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Auxin modulates root-hair growth through its signaling pathway in citrus

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ABSTRACT

Root-hairs are tip-growing extensions from the root epidermis, and can be affected by phytohormones, such as auxins. A sand culture study was performed to analyze the effects and mechanisms of 3-indolebutyric acid (IBA) and 2-naphthoxyacetic acid (2-NOA, auxin inhibitor) on root-hair growth in trifoliate orange, a widely used rootstock for citrus. The results showed that IBA increased root-hair growth while 2-NOA had the inhibitory effect. A positive correlation was observed between the endogenous auxin level and root-hair number in the root-hair zone. The IBA treatment increased the endogenous IAA content in the root-hair zone of lateral roots by 22.8% while 2-NOA reduced it by 21.4%, compared with control group. Interestingly, 2-NOA increased the endogenous IAA level compared with IBA and control groups in the root tip zone, while it had no distinct difference from the control group in the stele zone of lateral roots. Thus, 2-NOA blocked IAA fluxing from root tips to root-hair zones. Furthermore, IBA up-regulated expression of auxin biosynthesis and transport genes, thus increasing the endogenous IAA level in the root-hair zone which may promote root-hair growth. However, application of 2-NOA had the opposite effect. These results indicated that IBA and 2-NOA regulated auxin biosynthesis and transport toward the root-hair zone, where it achieved a local alteration of the auxin signaling pathway influencing root-hair growth.

1. Introduction

As extensions from the root epidermis, root-hairs can increase the root surface area and play important roles in nutrient and water absorption, the adhesion of the growing root to the rhizosphere, and the interaction of plants and soil microorganisms, such as arbuscular mycorrhizal (AM) fungi and nitrogen-fixing bacteria (Grierson and Schiefelbein, 2002; Libault et al., 2010; Tanaka et al., 2014; Li et al., 2017; Vincent et al., 2017; Zou et al., 2017). Swarup et al. (2005) reported that the expansion of epidermal cells has a response to an auxin gradient. Auxins induce signaling pathway for root epidermal hair cells (Nakamura et al., 2017). Besides auxin, epidermal hair cell development is also influenced by other plant growth regulators (ethylene, jasmonic acid, strigolactone, etc) (Grierson and Schiefelbein, 2002; Zhu et al., 2005; Kapulnik et al., 2011; Zhang et al., 2013, 2016). In particular, auxins are considered the main signaling molecules involved in regulating root-hair growth (Pitts et al., 1998; Muday et al., 2012). In *Arabidopsis*, the auxin response mutant *aux1* has fewer and shorter root-hairs, but root-hair growth can be dramatically promoted by exogenous auxins, such as 1-naphthylacetic acid (NAA) (Pitts et al., 1998; Rahman et al., 2002; Muday et al., 2012). Bruex et al. (2012) found that 90% of genes related to root-hair growth can be positively regulated by auxins based on transcriptome sequencing data analysis.

Furthermore, many researchers proposed that auxins play an important role in root-hair initiation and elongation (Rahman et al., 2002; Cho et al., 2007; Jones et al., 2009;Strader and Bartel, 2009; Ganguly et al., 2010). Auxins are primarily synthesized at the shoot apex, transferred towards the root tip by the vascular tissues of the stem, and moved basipetally to the elongation zone through peripheral root tissues (Rigas et al., 2013). Such transportation can be blocked by auxin transport inhibitors, such as 1-naphthoxyacetic acid (1-NOA), 2-naphthoxyacetic acid (2-NOA), and 3-chloro-4-hydroxyphenylacetic acid (CHPAA) which regulate overall auxin transport (LaňKová et al., 2010). In addition, auxin fluxes at the root apex are mainly controlled by various Pin-formed (*PIN*) auxin efflux carriers, in addition to *AUXIN RESISTANT 1* (*AUX1*), *LIKE AUX1* (*LAX*) auxin influx carriers, and some members of the *ATP-BINGING CASSETTE B* (*ABCB*) transporters (auxin efflux genes) (Yang and Murphy, 2009; Tromas and Perrot-

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Rechenmann, 2010; Swarup and Péret, 2012). Besides auxin transport, its synthesis is controlled by other genes, such as tryptophan aminotransferase related (*TARs*) and flavin-containing monooxygenase (*YUCs*) (Mano and Nemoto, 2012). Understanding auxin biosynthesis and transport is an important factor in understanding root-hair growth.

Citrus is an important economic plant, which is generally considered to have few or even no root-hairs in orchards. However, trifoliate orange (*Poncirus trifoliata* L. Raf.), a widely used rootstock for citrus, can grow some root-hairs in sand culture (Cao et al., 2013; Zhang et al., 2013, 2016). These root-hairs are strongly affected by the exogenous auxins (Zhang et al., 2013). However, the relevant mechanisms may differ from *Oryza sativa* or *Arabidopsis* and still remain unclear. The objective of the present work was to evaluate the effects and mechanisms of auxins on root-hair growth in trifoliate orange.

2. Materials and methods

2.1. Plant material and growth conditions

The experiment was conducted in a greenhouse of Yangtze University, where the photosynthetic photon flux density was 900 μmol/m^2/s, day/night temperature 28/21 °C, and relative humidity 85%. The sand used in this experiment was washed 6 times with pure water and sterilized at 121 °C/0.1 MPa. Uniform size seeds of trifoliate orange were sterilized with 75% ethyl alcohol for 15 min, followed by pre-germination treatment in a constant temperature incubator at 28 °C/80% (T/RH) without light. When germinated seeds were 1 cm long, they were sown in round pots (Height × Diameter = 30 × 20 cm) filled with sand and placed in the greenhouse. All seedlings were irrigated with full Hoagland solution containing the corresponding growth regulators (100 mL) once every 3 days. After the seedlings were irrigated 15 times, the containers were dismantled, and the sand was carefully dislodged. Subsequently, the roots of seedlings were collected and gently washed 3 times with pure water to remove the fine sand from the surface of the roots.

The basic culture Hoagland solution (Hoagland and Arnon, 1950; Zhang et al., 2013, 2016) was as follows: 4.00 mmol/L (mM) Ca$(NO_3)_2{\cdot}4H_2O$, 6.00 mM KNO_3, 2.00 mM $MgSO_4{\cdot}7H_2O$, 1.00 mM $NH_4H_2PO_4$, 46.00 μM H_3BO_3, 9.20 μmol/L (μM) $MnCl_2{\cdot}4H_2O$, 0.77 μM $ZnSO_4{\cdot}7H_2O$, 0.32 μM $CuSO_4{\cdot}3H_2O$, 0.12 μM H_2MoO_4, and 50 mM EDTA-Fe, at 5.85–6.00 pH.

2.2. Experimental design

The seedlings were treated with growth regulators (Shanghai Yuanye Biological Technology Co., Ltd., China) in 3 different ways: the control group, 1.0 μM IBA treatment (IBA), and 50.0 μM 2-NOA treatment (2-NOA). Each treatment had three replicates of 9 pots of seedlings with 3 seedlings in each pot.

2.3. Scanning electron microscopy was used to observe root-hair growth

According to the protocol (Zhang et al., 2016), 1.0-cm-long root-hair zones (2 cm from the root tip) of the lateral roots were selected for scanning electron microscopy (SEM, model JSM-6390LV, JEOL Co., Japan) to investigate the density, length, and diameter of the root-hairs. For each treatment, 9 photos from different roots at 100× were randomly chosen for the measurement of the root-hair density and 9 photos from different roots at 400× were also randomly chosen for the measurement of their length and diameter using ImageJ software (National Institutes of Health, USA) (http://rsb.info.nih.gov/ij/).

2.4. Lateral root IAA fluxes

A non-invasive micro-test technique (NMT) was used to determine root IAA fluxes in the root-hair zone of lateral roots and the number of root-hairs was recorded by inverted microscope. NMT (NMT100 Series, Younger USA Science & Technology Corp, Amherst, MA01002, USA; Xuyue (Beijing) Sci. & Tech. Co., Ltd., Beijing, China) with Aset 2.0 (Science Wares, Falmouth, MA, USA) and iFluxes 1.0 software (Younger USA Science & Technology Corp, Amherst, MA 01002, USA) was used. Root preparation was according to Yan et al. (2017) and incubated in a balanced solution (pH 6.1) containing 0.2 mM KCl and 0.2 mM $CaCl_2$ for 10 min. An IAA-sensitive microsensor (Φ2 ± 4 μm, XY-DJ-600, Younger USA) was polarized at +700 mV. The IAA sensor was placed near (2 μm) the surfaces of the roots. Before testing, the IAA electrode was calibrated with 0, 2, 4, 6, and 8 μM IAA and used only by means of a linear calibration slope ($R^2 > 0.99$). A typical plant from each pot was measured. The IAA flux was calculated by Fick's first law of diffusion: $J = -D \times D \times \Delta C/\Delta X$, where J is the free IAA flux (fmol/cm^2/s), D is the molecular diffusion coefficient (7×10^{-6} cm^2/s), ΔC is the auxin concentration gradient (gmol), and ΔX is the excursion distance for the microelectrode oscillation (30 μm). Here, J is a positive value for efflux and a negative value for influx.

2.5. Lateral root endogenous IAA concentration

To determine the content of endogenous IAA in lateral roots, the root-tip, the root-hair zone and the stele of root-hair zone were separated by surgucal knife, the epidermis of the root-hair zone were separated in scraping way by surgical knife blade. All tissues' endogenous IAA were analyzed by Enzyme-linked Immunosorbent Assays (ELISA) at the lab of Prof. Wang (Engineering Research Center of Pant Growth Regulator, Ministry of Education, China Agricultural University, China), as previously described (Chen et al., 2009). All measurements were performed in triplicate.

2.6. RNA extraction, RT-PCR and qRT-PCR

Total RNA was extracted from lateral roots (0.5 g) using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA with the PrimeScript RT reagent kit (TaKaRa, Japan). DNase I (TaKaRa, Japan) was added to remove genomic DNA. PCR primers of auxin biosynthesis and transport genes were designed based on *Clementina* cDNA sequences (http://www.phytozome.net/clementine) by Primer 5.0, while *β-actin* was used as an internal reference gene for the normalization of the data of quantitative RT-PCR (qRT-PCR, Supplementary Table S1). Relative expression was determined by qRT-PCR in an ABI Q7 Real-time PCR system (Applied Biosystems, USA) using the intercalation dye SYBR Green as a fluorescent reporter. Reactions were carried out in a final volume of 10 μL containing 20 ng of cDNA template, 5 μM of each primer, and 5 μL of SYBRPreMix Ex Taq™ (2x) (TaKaRa, Japan). The qRT-PCR was performed with the following protocol: one cycle at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s. The relative expression levels of the amplified products were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The qRT-PCR results are shown as the mean values of 4 independent biological replicates ± SD.

2.7. Data analysis

The data were statistically analyzed for variance (ANOVA) with SAS 8.1 software. Duncan's multiple range tests were used to compare significant differences among treatments at $P < 0.05$.

3. Results

3.1. Root-hair growth

The length and density of root-hairs were decreased by 2-NOA treatment in comparison with control treatment in lateral roots (Figs. 1