST) and cysteine (Cys) levels increased but the activity of glutathione reductase (GR) decreased. However, application of HBR increased the GSH pool and maintained their redox ratio by increasing the enzyme activities of GSH biosynthesis (γ-ECS and GS) and GSH metabolism (GR, GPX and GST). The results of present study are novel in being the first to demonstrate that exogenous application of HBR modulates the GSH synthesis, metabolism and redox homeostasis to confer resistance against Zn^{2+} induced oxidative stress.

1. Introduction

Zinc ($^{65.39}Zn_{30}$) a group IIB transition element (Garg and Kaur, 2013), is the second most abundant transition metal after iron (Fe) and plays an important role in plant growth and development. But at elevated levels it becomes toxic to plants (Broadley et al., 2007). Zinc (Zn^{2+}) is one of the major pollutants that are released into the environment as a result of industrial activities, mining, smelting, sewage sludge as well as persistent use of zinc fertilizers (White and Broadley, 2005; Mateos-Naranjo et al., 2008). Zinc toxicity symptoms include nutrient imbalances, growth inhibition, stunted growth, chlorosis, photosynthesis impairment, altered mitotic activity and membrane integrity at cellular level (Sagardoy et al., 2009; Gomes et al., 2013). Accumulated literature showed that, higher Zn^{2+} accumulation induces oxidative stress through an increased production of reactive oxygen species (ROS) i.e. superoxide radicals (O^{-}_2), hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2) (Lin and Aarts, 2012; Remans et al., 2012). The ROS thus generated posses strong oxidizing capacities that can attack nucleic acids, pigments, proteins, causing membrane lipid peroxidation and, altered activities of antioxidative enzymes and antioxidant content and finally leading to cell death (Gomes et al., 2013; Li et al., 2013).

Plants have developed different detoxification strategies to cope with excessive ROS under heavy metal stress. To protect the cells against oxidative damage, superoxide dismutase constitutes the first line of defense to funnel superoxide radicals into H_2O_2 and O_2. Subsequently, H_2O_2 is eliminated by conversion to water by the action of catalase and peroxidases (Gill and Tuteja, 2010). Of the various detoxification pathways, glutathione (GSH), its redox state (GSH/GSSG) and related enzymes [glutathione reductase (GR; EC 1.6.4.2), glutathione peroxidase (GPX; EC 1.11.1.9) and glutathione-S-transferase (GST; EC 2.5.1.18)] were found to play an indispensable role in detoxification of ROS (Anjum et al., 2012). Glutathione is non-enzymatic antioxidant involved in scavenging of ROS either directly or indirectly via ascorbate-glutathione cycle, sulphur metabolism, regulation of cellular homeostasis and, signal transduction (through either glutathionylation of proteins or thiol bridge redox reactions) (Anjum et al., 2012) and is considered to be the major intracellular redox buffer (Foyer and Noctor, 2011). Regulation of activities of the enzymes related to GSH

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; Cys, cysteine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; GR, glutathione reductase; GPX, glutathione peroxidase; GSSG, glutatathione disulfide; GSH, glutathione; GS, glutathione synthetase; GST, glutathione-S-transferase; HBR, 28-Homobrassinolide; OPT, *o*-phthaldialdehyde; PC, phytochelatin; PMSF, phenyl methyl sulfonyl fluoride; PVP, polyvinylpyrolidone; Zn^{2+}, zinc; γ-ECS, gamma-glutamylcysteine synthetase.

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http://dx.doi.org/10.1016/j.envexpbot.2013.08.003

biosynthesis and metabolism and, the changes in GSH level, GSH/GSSG ratio, can enhance the resistance and adaptation of plants to various environmental stresses (Rausch et al., 2007). Glutathione is synthesized from L-glutamate, L-cysteine and glycine in two ATP-dependent reactions catalyzed by gamma-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) and glutathione synthetase (GS; EC 6.3.2.3). Glutathione is oxidized to GSSG as part of its cellular antioxidant defense, and then, in turn, GSSG is reduced back to GSH by the action of GR using NADPH to maintain highly reduced state of GSH/GSSG (Foyer and Noctor, 2011; Seth et al., 2012). Glutathione peroxidase and GST use GSH pool as substrate to detoxify the hydrogen peroxide and xenobiotics by catalyzing their conjugation with GSH. Apart from that, plants synthesize sulphur-containing defense compounds *viz.* Phytochelatins (PCs), metallothioneins (MTs) and GSH which form complexes with heavy metals and subsequently sequestrated them to vacuoles (Cobbett and Goldsbrough, 2002; Yadav, 2012).

Brassinosteroids (BRs) comprises a class of plant specific polyhydroxylated derivatives of 5α-cholestane, structurally that resembles animal steroid hormones and ecdysteroids from insects. Brassinosteroids were classified as essential plant hormones nearly after the discovery of brassinolide (the first brassinosteroid) in the rape (*Brassica napus* L.) pollen. They are distributed ubiquitously throughout the plant kingdom (Bajguz and Hayat, 2009; Clouse, 2011). Up to date, 70 compounds belonging to the class of BRs are characterized, among them 65 are in free form and 5 in conjugated form (Bajguz and Tretyn, 2003). Brassinosteroids regulate various aspects of plant growth and development, including cell elongation, photomorphogenesis, xylem differentiation, seed germination, leaf bending and epinasty, proton pump activation, regulation of gene expression, nucleic acid and protein synthesis and photosynthesis (Clouse, 2011; Yang et al., 2011). As indicated by numerous studies, BRs can reduce negative impacts of various abiotic and biotic environmental stresses induced by drought, high temperature, chilling, salinity, and heavy metals (Vardhini et al., 2010; Vriet et al., 2012). Some reports suggest that BRs help the plants to withstand the toxic effects of pathogen infection and, residues of pesticides and herbicides (Bajguz and Hayat, 2009; Xia et al., 2009). Although, BRs utilization has been increasing in agriculture and horticulture as a plant growth regulator to scale up the crop production, the underlying mechanisms by which BRs influences plant growth and development, and stress tolerance are poorly understood (Xia et al., 2009).

Previous studies have demonstrated that 28-Homobrassinolide (HBR) enhances the growth and the antioxidant responses of plants subjected to abiotic stresses like salinity (Arora et al., 2008; Hayat et al., 2010) and heavy metal toxicity (Sharma et al., 2008, 2011; Fariduddin et al., 2009; Yusuf et al., 2011). Little attention has been focused on glutathione metabolism under heavy metal stress especially under zinc stress. In the present work, our objective was to demonstrate that the exogenous application of 28-Homobrassinolide under Zn^{2+} toxicity conditions may provides an effective strategy for combating this ionic stress, by stimulating GSH synthesis, metabolism and maintaining redox homeostasis. To best of our knowledge, the data presented in the present work are novel in being the first to demonstrate that exogenous application of HBR regulates the GSH synthesis and metabolism in the seedlings exposed to excess Zn^{2+}.

2. Materials and methods

2.1. Experimental material

Radish (*Raphanus sativus* L. cv. 'Japanese white') seeds used in the present investigation were obtained from National Seed Corporation, Hyderabad, India. 28-Homobrassinolide (HBR) was procured from CID Technologies Inc., Brampton, Ontario, Canada.

2.2. Growth conditions

Seeds were surface sterilized by incubation in 0.5% (v/v) sodium hypochlorite for 5 min, followed by five washes with double distilled water. A dose-response curve with radish was established for a workable concentration of Zn^{2+} (1, 2.5, 5, 7.5 and 10 mM as $ZnSO_4{\cdot}7H_2O$) and at an IC_{50} of 5 mM Zn^{2+} concentration, the germination and seedling growth was found inhibited substantially but not completely. The seeds were placed in autoclaved 90 mm petri dishes lined with Whatman # 1 filter paper wetted with 5 mL of treatment solution. The treatments were divided into (1) distilled water (control), (2) 0.5 μM, 1 μM and 2 μM 28-Homobrassinolide solutions, (3) 5 mM Zn^{2+} (stress control), (4) 5 mM Zn^{2+} solution supplemented with 0.5 μM, 1 μM and 2 μM 28-Homobrassinolide solutions. The seeds were allowed to germinate at 25 ± 1 °C in dark in a growth chamber. Experiment was repeated twice with five replicates each with 20 seeds for every treatment. However, 3 mL test solutions were added on 4th day of the experiment. Seven day old seedlings were employed for analytical purpose.

2.3. Zinc (Zn^{2+}) analysis

Zinc metal in seedlings was determined by tri-acid mixture (H_2SO_4, HNO_3 and $HClO_4$ in a 1:5:1 ratio) with atomic absorption spectroscopy (Model AA-6200, Shimadzu, Japan) as described by Allen et al. (1976). The bioconcentration factor (BCF) was calculated by dividing the metal concentration in the tissue (mg kg^{-1}) at the harvest by the metal concentration in the applied external solution (mg L^{-1}).

2.4. Concentration of MDA and H_2O_2

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content using the thiobarbituric acid (TBA) method as described by Heath and Packer (1968). The MDA contents were calculated based on A_{532}–A_{600} with an extinction coefficient of 155 mM^{-1} cm^{-1}.

The hydrogen peroxide (H_2O_2) content was estimated by measuring the absorbance of titanium hydroperoxide complex at 415 nm. The H_2O_2 content was calculated from a standard curve according to the protocol of Mukherjee and Choudhari (1983).

2.5. Extraction and assay of enzyme activities

Fresh seedlings were homogenized with 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, and centrifuged 15 min at 10,000 × g (0 °C) and extract was used for determination of γ-ECS and GS activities.

Activity of γ-ECS was determined using the method of Rüegsegger and Brunold (1992). The reaction was initiated by adding the extract (150 μL) to give 500 μL assay mix containing 100 mM HEPES (pH 8.0), 50 mM $MgCl_2$, 20 mM glutamate, 1 mM cysteine, 5 mM ATP, 5 mM phosphoenolpyruvate, 5 mM DTT, 10 units mL^{-1} pyruvate kinase. The reaction mixture was incubated for 45 min at 37 °C and the reaction was stopped by addition of 100 μL of 50% TCA. The mixture was centrifuged and the supernatant was used for estimation of phosphate content by the phosphomolybdate method.

The activity of GS was determined according to the method of Cobbett et al. (1998). The reaction mixture contained 100 mM Tris–HCl (pH 8.0), 50 mM KCl, 20 mM $MgCl_2$, 1 mM γ-EC, 2 mM glycine, 5 mM ATP, 5 mM phosphoenolpyruvate, 5 mM DTT, 10 units mL^{-1} pyruvate kinase. The reaction was started by adding