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Abscisic acid and nitric oxide modulate cytoskeleton organization, root hair growth and ectopic hair formation in *Arabidopsis*

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ABSTRACT

Abscisic acid (ABA) and nitric oxide (NO) are two plant growth regulators that participate in many signaling cascades in different organs all along the plant life. Here, we were interested in deciphering the effects of ABA and NO on the cytoskeleton organization in a model of polarized cell growth like root hairs. *Arabidopsis* roots were exposed to different concentrations of ABA, and the length of primary root, epidermal cells and root hairs were measured. The NO concentration was detected with the NO-specific fluorescent probe DAF-FM DA. To quantify the effects of ABA and NO on cytoskeleton, Arabidopsis seedlings expressing GFP-MAP4 were used to analyze microtubules (MTs) orientation. Changes in cytoplasmic streaming were quantified through fluorescence recovery after photobleaching (FRAP) experiments using confocal laser scanning microscopy (CLSM) and the probe fluorescein diacetate (FDA). Results indicate that ABA decreases root hair length and induces the differentiation of atrichoblasts into trichoblasts, increasing root hair density. ABA also triggers an increase of NO level in root hairs. Both, ABA and NO affect MT organization in root hairs. While root hairs show MT orientation close to the longitudinal axis in control roots, ABA and NO treatments induce the oblique orientation of MTs. In parallel, cytoplasmic flow, executed by actin cytoskeleton, is enhanced by NO, in an ABA-independent manner. For all experimental conditions assayed, basal levels of NO are required to keep MT organization and cytoplasmic streaming. Our findings support ABA and NO as key modulators of growth and ectopic formation of root hairs through actions on cytoskeleton functions.

1. Introduction

Root hairs are tip-growing tubular outgrowths that emerge from specialized root epidermis cells known as trichoblasts, and expand locally at their apical dome [1]. Their functions are anchoring and increasing the area of soil exploitable by the plant. Rapid tip growth depends on several processes such as Ca^{+2} influx, MAPK cascades, microtubule (MT) and actin arrangements, vacuolar development, nucleus migration and vesicular trafficking, among others [2–7].

Cytoskeleton plays an important role in tip growth. As in other cells, cytoskeleton functions depend on distribution and arrangement of MTs and microfilaments. During tip growth, actin cytoskeleton is responsible for vesicle delivery and nuclear and organelle dynamics [3,8], while MTs are required to maintain the direction of the tip growth but not for sustaining the growing process *per se* [9].

MTs are negatively charged polyelectrolytes [10]. The organization and dynamics of MTs are controlled by Microtubule-associated proteins (MAPs) interacting with MT surface electrostatically through positively charged amino acid stretches [10]. MAPs bind alongside MTs, stabilize them against disassembly and mediate the interaction of MTs with other cellular components [11]. In growing root hairs, the cortical MT-system consists mainly of net-axially, parallel-aligned MTs that are absent from the tip of the root hair [12].

The actin side of cytoskeleton also plays essential roles in plant tip growth. F-actin bundles are arranged longitudinally in root hairs, and, together with myosin, are thought to cause cytoplasmic streaming that transports vesicles to the apex [13]. Organelles move actively in the cytoplasm, inducing a flow in the surrounding medium, called

Abbreviations: BDM, (2,3-butanedione monoxime); CLSM, (Confocal laser scanning microscopy); cPTIO, (carboxy PTIO); DAF-FM DA, (3-amino,4-aminomethyl-2,7-difluorofluorescein diacetate); GFP–MAP4, (Green fluorescent protein-Microtubule-associated protein 4); FDA, (Fluorescein diacetate); FR, (Fluorescence recovery); FRAP, (Fluorescence recovery after photobleaching); Ft, (Fluorescence.time^{-1}); MTs, (Microtubules); SNAP, (S-nitroso N-acetyl penicillamine)

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hydrodynamic flow [14].

Nitric oxide (NO) is a small and diffusible bioactive molecule, implicated in many plant physiological processes [15,16]. In roots, NO is involved in auxin-induced lateral root formation [17,18], adventitious root development [19], and also in root hair growth, through the vesicle formation and transport [20]. In growing root hairs, NO is located inside the vacuole, and relocated in the cytoplasm in maturity. It has also been observed that plants depleted in NO present abnormal root hair phenotypes (swollen, short and/or ramified), suggesting that the cytoskeleton could be affected in those cells [20]. Analysis of Arabidopsis wild type plants treated with NO synthase (NOS) inhibitor, and mutants of nitrate reductase (NR), show that both enzymatic NO sources are involved in root hair development [20,21].

Abscisic acid (ABA), a plant hormone that regulates many aspects of plant physiology, is also involved in polar cell expansion. ABA participates in the regulation of pollen tube growth in maize [23] and eliciting the water-stress response in root hairs of Arabidopsis and rice [24].

The crosstalk between ABA and NO signaling was firstly demonstrated in mechanistic events associated with ABA-induced stomatal closure [25,26] and in responses to UV-B-induced stress [27]. ABA can also promote NO generation in roots associated with NIA/NR- and AtNOA1-enzymatic activities [28]. More recently, it has been demonstrated that ABA signaling can be negatively regulated by NO through the S-nitrosylation of the kinase OST1 in guard cells [29], or through posttranslational modification of the transcriptional factor ABI5 during early seedling development [30], suggesting a more complex role for NO in the ABA signaling, than previously envisaged [31].

In this work, we present evidence showing that MTs and actin cytoskeleton are targets of ABA- and NO-regulated signaling processes, changing the pattern of root hair growth and their ectopic formation.

2. Materials and methods

2.1. Plant materials and chemicals

Seeds of *Arabidopsis thaliana* ecotype Col-0, G′4,3 mutant (double mutant *nia1/nia2*, chlorate resistant) were obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University, Columbus. Transgenic *A. thaliana* Col-0 expressing green fluorescent protein (GFP)–MAP4 [32] were used for cytoskeleton studies. Seeds were surface sterilized by immersion in 70% (v/v) ethanol for 5 min and 20% (v/v) bleach for 20 min, followed by three rinses in sterile water. Seeds were washed with sterilizing solution: 30% (v/v) bleach with 20% (v/v) Triton X-100, and sown in Petri dishes with ATS medium containing 0.6 (w/v) agar, 1% (w/v) sucrose and mineral nutrients [33], kept for 24 h at 4 °C and then incubated in a chamber at 25 °C with a photoperiod of 14 h for 6 d (Col-0) and 8 d (mutant). Six days-old (Col-0) seedlings were transferred onto nutrient agar plates containing or not different concentrations of the NO donor S-nitroso N-acetyl penicillamine (SNAP), or Abscisic Acid (ABA), with or without 500 μM of the NO scavenger carboxy PTIO (cPTIO), for two days. Roots and root hairs of at least seven seedlings were analyzed for each treatment and genotype, with a Nikon C1 Confocal Laser-Scanning Microscope (CLSM) and Leica ATC 2000 Light Microscope (LM) and/or Nikon Eclipse 200 (Tokyo, Japan) Fluorescence Microscope (FM), according to the experiments. All chemicals were purchased from SIGMA (SIGMA-ALDRICH Co., USA). Data is the result of at least three independent experiments performed for each assayed approach.

2.2. Measurement of primary root and root hair length, and root hair density

As stated above, Arabidopsis seedlings were treated with different concentrations of ABA, SNAP and/or 500 μM cPTIO, or nothing. Seedlings were removed from the agar and placed in water, and the root hairs were gently combed with the help of a brush. For LM, roots were stained with toluidine blue O (TBO) and mounted on a slide with water. Primary root, epidermal cells and root hair lengths (mm), and root hair density (hairs/mm) were measured with a graduated microscope. Root hair length was measured in the mature hair zone, more than 3 mm above the root tip, in the zone of mature root hairs where they have reached their maximum length. For density, root hairs were considered in dome, mature and elongation stages, and all of them counted in a microscope field from the immature zone of the root (1–2 mm above the tip root, where the epidermal cells are already defined) until the mature zone (beyond 3 mm above the tip root). For each experiment, at least 12 roots were measured, and at least 6 root hairs of each root were measured.

2.3. Detection and relative quantification of endogenous NO

The level of NO was monitored in root hairs using NO-specific cell-permeable fluorescent probe 3-amino, 4-aminomethyl-2,7-difluorofluorescein diacetate (DAF-FM DA, excitation at 490 nm, emission at 525 nm; Calbiochem, San Diego, CA, USA). Arabidopsis root hairs without any treatment or treated with 10 μM ABA and/or 500 μM cPTIO were incubated in 10 μM DAF-FM DA in 20 mM HEPES-NaOH (pH 7.5) for 1 h. Thereafter, roots were washed three times with fresh buffer and examined with FM. Photographs of DAF FM-loaded root hairs were captured with a Nikon 900 camera. The fluorescence was registered in fifteen squares of 6.25 μm^2 each along the root hair tube. Fluorescence was quantified with ImageJ program and expressed in arbitrary units (AU). For each experiment, at least 14 roots were measured, and at least 12 root hairs of each root were measured.

2.4. Microtubule (MT) organization

Seedlings of Arabidopsis Col-0 expressing MAP4-GFP were treated with ABA, or SNAP and/or cPTIO, as stated above, and the effects on the MT organization were studied during the root hair growth. Pictures of developing root hairs were taken with CLSM. Angles between 0 and 180° (in 20° ranges) of cortical MT inclination in respect the root hair longitudinal axis were measured with ImageJ program and quantified in percentages as described [6,34]. The angles were grouped into intervals of 20°, from 0° to 180° and they were expressed as percentages. To differentiate the angles lower and higher of 90° for their analysis by CLSM, all root hairs were positioned with the hair protrusion at the base of the trichoblast (oriented towards the root apex). For each experiment, at least 13 roots were measured, and at least 3 root hairs of each root were measured.

2.5. Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed using CLSM. Seven-days-old Arabidopsis seedlings, treated or not with ABA, SNAP and/or cPTIO, and the Arabidopsis G′4,3 mutant, were marked with FDA (Fluorescein diacetate, Calbiochem) for 1 min, soaked and mounted in dH_2O. For experiments using the actin inhibitor 2,3-butanedione monoxime (BDM, Sigma), Arabidopsis Col-0 seedlings were mounted in BDM 5% (v/v). Pre- and post-bleaching periods were carried out using 488-nm laser line at 2–4% transmission. Photobleaching was performed at 100% transmission in an area of 10.51 μm^2, corresponding to the cytoplasmic zone at the tip of the root hair. Movies of each treatment were recorded and analyzed. Fluorescence intensity values were normalized and the slope of fluorescence recovery was used to compare the different treatments. For each experiment, at least 14 roots were measured, and at least 3 root hairs per root were measured.