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Research article

An ethylene and ROS-dependent pathway is involved in low ammonium-induced root hair elongation in *Arabidopsis* seedlings

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ABSTRACT

Root hairs are plastic in response to nutrient supply, but relatively little is known about their development under low ammonium (NH_4^+) conditions. This study showed that reducing NH_4^+ for 3 days in wild-type *Arabidopsis* seedlings resulted in drastic elongation of root hairs. To investigate the possible mediation of ethylene and auxin in this process, seedlings were treated with 2,3,5-triiodobenzoic acid (TIBA, auxin transport inhibitor), 1-naphthylphthalamic acid (NPA, auxin transport inhibitor), p-chlorophenoxy isobutyric acid (PCIB, auxin action inhibitor), aminoethoxyvinylglycine (AVG, chemical inhibitor of ethylene biosynthesis), or silver ions (Ag^+, ethylene perception antagonist) under low NH_4^+ conditions. Our results showed that TIBA, NPA and PCIB did not inhibit root hair elongation under low NH_4^+ conditions, while AVG and Ag^+ completely inhibited low NH_4^+-induced root hair elongation. This suggested that low NH_4^+-induced root hair elongation was dependent on the ethylene pathway, but not the auxin pathway. Further genetic studies revealed that root hair elongation in auxin-insensitive mutants was sensitive to low NH_4^+ treatment, but elongation was less sensitive in ethylene-insensitive mutants than wild-type plants. In addition, low NH_4^+-induced root hair elongation was accompanied by reactive oxygen species (ROS) accumulation. Diphenylene iodonium (DPI, NADPH oxidase inhibitor) and dimethylthiourea (DMTU, ROS scavenger) inhibited low NH_4^+-induced root hair elongation, suggesting that ROS were involved in this process. Moreover, ethylene acted together with ROS to modulate root hair elongation under low NH_4^+ conditions. These results demonstrate that a signaling pathway involving ethylene and ROS participates in regulation of root hair elongation when *Arabidopsis* seedlings are subjected to low NH_4^+ conditions.

1. Introduction

Root hairs differentiate from root epidermal cells to achieve increased surface area, nutrient and water uptake, volume of soil occupied, and anchorage in plants (Bibikova and Gilroy, 2003). In *Arabidopsis*, root hair morphogenesis can be divided into three major stages: root hair initiation, the transition to root hair tip growth, and tip growth (Dolan et al., 1994).

Many molecules are involved in root hair tip growth, including calcium ions, reactive oxygen species (ROS), and Rho-related GTPases and phospholipids (Wada et al., 2015). Of these, ROS are likely especially important in root hair tip growth. For example, diphenylene iodonium (DPI), an NADPH oxidase inhibitor, suppresses ROS production in wild type (WT) *Arabidopsis* and inhibits root hair elongation. Furthermore, short root hairs are seen in the *root hair deficient* (*rhd2*) mutant, which is deficient in the RHD2 NADPH oxidase involved in ROS production. These reports suggest that root hair tip growth is dependent on ROS produced by RHD2 and NADPH oxidase (Foreman et al., 2003). In addition, ROS are important in establishing a tip-focused Ca^{2+} gradient by stimulating Ca^{2+} channel activity (Foreman et al., 2003). Finally, ROS are required for cell wall integrity at the tip of the growing root hair, based on the observation that a subset of hairs in *rhd2* mutants burst soon after initiation (Macpherson et al., 2008).

Plant hormones are important in regulating root hair formation, particularly ethylene and auxin (Pitts et al., 1998). Treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) promotes root hair elongation and induces ectopic root hair formation (e.g., epidermal cells in non-hair positions develop into

Abbreviations: Ag^+, silver ions; AVG, aminoethoxyvinylglycine; DMTU, dimethylthiourea; DPI, diphenylene iodonium; N, nitrogen; NH_4^+, ammonium; NPA, 1-naphthylphthalamic acid; PCIB, p-chlorophenoxy isobutyric acid; ROS, reactive oxygen species; TIBA, 2,3,5-Triiodobenzoic acid.

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root hairs), while inhibitors of ethylene biosynthesis and function decrease root hair elongation and reduce root hair formation (Tanimoto et al., 1995). In addition, root hair development is dependent on the auxin signaling pathway (Lee and Cho, 2013). For example, the auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1) mutant *tir1* has less root hair formation (Dharmasiri et al., 2005). Trichoblast-specific overexpression of the auxin efflux transporters PINFORMED3 and P-glycoprotein 4 resulted in increased auxin efflux from these cells, dramatically depleting auxin in root hair cells and inhibiting root hair growth (Cho et al., 2007; Lee and Cho, 2013).

Extensive research has shown that the soil nutrient status is important in regulating root hair development (Ma et al., 2001). For example, iron (Fe), phosphorus (P), potassium (K), manganese (Mn), and magnesium (Mg) deficiencies have been reported to induce root hair growth (Müller and Schmidt, 2004; Shin et al., 2005; Yang et al., 2008; Jung et al., 2009; Niu et al., 2014). Previous studies have also shown that root hairs were dramatically influenced by the Nitrogen (N) source and concentration (Bloch et al., 2011; Yang et al., 2011). For example, high NH_4^+ conditions stimulated branched root hair formation and inhibited root hair elongation in *Arabidopsis* seedlings (Yang et al., 2011). Bloch et al. (2011) demonstrated that the interaction between the N source and ROP signaling was important in regulating root hair tip growth in *Arabidopsis* seedlings.

Plant hormones, particularly ethylene and auxin, act in tandem with environmental signals to regulate root hair development (Martín-Rejano et al., 2011; Nagarajan and Smith, 2012; Lee and Cho, 2013). For example, auxin transport and signaling system is partially modulated by P deficiency. Also, ethylene synthesis and signaling is influenced by many nutrient deficiencies (González-Mendoza et al., 2013; García et al., 2015). Under low P conditions, treatment with the ethylene precursor ACC promoted root hair elongation, while ethylene synthesis and action inhibitors decreased root hair elongation (Zhang et al., 2003). In addition, K^+ deficiency- and low boron-induced root hair development have been linked to an ethylene-dependent pathway in *Arabidopsis* (Jung et al., 2009; Martín-Rejano et al., 2011). However, only a few studies investigated the interaction of N availability and plant hormone on root hair development. Yang et al. (2011) reported that exogenous methyl jasmonate treatment exacerbated high NH_4^+-induced branched root hair formation, while ethylene inhibited branched root hair formation.

ROS were reported to regulate the N availability-mediated root hair development (Shin et al., 2005). Moreover, there is an increasing body of evidence suggests that ROS are involved in a variety of ethylene-mediated responses in plants. For example, ethylene regulates stomatal closure via ROS production (Xia et al., 2015). So, in this study, we investigated the involvement of auxin, ethylene, and ROS under low NH_4^+ conditions in root hair development and found that ethylene and ROS were involved in low NH_4^+-induced root hair elongation in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of WT *Arabidopsis* (ecotype Columbia) and its mutants *tir1-1*, *aux1-7*, *axr1-3*, *eir1-1*, *etr1-1*, *etr1-3* and *eto1-1* (Columbia background) were stored under dry conditions at 4 °C until use. The basic culture medium contained 2 mM $MgSO_4$, 2 mM $CaSO_4$, 2.5 mM KH_2PO_4, 70 μM H_3BO_3, 14 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 10 μM NaCl, 0.2 μM Na_2MoO_4, and 40 μM Fe-EDTA and was solidified with 0.9% (w/v) agar. Sucrose (43 mM) and 2-(N-morpholino)-ethanesulfonic acid (4.7 mM) were added, and the pH was adjusted to 5.5. The control culture medium was basic culture medium supplemented with 5 mM KNO_3. The NH_4^+ treatment medium was basic culture medium supplemented with different concentrations of NH_4Cl as the sole N source, and the K^+ concentration was compensated for by adding the appropriate amount of KCl.

Seeds were surface-sterilized by immersion in 0.1% (v/v) $HgCl_2$ for 10 min and then rinsed six times in sterile water. The sterilized seeds were placed in Petri dishes containing control culture medium and incubated at 4 °C in the dark for 1 day. The Petri dishes were incubated vertically in a controlled environment under a 16 h/8 h light/dark cycle at 23 °C/20 °C and a light intensity of 150 μmol m^{-2} s^{-1} of photosynthetic active radiation.

2.2. Application of chemicals

2,3,5-triiodobenzoic acid (TIBA, auxin transport inhibitor), 1-naphthylphthalamic acid (NPA, auxin transport inhibitor) and p-chlorophenoxy isobutyric acid (PCIB, auxin action inhibitor) were dissolved in 1 mL ethanol and diluted with distilled water to a final stock concentration of 1 mM. ACC, aminoethoxyvinylglycine (AVG, chemical inhibitor of ethylene biosynthesis), and $AgNO_3$ (Ag^+, ethylene perception antagonist) were dissolved in distilled water to yield a stock solution of 1 mM. DPI (NADPH oxidase inhibitor) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were dissolved in DMSO and diluted with distilled water to final stock concentrations of 0.1 mM and 50 μM, respectively. Dimethylthiourea (DMTU, ROS scavenger), phenazine methosulphate (PMS), and methyl viologen (MV) were dissolved in distilled water to final stock concentrations of 1 mM, 5 mM and 1 mM, respectively. The concentrations of ethanol (0.001%–0.005% v/v) and DMSO (0.01%–0.005% v/v) used to dissolve these chemicals, were included in the control solution. All chemicals were purchased from Sigma-Aldrich. Chemicals were added to the culture media at 45–50 °C.

2.3. Measurement of root hair length

Seedlings grown in Petri dishes were placed on the stage of a stereomicroscope (MZFLIII, Leica Microsystem, Wetzlar, Germany), and images were captured of the apical segment within 1 cm of the primary root apex for all 25–30 seedlings. Root hair length was calculated by analyzing the digital images using Motic Images Plus 2.0 (Motic China Group Co., Ltd).

2.4. Detection of ROS formation in the root hair tip and root differentiation zone

ROS levels were detected using the dye H_2DCFDA (Foreman et al., 2003). After 24 h of treatment under low NH_4^+, seedling roots were incubated in 20 μM H_2DCFDA at 4 °C for 1 h and then washed with 0.1 mM KCl and 0.1 mM $CaCl_2$ (pH 6.0) at 22 °C for 1 h before observation. Roots stained with H_2DCFDA were imaged using the Perkin Elmer Confocal System-UltraVIEW VoX with excitation at 488 nm and bandpass detection of 500–530 nm.

2.5. Data analysis

The results were expressed as means ± SD of at least three independent experiments (n = 25–30). Statistical analyses were performed using SPSS (ver. 17.0, SPSS Inc., Chicago, IL, USA). Student's *t*-test ($P < 0.05$) or Duncan's multiple range test ($P < 0.05$) was used as appropriate.