Journal of Inorganic Biochemistry 103 (2009) 1497–1503

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

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

Effect of phosphate on aluminium-inhibited growth and signal transduction pathways in *Coffea arabica* suspension cells

Leticia Chee-González [a], J. Armando Muñoz-Sánchez [a], Graciela Racagni-Di Palma [b], S.M. Teresa Hernández-Sotomayor [a,*]

[a] Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43, No. 130, Col. Chuburná de Hidalgo, 97200 Mérida, Yucatán, Mexico
[b] Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, Río Cuarto 5800, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 8 April 2009
Received in revised form 29 May 2009
Accepted 29 July 2009
Available online 15 August 2009

Keywords:
Phosphate deficiency
Aluminium toxicity
Coffea arabica
Phospholipase C

ABSTRACT

In acid soils, aluminium (Al) toxicity and phosphate (Pi) deficiency are the most significant constraints on plant growth. Al inhibits cell growth and disrupts signal transduction processes, thus interfering with metabolism of phospholipase C (PLC), an enzyme involved in second messenger production in the cell. Using a *Coffea arabica* suspension cell model, we demonstrate that cell growth inhibition by Al toxicity is mitigated at a high Pi concentration. Aluminium-induced cell growth inhibition may be due to culture medium Pi deficiency, since Pi forms complexes with Al, reducing Pi availability to cells. Phosphate does not mitigate inhibition of PLC activity by Al toxicity. Other enzymes of the phosphoinositide signal transduction pathway were also evaluated. Aluminium disrupts production of second messengers such as inositol 1,4,5-trisphosphate (IP_3) and phosphatidic acid (PA) by blocking PLC activity; however, phospholipase D (PLD) and diacylglycerol kinase (DGK) activities are stimulated by Al, a response probably aimed at counteracting Al effects on PA formation. Phosphate deprivation also induces PLC and DGK activity. These results suggest that Al-induced cell growth inhibition is not linked to PLC activity inhibition.

1. Introduction

Highly acidic soils present several limiting factors for plant growth, including toxic aluminium (Al) levels and deficiencies in essential elements such as phosphate (Pi). These particular constraints are significant because of their ubiquity and acute impacts on plant growth [1]. When soil pH drops below 5, rhizotoxic Al species are solubilised into the soil solution at levels which inhibit root growth and function. Al toxicity occurs through binding with the cell wall, DNA and the cell membrane [2]. Micromolar concentrations of Al^{3+} in soil solution inhibit root development at the organ, tissue and cellular levels, limiting water and nutrient uptake [3].

Several plant species that grow in acid soils have developed metabolic adaptations to resist high Al^{3+} availability in the soil solution [4]. Higher plants are stationary and cannot escape stress, and have therefore developed specific strategies to mitigate exposure to some soil toxins [5]. Plants have evolved numerous adaptive strategies in response to soil Pi deficiencies and toxic Al concentrations. For example, exudation of organic acids such as citrate and malate sparingly mobilize available forms of Pi and chelate toxic Al^{3+} [1,6,7]. However, organic acid exudation induced by Pi deficiency differs from that induced by Al toxicity [8–10].

Phosphate probably plays a role in plant Al tolerance strategies. In wheat, Pi efflux may be a potential Al tolerance mechanism [11], and Tan and Keltjens [12] found that increasing Pi supply may mitigate Al phytotoxicity, possibly by improving Pi uptake and root growth. Chelation of apoplastic and rhizospheric Al by Pi apparently contributes to Al exclusion in certain cultivars and species. In buckwheat, root apex Pi content has been significantly correlated with Al immobilization and detoxification [11,13–16]. Using soybean, Liao et al. [17] reported that two P-efficient genotypes exhibited higher Al tolerance than two P-inefficient genotypes when treated with high Pi levels, and that Pi supplementation of the media significantly decreased Al accumulation in the root tips of both P-efficient and P-inefficient genotypes.

In plant tissues and cells, Al is known to bind to the cell wall [18,19], induce changes in plasma membrane properties [20], cause peroxidation of membrane lipids [20,21] and influence membrane integrity [22]. However, the steps that initiate the plant signalling response to Al are not yet fully understood. Research using wheat roots [23] and coffee suspension cells [24,25] has shown that Al alters the phospholipids signal transduction pathway, specifically reducing phospholipase C (PLC) activity. The PLC enzyme is involved in processes such as growth, development

* Corresponding author. Tel.: +52 999 981 3966; fax: +52 999 981 3900.
E-mail address: ths@cicy.mx (S.M. Teresa Hernández-Sotomayor).

0162-0134/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2009.07.020

and differentiation [26], and catalyses hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [27]. DAG can be phosphorylated by diacylgycerol kinase (DGK) to produce phosphatidic acid (PA), which, together with IP_3, is involved in calcium homeostasis regulation, protein kinase activation and membrane channel activation [27–31]. Al also inhibits PA formation by blocking PLC activity [25], although it is generated by both the PLC/DGK pathway and phospholipase D (PLD) activity [29]. In coffee suspension cells, Al toxicity increases lipid kinase activity, responsible for formation of the phospholipids membrane, and Al may also modify some enzymes involved in this metabolism [22]. Levels of enzymes such as DGK, phosphatidylinositol 4-kinase (PI4K) and phosphatidylinositol 4-phosphate 5-kinase (PIP5K), which produce PA, phosphatidilinositol 4-phosphate (PIP) and PIP_2, respectively, increase in the presence of different Al concentrations. Recent research suggests that Al affects other signalling pathways as well [32].

Deficiencies in Pi levels affect the phosphoinositides signal transduction pathway. Phosphate deprivation leads to decreases in the total content of phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (which constitute ~30% of total Pi storage molecules in plants), and concomitant increases in the synthesis of non-phosphorus lipids such as galactolipid digalactosylglycerol (DGDG) and sulfolipid sulfoquinovosyldiacylglycerol (SQDG), presumably to preserve membrane integrity [33–35]. Galactolipids are synthesized through transfer of galactose from UDP-galactose to diacylglycerol (DAG) by monogalactosyldiacylglycerol synthase (MGDG) and DGDG synthase [36]. A portion of the DAG required to increase non-phosphorus lipids biosynthesis is probably obtained by hydrolysis of phospholipids, although this has not been demonstrated experimentally. Two alternative pathways for DAG production from phospholipids have been proposed: direct hydrolysis by PLC; and an indirect two-step reaction involving PLD which yields PA and PA phosphatase (PAP) releases DAG and Pi. The possible involvement of these two pathways implies the existence of transcriptionally-induced phospholipases which may degrade phospholipids. A recent study reports increases in PC-hydrolyzing activity under Pi deprivation conditions as well as the presence of a novel phospholipase C gene (NPC4), which is transcriptionally- activated [37]. In addition, Cruz-Ramírez et al. [38] reported that expression of *PLDZ2* is specifically regulated by Pi availability and that it actively participates in hydrolysis of PC and PE to release Pi from phospholipids and provide DAG for DGDG biosynthesis.

The study objective was to evaluate the effects of different Pi concentrations on Al-induced inhibition of cell growth and the phosphoinositide signal transduction pathway to better understand the physiological and biochemical responses induced by Pi deficiency and Al toxicity in *Coffea arabica* suspension cells.

2. Materials and methods

2.1. Plant materials and growth conditions

Cell suspensions of an Al-sensitive *C. arabica* cv. Catuai line (L2) were cultivated in modified MS medium at half ionic strength [39] containing 3% sucrose (macronutrients: 9.93 mM KNO_3, 10.24 mM NH_4NO_3, 1.49 mM $CaCl_2$, 759 μM $MgSO_4$; and micronutrients: 51.12 μM $FeSO_4$, 59.17 μM Na_2-EDTA, 2.5 μM KI, 50 μM H_3BO_4, 46.15 μM $MnSO_4$, 14.9 μM $ZnSO_4$, 0.68 μM Na_2MoO_2, 0.05 μM $CuSO_4$ and 0.052 μM $CoCl_2$), and supplemented with inositol, 30 μM thiamine-HCl, 13.6 μM 2,4-dichloro-phenoxyacetic acid (2,4-D) and 4.4 μM 6-benzyl-aminopurine (6-BAP). Media were prepared at one of four KH_2PO_4 concentrations (100, 250, 625 or 1250 μM), and pH was adjusted to 4.3 before autoclaving. Suspension cells were grown in darkness at 100 rpm and subcultivated every 14 days for maintenance.

2.2. Treatments

The effects of Al on cell growth were evaluated by supplementing sterile medium with 100 μM $AlCl_3$ and measuring fresh growth at day 14 of the culture cycle by calculating total cellular weight per flask per treatment. PLC, PLD and lipid kinase activities were determined at day 14 by treating the cells with 100 μM $AlCl_3$ for 1 h, filtering them by vacuum filtration and then quick freezing in liquid nitrogen.

2.3. Cell extracts

Both treated and untreated (control) cells were quick frozen in liquid nitrogen, macerated and homogenized with a polytron in buffer A [1 g of tissue in 2.5 mL; containing 50 mM NaCl, 1 mM EGTA, 50 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM sodium pyrophosphate and 0.2 mM sodium-orthovanadate]. Extracts were centrifuged at 13,000*g* for 30 min at 4 °C, the supernatant recovered, quick frozen in liquid nitrogen and stored at −80 °C for later determination of PLC and PLD activity. Sample protein content was measured using a bicinchoninic acid protein assay reagent with bovine serum albumin (BSA) as a standard [40].

2.4. PLC assay

Hydrolysis of [^{3}H]-PIP_2 was measured as described by Hernández-Sotomayor et al. [41] in a reaction mixture (50 μL) containing 35 mM NaH_2PO_4 (pH 6.8), 70 mM KCl, 0.8 mM EGTA, 0.8 mM $CaCl_2$ (final concentration 25 μM), 200 μM PIP_2 (~333.33 Bq) and 0.08% deoxycholate. The reaction was stopped with 100 μL of 0.1% (w/v) BSA and 250 μL of 10% (w/v) trichloroacetic acid (TCA). Precipitates were removed by centrifuging (13,500*g* for 10 min) and the supernatants collected for quantification of released [^{3}H]-IP_3 by liquid scintillation counting (ACS, Amersham Ltd.). PLC activity was reported as specific activity (pmol min^{-1} mg^{-1} protein), using from 10 to 20 mg protein per assay.

2.5. PLD assay

Hydrolysis of [^{3}H]-PC was measured in a reaction mixture (100 μL) containing buffer acetate (40 mM $NaCH_3COOH$, 0.8 mM $MgCl_2$, 32 mM KCl, pH 5), 1 mM EGTA, 10 mM $CaCl_2$, 414 μM PC (436.8 Bq) and 0.02% deoxycholate. The reaction was begun by adding 40 μL cell extract, allowed to incubate at 37 °C for 10 min, and stopped with 100 μL of 0.1% (w/v) BSA and 250 μL of 10% (w/v) TCA. Precipitates were removed by centrifuging (13,500*g* for 10 min) and the supernatants collected for quantification of released [^{3}H]-choline by liquid scintillation counting (ACS, Amersham Ltd). PLD activity was reported as specific activity (pmol min^{-1} mg^{-1} protein).

2.6. Aluminium measurement

Cells were incubated with 100 μM $AlCl_3$ for 1 h, collected from the media by vacuum filtration on filter paper (medium pore size), oven-dried at 65 °C for 24 h, and then furnace ashed at 200 °C for 1 h and 500 °C for an additional 3 h. The ash was dissolved in 40% HCl and completely evaporated on a hot plate. When the crucibles were cold, the solubilised ashes were recovered by adding 1 mL pure HCl and 4 mL water, and transferred to previously acid-washed test tubes. Aluminium concentration was measured