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Proteome profile analysis of boron-induced alleviation of aluminum-toxicity in *Citrus grandis* roots

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ABSTRACT

Aluminum (Al)-toxicity and boron (B)-deficiency are two major factors limiting crop production in tropical and subtropical areas. Elevating B supply can alleviate the Al-induced inhibition of growth in *Citrus grandis*. Seedlings of *C. grandis* were irrigated for 18 weeks with nutrient solutions containing two B levels (2.5 and 20 μM H_3BO_3) and two Al levels (0 and 1.2 mM $AlCl_3{\cdot}6H_2O$). By using 2-dimensional electrophoresis (2-DE) based MALDI-TOF/TOF-MS method, this study successfully identified and quantified sixty-one differentially abundant proteins in *Citrus* roots in response to B-Al interactions. The mechanisms underlying the B-induced alleviation of Al-toxicity unveiled by 2-DE technique could be summarized as follows: a) remodeling of cell wall by reducing the synthesis of lignin (sugar ATP Binding Cassette (ABC) transporter ATPase and cinnamyl alcohol dehydrogenase) and increasing the modification of cell wall (UDP-forming); b) enhancing the abundances of proteasomes and turnover of dysfunctional proteins (proteasome or protease); c) increasing the abundance of stress response proteins, such as alcohol dehydrogenase, S-adenosylmethionine synthetase (SAMS) and glycosyl hydrolase; d) reinforcing cellular biological regulation and signal transduction (calreticulin-1). For the first time, some proteins, such as cell division protein 48 (CDC48), calreticulin and phospholipase, which might be involved in the downstream signaling of Al in *Citrus* plants, were successfully identified.

1. Introduction

Aluminum (Al) is the most abundant metallic element in earth crust. When soil pH is higher than 5.0, Al exists as non-phytotoxic compounds such as aluminosilicates and precipitates. While soil pH is lower than 5.0, it exists mainly as Al^{3+}, which is phytotoxic to most of crop plants. The apoplast of root apices is the primary site of Al^{3+} toxicity and several micromole concentration of Al^{3+} can rapidly inhibit the elongation of primary roots and the development of lateral roots, leading to poor uptake of nutrients and water and cessation of root growth (Barceló and Poschenrieder, 2002). Boron (B)-deficiency is another worldwide problem for many agricultural crops including *Citrus* in many countries, especially in acid soil regions (Jiang et al., 2009). The role of B in plants is its cross-linking with pectic polysaccharide rhamnogalacturonan II (RG-II) in cell walls by the formation of RG-II-B diolester, which is essential for cell wall structure and function (Ryden et al., 2003).

Due to the similarity of B-deficiency and Al-toxicity induced symptoms in plants, it was proposed that Al executed its toxic effects by inducing B-deficiency in plants. However, subsequent studies demonstrated that Al treatment did not reduce B uptake or concentration both in roots and leaves (Jiang et al., 2009). This gave rise to the possibility that B-Al competition could affect normal function of B in plant cells and supplementation of B beyond the metabolic need of the plant could ameliorate the Al-induced growth impairments. Yu et al. (2009) proposed that the possible role of B in alleviating Al-toxicity was that sufficient B reduced unmethylesterified pectin in root cell walls and improved the cross-linking of RG-II which resulted in a stable network of cell walls with decreased pore sizes. Li et al. (2017) revealed that abundant B supply to pea roots enhanced the immobilization of Al in

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alkali-soluble pectin, thus most likely reducing the entry of Al^{3+} into the symplast from the surroundings. The alleviation of Al-induced inhibition in plant growth and development due to increased B levels in nutrient solution has been observed in some higher plants, including pea (Yu et al., 2009), flax (Heidarabadi et al., 2011) and *Citrus* (Zhou et al., 2015; Wang et al., 2015; Jiang et al., 2009).

Previous studies reported that increased B applications to Al-toxicity plants could stimulate the synthesis and accumulation of the antioxidant compound like ascorbate and reduced glutathione, thereby scavenging reactive oxygen species (ROS) more effectively and enhancing root growth (Riaz et al., 2018). Heidarabadi et al. (2011) suggested that adequate B resulted in the decrease of enzyme activities involved in phenolic compounds, lignin content and wall-bound phenols under Al stress, thereby ameliorating Al toxicity in flax plants. Jiang et al. (2009) showed that the antagonistic actions of B against Al-toxicity on *C. grandis* root was probably due to the B-induced alterations of Al speciation and/or sub-cellular compartmentation, and that the B-induced alleviation of shoot growth and photosynthesis inhibition could be due to lower Al accumulation in shoots. More recently, Wang et al. (2015) and Zhou et al. (2015) reported that the differentially expressed genes related to detoxification of ROS, cell transport, energy production, calcium (Ca) signal and hormone, cell wall modification might play a role in the B-induced alleviation of Al-toxicity in *C. grandis* seedlings.

Citrus plants are susceptible to Al-toxicity and B-deficiency. Although previous studies revealed that application of magnesium (Mg; Rengel et al., 2015), Ca (Rengel, 1992), silicon (Si; de Freitas et al., 2017), sulfur (S; Guo et al., 2017) and salicylic acid (Liu et al., 2012) could ameliorate Al toxicity by suppressing Al uptake, restoring root membrane integrity, reducing ROS level and ROS induced oxidative damage and regulating the activities of antioxidant enzymes, some μM B could apparently alleviate Al toxicity induced growth inhibition, whereas some mM Ca or Mg need to be used to take effects. So, if necessary, application of B is a more economic way than Ca, Mg, Si etc. Although several studies mentioned above have investigated the possible alleviative mechanisms of B on Al-toxicity in some details, the molecular mechanisms on the B-induced alleviation of Al-toxicity are still pending. Based on our previous work (Jiang et al., 2009; Tang et al., 2011), we selected two B (2.5 and 20 μM H_3BO_3) levels and two Al (0 and 1.2 mM $AlCl_3$) levels to generate different interaction treatments. After treatment, we used 2-dimensional electrophoresis (2-DE) technique based MALDI-TOF/TOF-MS method to identify and quantify the differentially abundant proteins that might play key roles in the alleviative effects of B on Al-toxicity in *C. grandis* roots. Furthermore, the possible mechanisms on the B-induced alleviation of Al-toxicity in other plants were also pointed out.

2. Material and methods

2.1. Plant material and treatments

C. grandis seeds were germinated in plastic trays containing clean river sands and kept in moisture. After germination, tender seedlings were irrigated with adequate nutrient solution every two days for 5 weeks before being transplanted into 6 L pots containing clean river sand and grown in a greenhouse under natural photoperiod. Six weeks after transplanting, seedlings were supplied with nutrient solution. The nutrient solution contained 1 mM $Ca(NO_3)_2$, 1 mM KNO_3, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.065 μM $(NH_4)_6Mo_7O_{24}$, and 20 μM Fe-EDTA. There were four treatment combinations containing two B (2.5 and 20 μM H_3BO_3) × two Al (0 and 1.2 mM $AlCl_3{\cdot}6H_2O$) levels and 20 pots (two seedlings per pot) for each treatment in a completely randomized design. We used 2.5B (2.5 μM H_3BO_3 + 0 mM $AlCl_3{\cdot}6H_2O$), 2.5B + Al (2.5 μM H_3BO_3 + 1.2 mM $AlCl_3{\cdot}6H_2O$), 20B (20 μM H_3BO_3 + 0 mM $AlCl_3{\cdot}6H_2O$) and 20B + Al (20 μM H_3BO_3 + 1.2 mM $AlCl_3{\cdot}6H_2O$) to represent the four treatment combinations hereafter, respectively. Ten weeks after transplanting, each pot was irrigated daily with nutrient solution (500 mL) until dipping for 18 weeks. All the nutrient solutions were freshly prepared before use and the pH of the nutrient solutions were adjusted to 4.1–4.2 using HCl or NaOH. At the end of treatment, about 0.5 cm newly-grown root apices were excised and immediately frozen in liquid nitrogen and stored at − 80 °C until extraction.

2.2. Plant dry weight (DW), B and Al concentrations

Ten plants per treatment (10 replicates) from different pots were harvested and sliced into shoots and roots. The harvested tissues were fixed at 120 °C for 20 min, dried at 70 °C for 48 h and DW were measured.

Dried roots and leaves were ground into fine powder with a pulverizer and sifted by 0.5 mm sieve. B was assayed by inductively-coupled plasma mass spectrometry after samples were ashed at 500 °C for 5 h, and dissolved in 0.1 M HCl. Al was assayed by the aluminon method after samples were digested in a mixture of HNO_3: $HClO_4$ (5: 1 v/v). There were five replicates for B and Al measurements.

2.3. Assays of thiobarbituric acid-reactive substances (TBARS), H_2O_2, callose, citrate and isocitrate

TBARS was extracted and measured according to the methods described by Hodges et al. (1999)·H_2O_2 production was measured according to Chen et al. (2005). Briefly, eight to ten root apices were incubated into 1.5 mL 50 mM K_2HPO_4-KH_2PO_4buffer solution (pH 7.0) containing 0.05% (w/v) guaiacol and 2 U horseradish peroxidase for 2 h at room temperature in the dark. Absorbance was measured under 470 nm with a spectrophotometer. The content of H_2O_2 was calculated with an extinction coefficient of $\varepsilon = 26.6\ cm^{-1}\ mM^{-1}$. Callose was measured according to the method described by Jones et al. (2006). The fluorescence signal was read on an Agilent Cary Eclipse Fluorimeter (excitation 400 nm, emission 510 nm, slit width 10 nm). There were four replicates for TBARS, H_2O_2 and callose assays.

Citrate and isocitrate of *C. grandis* roots were extracted and measured according to Tang et al. (2011). Citrate was measured in 1 mL reaction mixture containing 100 mM Tris–HCl (pH = 7.6), 0.2 mM NADH, 7 U lactate dehydrogenase (LDH), 14 U NAD-malate dehydrogenase (NAD-MDH), 0.5 U citrate lyase and 200 (roots) or 50 (leaves) μL extract. Isocitrate was measured in 1 mL mixture containing 100 mM Tris–HCl (pH7.6), 3.3 mM $MnSO_4$, 0.15 mM NADP, 0.1 U NADP-isocitrate dehydrogenase (NADP-IDH) and 200 μL extract. The experiment was conducted with four replicates.

2.4. Total protein extraction, 2-DE and protein identification

Root tips of six plants from different pots were mixed as a biological replicate. Equal amounts of root tips were collected from each plant. There were three biological replicates for each treatment (total of 18 plants from 18 pots). Total proteins were extracted from *C. grandis* roots by using phenol-acetone extraction as described by Yang et al. (2013). The protein concentration was measured by using Bradford protein assay with bovine serum albumin (BSA) as the standard material (Bradford, 1976).

Protein samples (1.0 mg) were diluted with appropriate amount of rehydration solution [8 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% (v/v) IPG (immobilized pH gradient) buffer, 13 mM dithiothreitol, 0.002% (w/v) bromophenol blue] to the total volume of 450 μL and then applied to 24 cm Immobiline DryStrips with a linear pH gradient of 4–7 (GE Healthcare, Uppsala, Sweden). The isoelectric focusing electrophoresis, SDS-PAGE, gel images acquisition were performed according to the methods as described by Sang et al. (2017). Image analysis was performed with PDQuest software (Version 8.0.1, Bio-Rad,