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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.

3. Results

3.1. ABA alters root hair growth and development, and increases NO levels in Arabidopsis

To test the effect of ABA on the root system, Arabidopsis seedlings were exposed to different ABA concentrations (1, 5, 10, 100 and 400 μM) for two days, and several parameters of root growth were analyzed. The length of the primary root was affected by ABA in all tested concentrations (Fig. 1A). One μM ABA caused 20% decrease of the primary root length, whereas 400 μM ABA resulted in the strongest inhibitory response (Fig. 1A). ABA concentrations between 1 and 100 μM diminished almost 50% the length of epidermal cells and root hairs, while the higher tested ABA concentration (400 μM) had an almost toxic effect since it inhibited 75% the epidermal cells length and more than 90% the length of the root hairs (Fig. 1B and C, respectively). The treatments between 1 and 100 μM ABA increased the root hair density, being ectopic most of them and not merely a consequence of epidermal cell shortening (Fig. 1D and E). The increase of the number of root hairs per area of primary root represents, thereby, a genuine differentiation of atrichoblast into trichoblast induced by ABA.

To explore the involvement of NO in ABA-triggered responses in root hairs, 6-days-old Arabidopsis Col-0 seedlings were treated with 10 μM ABA with or without 500 μM of the NO scavenger carboxy PTIO (cPTIO) for two days. Roots were loaded with the fluorescent probe DAF-FM DA and analyzed for detecting the presence of NO in roots and root hairs (Fig. 2A and insets, respectively). Root hairs were near 50% shorter than control in ABA-treated roots and cPTIO treatment results in root hairs shorter than wild-type and ABA-treated (Fig. 2B). The quantification of fluorescence indicates that ABA-treated root hairs have 60% more NO-fluorescence than control, and cPTIO treatment decreased more than 70% the fluorescence intensity, both in the presence or absence of ABA (Fig. 2C).

In addition, the NO scavenger cPTIO was not able to completely block the capacity of ABA to form new root hairs, indicating that a complex network of signals acts downstream ABA and NO to induce the new root hair formation in Arabidopsis (Supplemental Fig. S1, supplementary information).

3.2. ABA and NO change the microtubule (MT) orientation

Since root hairs are epidermal cells with polarized growth where cytoskeleton plays a pivotal role, here we had interest in looking for a nexus between ABA and NO that could modulate cytoskeleton organization during root hair growth. As a tool for analyzing alterations in the MT orientation, we set up the angles of MT inclination respect to the longitudinal axis in root hairs, in ranges of 20° between 0 and 180° (Fig. 3A). Ranges of angles of 0–20° and 161–180° (0–20°//161–180°) between MTs and the longitudinal axis of root hairs have the same inclination, as well as those of 21–40° and 141–160° (21–40°//141–160°), and 41–60° and 121–140° (41–60°//121–140°). Ranges of angles close to perpendicularity (90°) (61–80°//81–100°//101–120°), are opposite to the frequent position of MTs, which are oriented all along the longitudinal axis of root hairs.

The effect of NO and ABA on MTs orientation was analyzed by CLSM in Arabidopsis root hairs expressing green fluorescent protein (GFP)–MAP4. Pictures of z-stack register were used for measuring angles between MT and the longitudinal axis of root hairs. Roots were treated or not with ABA, with or without the addition of cPTIO, for two days. Pictures of different planes were taken and angles of MTs measured with the ImageJ program. While control root hairs presented MTs with angles close to the root hair's longitudinal axis (63% in 0–20°//161–180°; and 33% in 21–40°//141–160°), root hairs treated with cPTIO, with or without ABA, showed MTs with random organization (34% in 0–20°//161–180°; 36% in 21–40°//141–160°; 21% in 41–60°//121–140° and 9% in intermediate ranges) (Fig. 3B). This is consistent with the high percentage of root hairs with abnormal phenotype (sinuous, branched, double hair) found in Arabidopsis root hairs treated with cPTIO [20]. MTs of ABA-treated root hairs displayed a random organization with a high representation of the angles between 21 and 40°//141–160° (10% in 0–20°//161–180°, 59% in 21–40°//141–160°, 14% in 41–60°//121–140° and 5% in intermediate ranges).

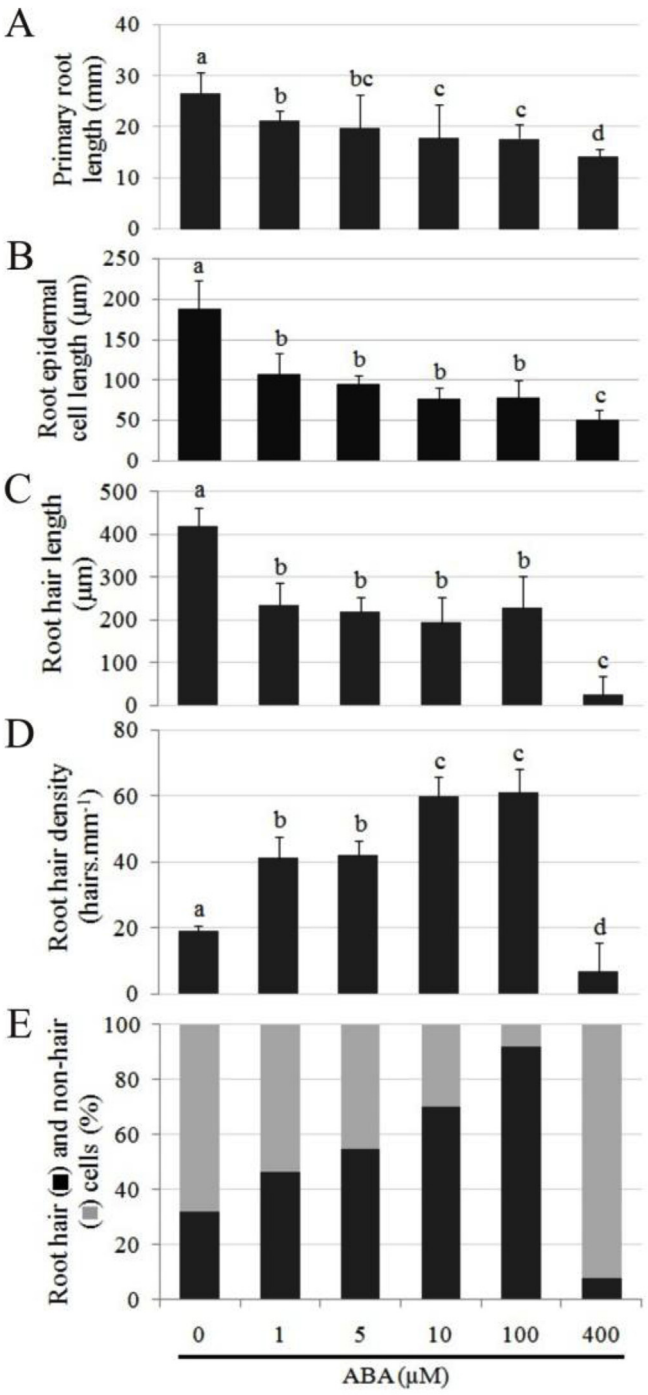

Fig. 1. Abscisic acid (ABA) influences root and root hair growth and development in Arabidopsis. Arabidopsis Col-0 seedlings were treated or not with 1, 5, 10, 100 and 400 μM ABA for two days. Primary root length (A), root epidermal cell length (B), root hair length (C), root hair density (D) and percentage of root hair and non-hair cells (E) were analyzed. Different letters mean significant differences (ANOVA, Tukey's test, A: $p < 0.01$, C and D: $p < 0.05$, Dunn's test, B: 0.05). The results are representative of at least three independent experiments.

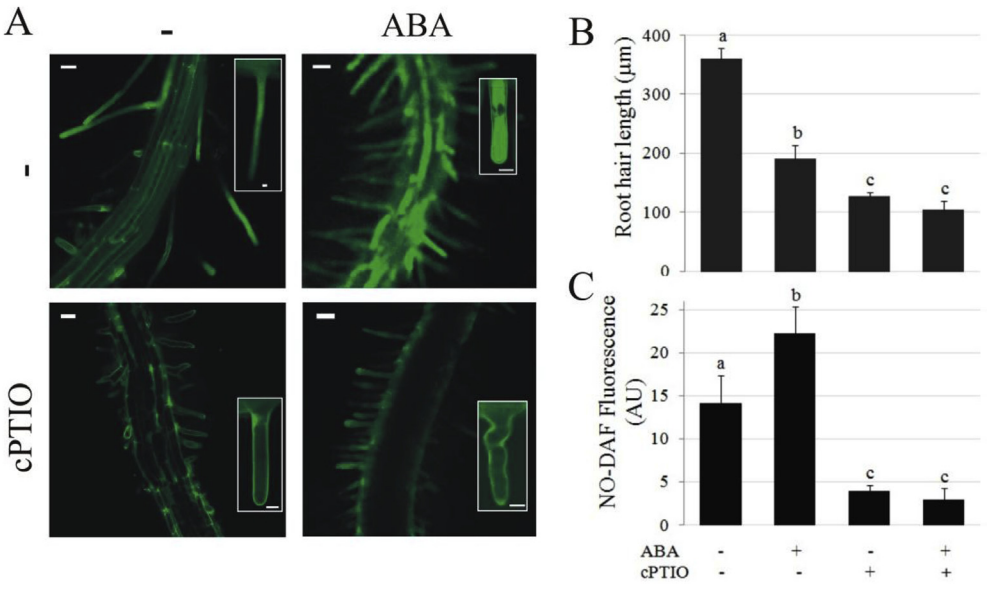

Fig. 2. ABA triggers nitric oxide (NO) increase in Arabidopsis root hairs. Arabidopsis Col-0 seedlings were treated with 10 μM ABA and/or 500 μM of the NO scavenger cPTIO, or nothing for two days. (A) Arabidopsis roots were loaded with the NO sensitive fluorescent dye DAF-FM DA. Pictures of roots (scale bar = 50 μm) and root hairs (inset, scale bar = 10 μm) were taken with a Nikon 900 Camera and FM. Root hairs length was measured (B) and Fluorescence intensity of root hairs was quantified with ImageJ and expressed in arbitrary units (AU) (C). Different letters mean significant differences. ANOVA, Dunn's test $p < 0.05$ (A) and Tukey test $p < 0.01$ (B). Results are representative of at least three independent experiments.

We also summarized the changes in percentages of MTs displaying different orientation in root hairs after the treatment with different concentrations of ABA and NO (Supplemental Figs. S2 and S3, supplementary information). While low concentrations of ABA may already affect the MT inclination, 40 μM ABA seems to induce the more oblique MT arrangement (Supplementary Fig. S2). A positive correlation between the increase of MT percentage with oblique orientation and the increase of the NO concentration could be found (Supplemental Fig. S3). On the other hand, the NO depletion resulted in the highest percentages of MT with oblique orientation (Fig. S3).

3.3. NO affects root hair length and MT organization in a dose-dependent manner

To test the response of root hair to NO, Arabidopsis Col-0 seedlings were treated with 0, 1, 10, 50, 100, 200 or 500 μM of the NO donor SNAP for two days. The length of root hairs was not affected between 0 and 100 μM SNAP (Fig. 4A). Higher concentrations of SNAP caused reduction in the root hair length, being 17% and 60% shorter at 200 μM and 500 μM SNAP, respectively. As expected, cPTIO treatment also generated root hair 78% shorter than control (Figs. 2A and 4A), indicating that high NO levels as well as NO depletion resulted in the partial inhibition of root hair growth.

Representative pictures depicting the MT organization in NO-treated root hairs were taken (Fig. 4B, left panel). The percentages of MT showing different angles with respect to longitudinal axis of root hairs were quantified and presented in diagrams (Fig. 4B, right panel). As shown, microtubular angles were close to longitudinal axis in control, while cPTIO presented a random organization (Fig. 4B). The treatment with the NO donor SNAP resulted in a change of the MT organization. There was found a change in the percentages of MT that changed their orientation for ranges of angles between 0 and 20°// 161–180° and 21–40°//141–160° when root hairs were treated with 0, 1 or 100 μM SNAP (Fig. 4C). Between 0 and 100 μM SNAP, NO induced a dose-responsive change in the orientation of MTs, by decreasing the percentages of MTs with longitudinal orientation and increasing the percentages of MT with slight oblique orientation (Fig. 4C). In this range of NO concentrations, there was a slight change of MT orientation without affecting the length of the root hair (Fig. 4A), suggesting that an impaired root hair growth is probably associated with a more severe MT disorganization.

3.4. Effects of ABA and NO on the cytoplasmic streaming

Experiments using the Fluorescence Recovery after Photobleaching (FRAP) technique were performed with CLSM for studying the effects of ABA and NO on the cytoplasmic flow in Arabidopsis root hairs. Fluorescein diacetate (FDA) is a molecule that becomes fluorescent when goes across plasma membrane and is hydrolyzed by a number of enzymes, such as proteases, lipases, and esterases. The product of the enzymatic reaction is fluorescein, which can be visualized within cells by FM. In our experimental system, fluorescein is part of the free components dragged during active transport of organelles in the cytoplasm of root hairs.

A small area of each FDA-loaded root hair was photobleached, the time of re-appearance of the fluorescent signal was recorded (Fig. 5A), and rates of fluorescence recovery (FR) calculated (Fig. 5B). Several pictures were taken before, during and after photobleaching, and representative photos corresponding to different treatments and Arabidopsis genotypes are shown in Fig. 5A (upper).

In control root hairs, the FR rate [Fluorescence.time^{-1}, Ft] was 3.45 ± 1.4 (Fig. 5B). The inhibitor of actin transport 2,3-butanedione monoxime (BDM) was used as a control and it inhibited 50% the FR respect to the control. Noteworthy, cPTIO-treatment and the Arabidopsis G′4,3 mutant, impaired in NR activity and NO production, showed even lower values (0.5 ± 0.25 and 0.4 ± 0.4 Ft, respectively) than that obtained with BDM (Fig. 5A, bottom). Treatment with ABA presented a higher FR rate than control, but with a non-significant difference (5.2 ± 3 Ft). When scavenging NO with cPTIO, ABA presented a FR value (0.25 ± 0.15 Ft) close to cPTIO treatment and G′4,3 mutant, suggesting that NO is fully required for a correct cytoplasmic flow. Furthermore, treatments with 50 and 100 μM SNAP increased 3 and 4 times, respectively, the cytoplasmic streaming, indicating that NO not only is required for sustaining the cytoplasmic streaming, but even can accelerate it (Fig. 5C).

4. Discussion

4.1. ABA and NO crosstalk in root hair growth and development

Many evidences support ABA as a hormone associated with stress responses and growth processes in plants [35]. Furthermore, ABA participates on different aspects of the cell dynamic in concerted actions with other hormones [36–38]. Here, we show that ABA influences root

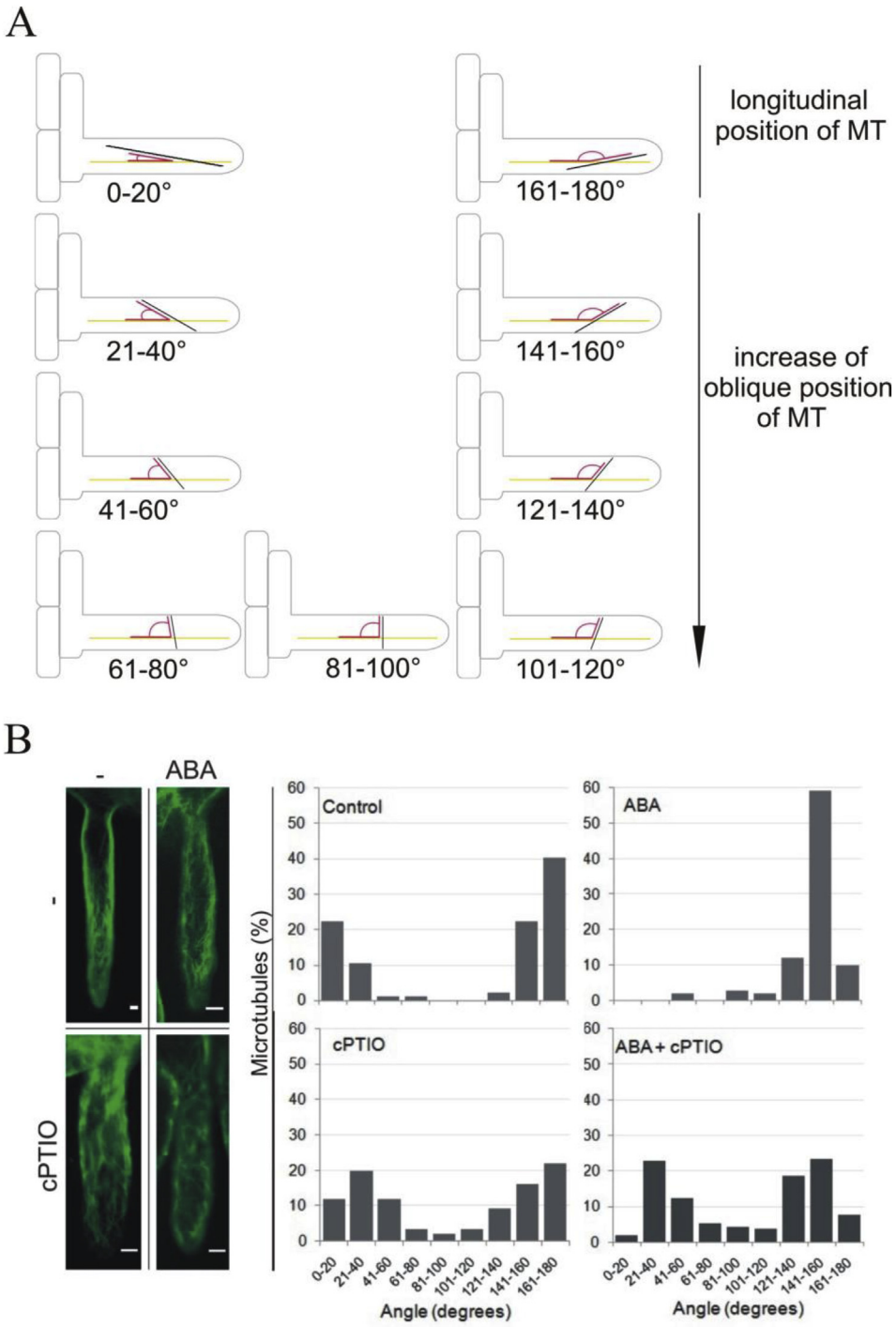

Fig. 3. ABA and NO modulate the microtubule (MT) orientation. A, Scheme showing ranges of MT inclination between 0 and 180° with respect to the root hair longitudinal axis. 0–20°// 161–180° ranges correspond to longitudinal position, and 61–80°//81–100°//101–120° correspond to position of extreme obliquity of MT in root hairs. A representative angle for each range is pictured with a red symbol together to longitudinal axis in yellow. B, Arabidopsis seedlings expressing MAP4-GFP were treated or not with 10 µM ABA and/or 500 µM cPTIO, or nothing. Pictures of MTs were taken with CLSM (left). MTs with different orientation according to the established angular ranges were recorded as percentages (%) (right). Values represent the results of at least three independent experiments. Scale Bars: 5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cell expansion and promotes shortening of: (i) primary root and epidermal root cells (Fig. 1A and B), and (ii) root hair length (Fig. 1C). Contrasting effects of ABA on root hair growth have been recently reported in Arabidopsis and rice. While ABA affects root hair growth via the transcriptional regulator OBP4 and repression of Root Hair Defective 6-Like2 (RSL2) in Arabidopsis [39], in rice ABA regulates auxin homeostasis and promotes root hair elongation [40]. These results indicate the inconvenience of comparing root responses to growth regulators in different plant species and different conditions.

The cross-talk between ABA and NO was demonstrated in several plant responses to abiotic stresses [27,29,41–43]. We have previously demonstrated that: (i) NO is a non-traditional plant regulator modulating root hair growth, including the promotion of root hair differentiation in lettuce [21]; (ii) the NO scavenger cPTIO inhibits root hair growth in a dose-dependent response [21], and (iii) exogenous NO can revert the root hair phenotype in roots of G′4,3 mutant impaired in NO production [20]. Here, we show that in Arabidopsis, the inhibitory effect of ABA on root hair growth (Fig. 2A and B), is accompanied by a significant increase of the NO level (Fig. 2A and C). On the other hand, the depletion of NO with cPTIO also resulted in a severe inhibition of root hair growth (Fig. 2B), indicating that NO is required in a precise range of concentration for the promotion of root hair growth, otherwise it becomes a limiting factor. This was confirmed by the inhibition of root hair growth at high concentrations of NO (Fig. 4A). As previously

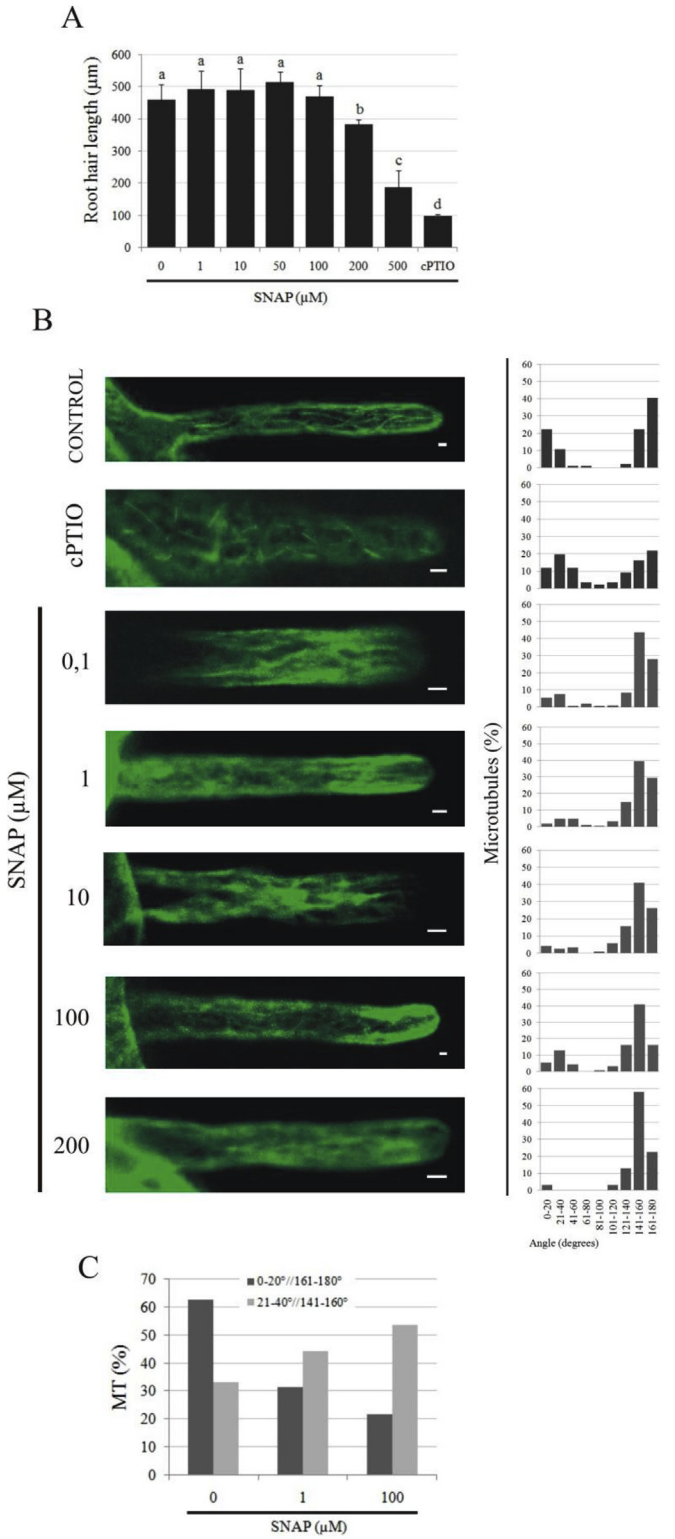

Fig. 4. NO is involved in root hair growth and MT organization in a dose-dependent manner. Arabidopsis seedlings expressing MAP4-GFP were treated or not with 1, 10, 50, 100, 200 and 500 µM of the NO donor S-nitroso N-acetyl penicillamine (SNAP) or 500 µM cPTIO for two days. (A) Root hair length measurements. (B) Pictures of MTs were taken with CLSM (left) and MT orientations were measured according to the established angular ranges (right). Changes in the orientation of MT corresponding to the 0–20°//161–180° and 21–60°//141–160° ranges of 1 and 100 µM SNAP treatments compared to control are shown (C). Values represent the results of at least three independent experiments (ANOVA, Tukey test $p < 0.01$). Scale Bars: 5 µm.

proposed, biological action of NO in plants were reminiscent of hormonal actions [44] and, like a hormone, at higher concentrations NO inhibits growth processes. Therefore, it can be postulated that the levels of NO required for the growth of root hairs must be between a minimum and a maximum threshold concentrations required to achieve the growth potential. Here, we also demonstrate that ABA has a strong promoter activity of the differentiation of root epidermal cells to ectopic root hair formation (Fig. 1D and E), resulting in a higher root hair density, and that NO is partially involved in this process.

4.2. ABA, NO and the regulation of microtubule orientation

An early report demonstrated that ABA affects cortical MTs orientation in hypocotyls cells of *Cucumis sativus* [45]. Later, Seung et al. [46] showed that ABA is involved in plant development and responses to environmental stress altering the formation and orientation of MT arrays in elongating cells. More recently, Takatani et al. [47] reported that ABA induces ectopic outgrowth in epidermal cells of Arabidopsis hypocotyls through cortical MT reorganization. The latter is a pivotal work that directly links ABA, ectopic outgrowth of epidermal cells and MT reorganization in hypocotyls. All these results demonstrate that MTs are target of ABA actions in response to different stimuli. Our findings extend the evidences of the modulating activity of ABA on the MT organization, now associating ABA effects to the change of predetermined atrichoblasts to trichoblasts in Arabidopsis roots (Figs. 1D and 3B).

We observed that beyond the minimum and maximum thresholds of NO concentrations required for root hair growth, the MT orientation is strongly affected in root hairs. The disruption of NO homeostasis by low exogenous NO addition changes the MT orientation, without affecting the growth of the hairs (Fig. 4B). The deviation of MTs orientation provoked by NO appears to be dose-dependent between 0 and 100 µM SNAP, from angles parallel to the longitudinal axis in untreated root hairs, to the oblique angles induced by higher NO doses (Fig. 4C). This provides further evidence that the physiology of MTs is, directly or indirectly, modulated by NO in root hairs [34]. In addition, we have already showed that NO depletion strongly affects the phenotype of root hairs, resulting in abnormal and aberrant forms, which are probably associated to cytoskeleton disorganization [20].

Here, for the first time, we present evidence of the link between ABA and NO on the MT physiology. This may have profound implications not only for understanding the fundamentals of the ABA connection with cell fate determination, but also in knowing how plants devise strategies to modulate the absorption surface of roots as a rapid physiological adaptation in response to environmental changes.

Many works reported effects of NO on MTs both in animals [48,49] and plants [34,50,51]. In eukaryotic cells, the best characterized regulation of protein activity mediated by NO is due to post-translational modifications, namely tyrosine nitration and S-nitrosylation of Cys residues. Tyrosine nitration of plant α-tubulin is a direct molecular mechanism induced by NO for regulating MT organization under physiological conditions, and responsible of the increased dynamic of MT [51–54]. Coincidently, NO production and cortical MT dynamics are part of the signaling system described in Arabidopsis epidermal cells [50,53,54] and guard cells [50,55].

4.3. ABA, NO and the modulation of the cytoplasmic streaming

Cytoplasmic streaming transports organelles, vesicles and solutes over longer distances in cells and especially toward the cell growing points [56]. The tip growth processes, like those occurring in pollen tubes and root hairs, are actin-dependent [2,57] and were shown to be regulated by NO [20,21,58]. Here, we present consistent evidence supporting that NO increase promotes cytoplasmic flow (Fig. 5C) and NO depletion causes a significant slowdown of circulation as shown in cPTIO treatment and in the NO-deficient Arabidopsis G′4,3 mutant

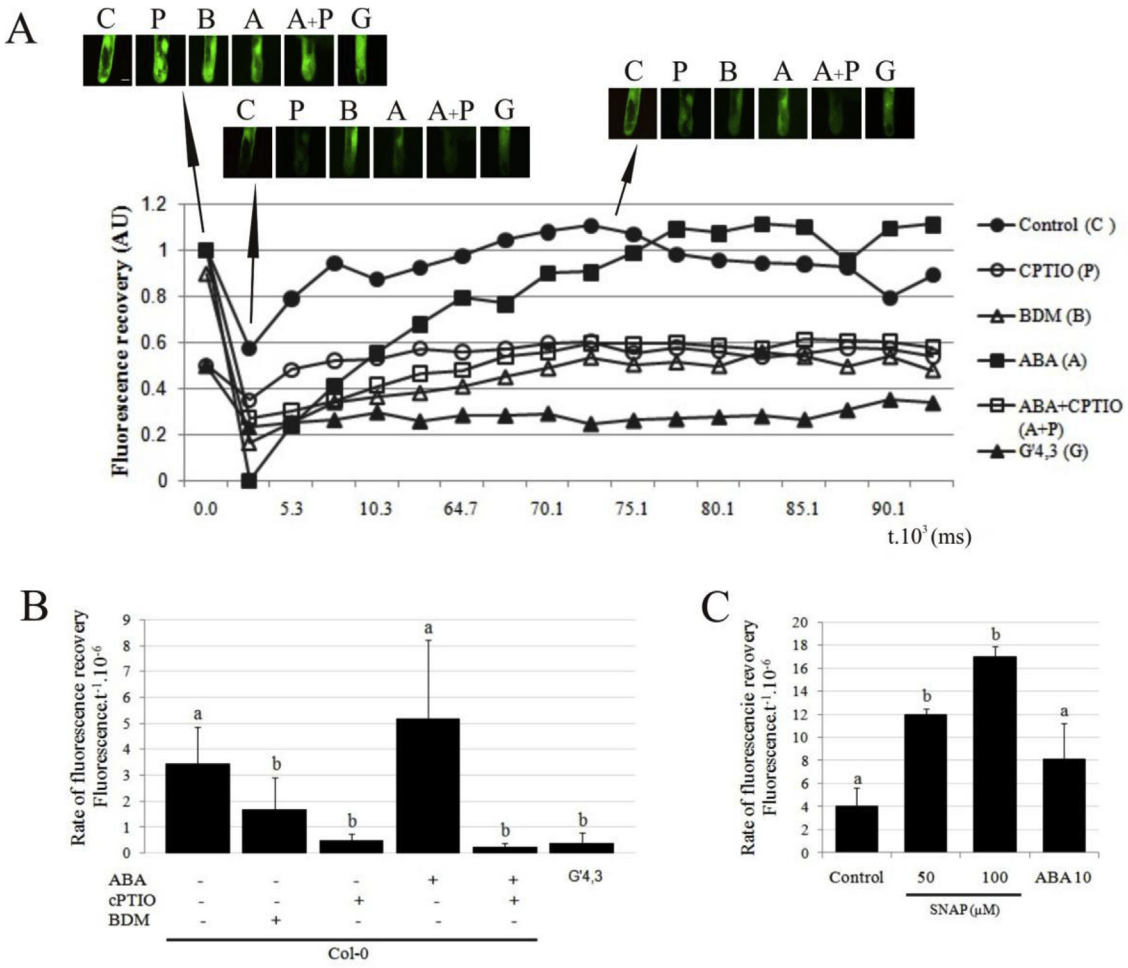

Fig. 5. Effects of ABA and NO on the cytoplasmic streaming. (A) Fluorescence Recovery After Photobleaching (FRAP) was used to quantify the cytoplasmic streaming in root hairs from: (i) Arabidopsis Col-0 treated or not with the actin inhibitor 2,3-butanedione monoxime (BDM), 10 µM ABA and/or 500 µM of the NO scavenger cPTIO, and (ii) G′4,3 Arabidopsis mutant. Cytoplasmic flow was followed after labeling with Fluorescein DA. Pictures of root hairs were taken before and after photobleaching, and during fluorescence recovery (pictures, insets); (B) slope of the fluorescence recovery. (C) Slope quantifications from FRAP assays of 50 and 100 µM of SNAP compared with control and 10 µM ABA. Values represent the results of at least three independent experiments. Different letters mean significant differences (ANOVA, $p < 0.01$). Root hairs are from at least two independent experiments.

(Fig. 5B). This suggests that the dynamic of the cytoplasmic flow appears to be NO-dependent in Arabidopsis root hairs. While the addition of NO boosts the rate of cytoplasmic flow, ABA has no apparent effect, indicating that both regulators have specificities in their actions and even in their common signaling pathways, depending on the cell biology process involved.

ABA has been implicated in actin structure formation [59]. It was reported that actin filaments of illuminated guard cells are radially organized and they are rapidly disassembled during ABA-induced stomatal closure [59]. On the other hand, actin cytoskeleton is a target of NO signaling through NO-driven post-translational protein modification [60]. Moreover, the NO-induced F-actin reorganization showed to have cell type-, cell development-, and subcellular domain-specificities in maize roots [52]. Here we show that cytoplasmic flow is affected by inhibiting myosin transport with BDM and, in a stronger way, by NO depletion (Fig. 5A and B).

4.4. Main conclusions

Recently it was shown that a pulse of transcription factors synthesis is governing the length of root hairs [61]. However, there is no a clear picture of how exactly are plant growth regulators participating in the root hair growth processes and the activation of molecular events balancing the formation of new, ectopic root hairs. Fig. 6 proposes a model that summarizes the linkage of signals promoted by ABA and NO associated with changes in cytoskeleton organization, and how they could determine root hair growth and development, and new ectopic hair formation in atrichoblasts positions.

Shortening the length and amplifying the number of root hairs could result in more robust and suitable organs for a better root fitness in dried soils, without affecting the total absorption surface of roots. Our results emphasize the central role played by cytoskeleton as target of ABA and NO for modulating root hair growing processes and they also demonstrate the effects of both regulators on the ability of the root to form new hairs and to adapt to a changing environment.

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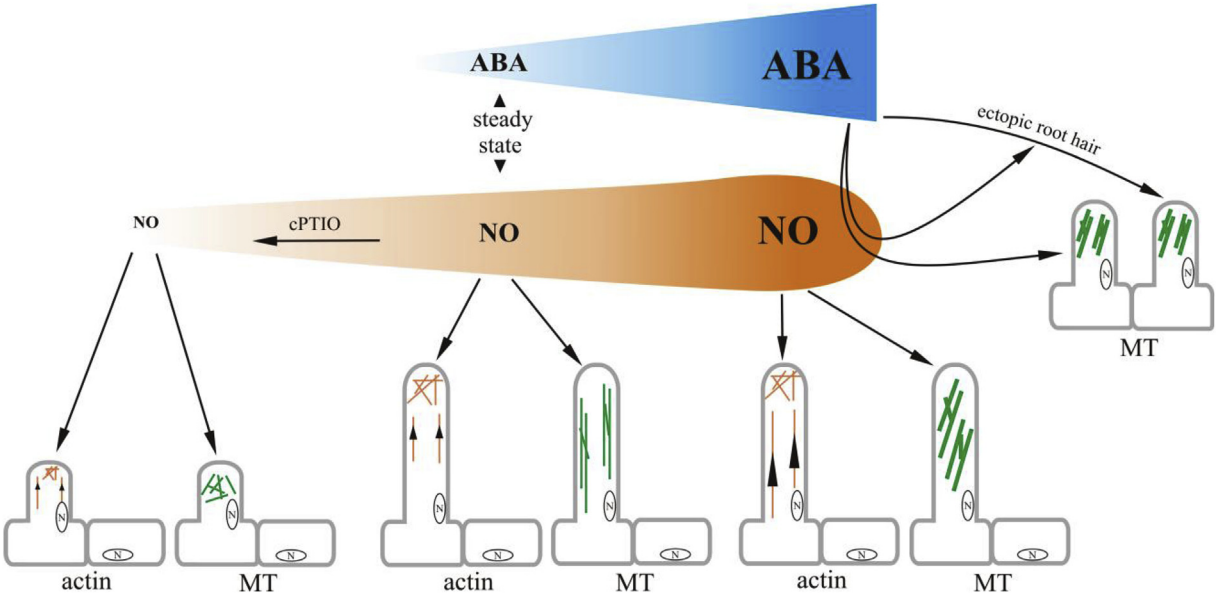

Fig. 6. Simplified model proposing changes modulated by ABA and NO on the cytoskeleton arrangement, and the growth and ectopic formation of root hairs in Arabidopsis. Under steady state conditions, ABA and NO contribute to keep basal homeostasis conditions for cytoskeleton functions, and normal growth of root hairs. The increase of ABA and NO concentrations disrupts the balance resulting in altered MTs (green) disposition/inclination, moving them from longitudinal to oblique sloping respect to root hair longitudinal axis. As a consequence of the ABA increase, new root hairs are formed from epidermal cells previously determined as atrichoblasts (ectopic root hairs). The increase of NO accelerates the cytoplasmic flow mediated by actin (red). On the contrary, by diminishing NO concentration with cPTIO, results in a slower cytoplasmic flow and disrupted MT organization with random disposition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2018.09.002.

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