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Boron deficiency and toxicity altered the subcellular structure and cell wall composition architecture in two citrus rootstocks

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ABSTRACT

Trifoliate orange and citrange are two important rootstock resources to citrus, and citrange has a stronger tolerance to boron (B) deficiency and toxicity than trifoliate orange. In this study, we described how B deficiency and toxicity depressed the variations of cell wall B location, subcellular structure, cell wall components and structure of the two citrus rootstocks, to evaluate the mechanisms of different B tolerance of rootstocks on the cellular and structural levels. The results showed that citrange had better growth and lower symptoms of B stresses than trifoliate orange. What is more, citrange had a stronger ability to allocate more B to cell walls than trifoliate orange at deficient-B level. Under B deficiency and excessive conditions, severe damages in subcellular structure with obvious irregular thickening of cell walls and higher accumulation of plastoglobulus were observed in leaf cells of both two rootstocks. Additionally, it also showed obvious variations in the mode of hydrogen bonding, and more accumulation of cellulose, phenols and carbonhydrates in cell walls of trifoliate orange leaf under B starvation, while lighter changes on cell wall components were observed in citrange. As for B toxicity, comparing to trifoliate orange, citrange showed a lighter damage on pectin crosslinking structure in cell walls of leaf tissue. These results gained some novel mechanisms of different citrus rootstocks to B stress tolerance, and provide a theoretical basis for cultivating improved citrus rootstocks.

1. Introduction

Boron (B) is an essential microelement for higher plants and B plays an important role in the structure of plant cell walls, and its deprivation causes a wide range of variations in the cell function, the physiology and biochemistry of cell walls, and plant metabolites (Pan et al., 2012; Dordas and Brown, 2005; Liu et al., 2014). It has been demonstrated that the accepted physiological function of B is to cross-link two chains of rhamnogalacturonan II (RG-II) by the formation of borate diol diester in pectin of cell wall, and then affect the biochemical and mechanical properties of the plant wall (O'Neill et al., 2004). Cell walls have an important influence on the structural integrity of the cells and determine the cells shape and size (Hayot et al., 2012).

Boron starvation is a widespread problem for many agricultural crops, including citrus (Shorrocks, 1997), is responsible for considerable loss of productivity and poor fruit quality in many citrus orchards (Xiao et al., 2007). Conclusive evidence shows that B deficiency results in curling of the leaves, leaf chlorosis, weakened photosynthesis, abnormal anatomic structure and metabolic disturbance in leaves (Liu et al., 2014; Han et al., 2009; Lu et al., 2017a,b; Dong et al., 2016). Application of B-enriched fertilizers is an effective solution for resolving B deficiency, but B toxicity is a more difficult problem to manage and the damage of B toxicity to plants is irreversible. At excessive B level, B tends to accumulate in leaf tips and leaf margins, causing margin damage (Reid et al., 2004; Shapira et al., 2013). Under such conditions, many plants show inhibited growth and the symptoms of B toxicity like chlorotic and necrotic patches, which are usually found on the margins and tips of older leaves (Nable et al., 1997; Han et al., 2009). Several studies have demonstrated the destruction of leaf structure, oxidative damage, and interference to synthesis of cell wall components with excessive B supply (Papadakis et al., 2004a; Shah et al., 2017; Mesquita et al., 2016).

Citrus is one of the most important economic crops in China, and the rootstocks have been used to optimize plant growth, and improved fruit production and quality (Xiao et al., 2007; Papadakis et al., 2004a). Early studies have indicated that citrus plants are sensitive to B deficiency or toxicity (Papadakis et al., 2003; Liu et al., 2011), and rootstocks vary greatly in their effects on tolerance to B deficiency (Boaretto et al., 2008) and toxicity (Papadakis et al., 2004a,b). Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and citrange [*Citrus sinensis* (L.) Osb. ×

Abbreviations: CK, control; BD, boron deficiency; BT, boron toxicity; FTIR, fourier-transform infrared spectroscopy; ^{13}C-NMR, ^{13}C nuclear magnetic resonance; TEM, transmission electron microscope; PBS, glutaraldehyde in phosphate buffer solution; CWM, cell wall material; Sg, starch grain; Pg, plastoglobulus; Tri, trifoliate orange; Cir, citrange

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Poncirus trifoliata (L.) Raf.] are two vital rootstock resources to citrus, and the two rootstocks have different tolerances to B stress (Forner-Giner et al., 2003; Liu et al., 2013). It has been reported that citrange has a stronger tolerance to B deficiency than trifoliate orange (Sheng et al., 2009) and evaluated the mechanisms of difference by studying cellular B allocation and pectin composition (Liu et al., 2013), structure and components of leaf (Lu et al., 2017a,b). Huang et al. (2014) investigated the changes on leaf photosynthesis, chlorophyll, plant B absorption and distribution induced by B toxicity, using two citrus species differing in B sensitivity, to elucidate the possible mechanisms of B tolerance in different citrus plants. Under high B conditions, better growth status was observed in "Newhall" plants grafted on citrange than in those grafted on trifoliate orange by Liu et al. (2011), which suggesting that plants grafted on citrange were more tolerant to B toxicity than the trifoliate orange-grafted plants. However, there have been few studies performed on the differences and changes in the cellular structure and the architecture of cell wall components in leaves of trifoliate orange and citrange rootstocks under B deficiency and excess conditions, the relationship between variations on cell walls and B-tolerant is not clear.

Fourier-transform infrared spectroscopy (FTIR) has been used to gather information about the chemical composition of almost all substances (Liu et al., 2014), and it is also extensively applied in analyzing plant cell walls (Abidi et al., 2014). In recent years, solid-state ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopy has become a powerful tool to identify the structure of organic compounds and provide comprehensive structural information (Mao et al., 2008). Despite its wide application in characterization of the structure of soil organic matter, wood lignin and cell wall polysaccharides, etc. (Rondeau-Mouro et al., 2003; Salati et al., 2008; Balakshin et al., 2016), the^{13}C-NMR technique has rarely been used to investigate the cell wall organic carbon structure of citrus under different B stress conditions.

Therefore, the trifoliate orange and citrange were selected as research materials, and chemical analysis methods combined with transmission electron microscope (TEM), FTIR and solid-state ^{13}C-NMR were conducted to examine (1) how B deficiency and toxicity altered B allocated in cell walls and cellular structure of the two citrus rootstocks; (2) varied changes on cell wall architecture and components betwwen trifoliate orange and citrange treated with deficient-B and toxic-B, and ultimately to gain the structural changes of cell walls in different citrus rootstocks under different B stresses.

2. Materials and methods

2.1. Plant culture and growth conditions

The experiment was conducted in a greenhouse located at Huazhong Agricultural University, Wuhan, China, from March to August 2016. The citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] and trifoliate orange [*Poncirus trifoliata* (L.) Raf.] seedlings used were uniform in size and obtained from a commercial nursery (Ganzhou, Jiangxi, China). After being washed with distilled water, plants were transplanted to 4-liter black plastic pots (three plants per pot), which had been immersed in 1 M HCl for 24 h and washed with distilled water. In this experiment, all the seedlings were cultured in a nutrient solution modified from Hoagland and Arnon (1950), containing the following macronutrients in mM: KNO_3, 2.00; Ca $(NO_3)_2$, 1.23; $MgSO_4$, 0.50; Na2HPO4, 0.14; NaH_2PO_4, 0.32; and the following micronutrients in μM: $MnCl_2$, 4.45; $ZnSO_4$, 0.80; $CuSO_4$, 0.16; Na_2MoO_4, 0.18; EDTA-Fe, 37.30. Two B contents were supplied at 10 μM (CK, control treatment) and 400 μM (BT, boron toxicity) using boric acid, accompanied with a treatment without B (BD, boron deficiency). The solution was aerated for 20 min at a 4-h interval and replaced once a week. The experiment was conducted in a completely randomized with six treatments, and each treatment was replicated six times with one replication contained one plant. The pH was adjusted to 5.8 ~ 6.2 every day with 0.5 M H_2SO_4 or 1 M NaOH.

2.2. Plant sampling and boron analysis

After completing 90-days, the plants were harvested and plant samples were divided into roots, stems, and leaves. All the leaves of each plant were further separated into three parts: one part was used for determination of B concentration, another part was stored for extraction of cell wall materials, and the third part was used for transmission electron microscope analyses. After drying to a constant weight at 75 °C, the dry weight of roots, stems and leaves were measured. Then the samples were ground to a fine powder and ashed at 500 °C for 5 h, followed by dissolving the ashes in 0.1 M HCl. The B contents in roots, stems and leaves were measured at 540 nm spectrophotometrically (Hitachi UV-3100 UV–vis; TECHCOMP, Shanghai, China), according to the curcumin colorimetric method (Dible et al., 1954).

2.3. Transmission electron microscope (TEM) analysis

The TEM slices were prepared by the method of Kong et al. (2013) with slight modification. The TEM analysis was performed as follows: briefly, leaves from the same parts under different B treatments were cut into small pieces (1 × 1 mm), then fixed in 2.5% glutaraldehyde in phosphate buffer solution (PBS) for 12 h at 4 °C. Next, the tissue blocks were rinsed four times with 0.1 M PBS (pH 7.4) and post-fixed for 2–3 h with 1% buffered osmium tetroxide, followed by rinsing three times with 0.1 M PBS (pH 7.4), and dehydration three times in an increasing acetone concentration series [50, 70 and 90 (three times)] and then in a mixture of 90% ethanol and 90% acetone for 15 min. After being stained with 2% uranyl acetate and lead citrate, ultrathin sections were examined with a JEM-100CX II transmission electron microscope.

2.4. Preparation of cell wall materials (CWM)

The cell wall was separated from the leaves as reported by Hu and Brown (1994) with some modifications. Firstly, the leaf samples were homogenized in liquid nitrogen with a mortar, after homogenizing in 10 volumes of ice-cold ultrapure water and centrifuging at 5000*g* for 10 min at 4 °C, the precipitate was washed with 10 volumes of ice-cold ultrapure water and centrifuged again. Next, the residue was washed three times with 10 volumes of 80% ethanol and once with 10 volumes of mixture of methanol/chloroform (1/1, v/v). Finally, the precipitate was washed with 10 volumes of acetone. The final insoluble pellet was dried at 50–60 °C, weighed and defined as CWM. The dry CWM was divided into two portions: one was dried to ashes at 500 °C for determining B allocated in cell walls as described above, and the other portion was used for FTIR and ^{13}C-NMR analyses.

2.5. Fourier transform infrared spectroscopy (FTIR) analyses of cell wall

According to the method described by Liu et al. (2014), disks for FTIR spectroscopy were prepared using a Graseby-Specac Press. A small amount of cell wall powder of leaves was mixed with KBr (1:100 m/m) and pressed into tablets. IR spectra (4000-400 cm^{-1}) were recorded using a VERTEX 70 spectrometer by a resolution of 4 cm^{-1} and 32 scans per sample. The 6 copies of the spectra of cell walls with different B treatments were normalized and baseline-corrected with OMNIC 32 software. Graphical data were processed with Microsoft Excel 2010 and Origin 8.6.

2.6. Solid-State nuclear magnetic resonance (^{13}C-NMR) spectroscopy analysis of cell wall

For the ^{13}C-NMR analysis, the cell wall of leaf samples was ground to fine powder and passed through a 0.2 mm sieve. The ^{13}C-NMR spectra were obtained on a fully automatic nuclear magnetic resonance