ATANN3 is involved in extracellular ATP-regulated auxin transport and distribution in *Arabidopsis thaliana* seedlings

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Research Article

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Abstract

Background

Extracellular ATP (eATP) exists in the apoplast of plants and plays multiple roles in growth, development, and stress responses. It has been reported that eATP stimulation suppresses growth rate and alters growth orientation of root and hypocotyls of *Arabidopsis thaliana* seedlings by affecting auxin accumulation and transport in these organs. However, the mechanism of eATP-stimulated vegetative organ growth remains unclear. Annexins are involved in multiple aspects of plant cellular metabolism, while the role of annexins in response to apoplast signal remains unclear. Here, by using loss-of-function mutants, we investigated the role of several annexins in eATP-regulated root and hypocotyl growth. Since mutant of *AtANN3* did not respond to eATP sensitively, the role of AtANN3 in eATP regulated auxin transport was intensively investigated.

Results

First, the inhibitory effect of eATP on root or hypocotyl elongation was weakened or impaired in *AtANN3* null mutants (*atann3*). Meanwhile, single-, double- or triple-null mutant of *AtANN1*, *AtANN2* or *AtANN4* responded to eATP stimulation in same manner and degree with Col-0. The abundance and distribution of Dr5-GUS and Dr5-GFP indicated that eATP-induced accumulation and asymmetric distribution of auxin in root tip or hypocotyl cells, which appeared in wild type controls, were lacking in *atann3* seedlings. Further, eATP-induced accumulation and asymmetric distribution of PIN2-GFP in root tip cells or PIN3-GFP in hypocotyl cells were reduced in *atann3* seedlings.

Conclusions

AtANN3 may be involved in eATP-regulated seedling growth through regulating auxin transport and accumulation in vegetative organs.

Background

Numerous signal molecules exist in the apoplastic matrix that are responsible for modulating cell metabolism, making the apoplast (including the cell wall and intercellular spaces) an essential modulator of plant cell growth and development. Among these signaling molecules, eATP plays an essential role. Intracellular ATP can leak through plasma membrane (PM) wounds, be secreted in secretory vesicles, or be released through specialized PM transporters [1–4]. During plant growth and development, eATP is involved in maintaining cell viability, regulating growth rate and direction of some vegetative organs (roots and hypocotyls) [5–9], and regulating reproductive processes [10, 11]. eATP is also involved in regulating stomatal movement and gravitropism [12–15]. In response to biotic or abiotic stresses (e.g. cold stress, salt stress, and pathogen attack), ATP secretion can increase, producing an eATP-stimulated

defensive or tolerant responses that act as a “danger signal” [1, 16–22]. As the main eATP hydrolyzing enzyme, extracellular apyrase is involved in terminating signal transduction and maintaining eATP level [23, 24].

To elucidate the mechanisms underlying eATP function, signal transduction of eATP has been extensively investigated over the past two decades. The first step of the eATP signaling pathway is the binding of eATP to its receptors. Two lectin-receptor kinases, P2K1 and P2K2, were identified in *Arabidopsis thaliana* and shown to be eATP receptors [21, 25]. The two P2K receptors have been shown to participate plant immune responses alone or cooperatively. Several signaling proteins in the PM, including heterotrimeric G proteins [7, 14, 26], NADPH oxidase [12, 14, 27], and ion channels [27–31] have been reported to be involved in eATP-stimulated physiological responses. These signal transducers are speculated to be governed directly or indirectly by eATP receptors and involved in eATP-stimulated generation of secondary messengers (e.g. Ca^{2+}, nitric oxide or reactive oxygen species) or intracellular signal transducing cascades [7, 9, 14, 27, 32–34]. After eATP stimulation, altered gene expression and protein synthesis had been observed and proposed to change plant growth & development in response to environmental signals [8, 25, 35].

Annexins are Ca^{2+}- and phospholipid-binding proteins that are located in the PM or inner membrane system, as well as in the cytoplasm of plant cells, that play multiple roles in plant growth, development, and stress responses [36–38]. Annexins are involved in seed germination and early seedling growth [39–41], the transition from the vegetative to the reproductive phase [42], pollen germination and tube growth [43], etc. Biotic stresses (fungal or viral pathogen attack) and abiotic stresses (cold, heat, salt, and drought) trigger or increase the expression of annexins in various plant species. The expression level of most annexins is positively correlated with plant cells’ tolerance to or defense against stresses [39, 40, 44–49].

Annexins are multi-functional proteins that are implicated in Ca^{2+} signaling, enzymatic metabolic reactions, and vesicle trafficking. Some annexins in maize and Arabidopsis have been shown to build reactive oxygen species-responsive Ca^{2+} or K^{+} channels [50–53]; these annexins are thought to be involved in stress-induced Ca^{2+} signaling. Some annexins showed enzymatic activity, including ATPase, GTPase, and nucleotide phosphodiesterase activity. The peroxidase activity of certain annexins has been shown to be involved in cellular redox reactions: when plants are exposed to stresses, annexins may suppress ROS accumulation, reduce lipid peroxidation, and protect cell activity [54, 55]. Annexins are membrane lipid or cytoskeleton binding proteins which localize to the PM and inner membrane where they participate in cytoplasmic vesicle trafficking and cell secretion [41, 43, 56, 57]. Annexins have also been observed in the nucleus where they are thought to regulate gene expression [58, 59].

As a multi-functional plant hormone, auxin plays essential roles in regulating growth and development. Plants responding to external stimuli (such as light, gravity, water, etc.) exhibit altered growth rate and orientation. Auxin accumulation and asymmetric distribution are responsible for regulating the elongation rate of plant cells in different parts of plant organs, which results in bending growth of these organs [60,

61]. Auxin transporters, especially PIN-FORMED transporters (PINs), play key roles in polar auxin transport [61–63]. Asymmetric distribution of PINs results in unidirectional auxin transport and asymmetric distribution. After stimulation, the subcellular PIN trafficking and PIN phosphorylation alter the localization of PIN proteins, which will alter auxin transport subsequently [64–68]. Small G protein- or clathrin-mediated vesicle trafficking is involved in PIN trafficking during photo- and gravity-tropic bending growth [69–72]. Most recently, two SNARE proteins were reported to be involved in auxin regulated seedling growth via regulating subcellular trafficking of auxin transporters [68]. In response to endogenous or exogenous stimuli, several amino acids in the central long hydrophilic ring of PINs can be phosphorylated, and the phosphorylation is sufficient to modulate the polar distribution, recycling, and ubiquitin-dependent turnover of PIN proteins [66, 67, 73]. There are 8 members in PIN family in *Arabidopsis thaliana*, each has distinct spatial-temporal expression and location profiles. In *Arabidopsis thaliana* seedlings, PINs-mediated auxin re-distribution play essential roles in tropic response of roots, hypocotyls to various stimuli, including gravity, light, salinity and water [60–62, 64].

eATP regulates auxin accumulation and asymmetric distribution in the roots of *Arabidopsis thaliana*, which alters the growth rate and direction of roots. PIN2 and PIN3 have been reported to be involved in eATP-regulated auxin transport [7, 8, 74]. However, the mechanism underlying eATP-regulated PINs abundance and re-location, which in turn alters auxin accumulation and asymmetric distribution, remains unclear. Some annexins have been shown to be necessary components in eATP signaling. Herein, to elucidate the role of annexins in eATP signaling, we investigated the effects of ATP supplementation on growth and auxin accumulation and distribution in seedlings of annexin-null mutants. Since AtANN3 mutants responded to eATP significantly differently from wildtype, the role of AtANN3 was intensively investigated.

Results

AtANN3 is involved in Arabidopsis seedlings' response to eATP

In our previous work, we showed that *Arabidopsis thaliana* seedlings responded to eATP by altering the growth rate and orientation of their roots and etiolated hypocotyls (Zhu et al. 2017, 2020). To verify the role of annexins in eATP signaling, the response of annexin-null mutant seedlings (*atann1*, *atann2*, *atann3* and *atann4*) to ATP addition was investigated.

When seedlings of wild type (Col-0) were transplanted onto the combined medium containing 0.5 mM ATP in the lower compartment, main roots showed a marked eATP avoidance response characterized by suppressed growth rate and altered growth direction as they approached the border between media (Fig. 1A). Root lengths of the 4 mutants were all significantly shorter than that of control ($p < 0.05$) (Fig. 1B). Root curvatures of *atann1*, *atann2* and *atann4* seedlings were all significantly larger than that of control, similar to the response of Col-0 (Fig. 1C). Conversely, the eATP avoidance response of *atann3* seedlings was significantly weaker than Col-0 (Fig. 1A). Although eATP effectively induced root bending

growth of *atann3* seedlings ($p < 0.05$), the curvature was significantly smaller than that of wild type ($p < 0.01$) (Fig. 1C). These results indicate that AtANN3 may be involved in eATP sensing and response.

To further verify the role of these 4 annexins in the eATP response, we examined hypocotyl growth rate and curvature of etiolated seedlings which were grown on 0.5 mM eATP-containing medium. Our results showed that *atann1*, *atann2* and *atann4* seedlings responded to eATP in same manner as Col-0, which showed strongly suppressed and markedly bent growth, while the response of *atann3* seedlings was significantly weaker than that of wild type (Fig. 2A). Data analysis showed that, compared to Col-0, eATP-treated *atann3* seedlings were much longer and less curved. Compared with the control, *atann3*-mutant hypocotyl length was only slightly shorter ($p > 0.05$) and hypocotyl curvature was slightly greater ($p > 0.05$) (Fig. 2B, C).

To verify whether there is redundancy in the function of AtANN1, AtANN2 and AtANN4, double- or triple-null mutants of the three annexins were used material, growth parameters of seedlings which were growing under light or in darkness were measured. Results showed that seedlings of the two double-null mutants, *atann1/atann2* and *atann1/atann4* responded to 0.5 mM ATP in same manner and degree with the wildtype, the root length and root curvature of seedlings which growing under light were not significantly different from that in Col-0, and the hypocotyl length and curvature of seedlings which were growing in darkness were in the same degree with that in Col-0 (Fig. S1, Fig.S2). Seedlings of the triple null mutants (*atann1/atann2/atann4*), either growing under light or in darkness, responded to 0.5 mM ATP as sensitively as the wildtype (Fig.S3).

AtANN3 is involved in eATP-induced auxin accumulation and distribution

To clarify the role of AtANN3 in eATP regulated seedling growth, *Dr5-GUS* and *Dr5-GFP* fusion genes were transformed into *atann3* mutants by hybridization. The transgenic seedlings were transplanted onto ATP-containing medium and then GUS staining and CLSM were used to investigate the effect of eATP on abundance and distribution of GUS or GFP in root tip cells and hypocotyl cells.

GUS staining in Col-0 seedlings (which were grown under light) showed that GUS was located mainly in root tip cells (especially in cells around the quiescent center (QC)). ATP treatment promoted GUS accumulation and caused the distribution of GUS to extend from the root tip to the meristematic and elongation zones, and especially in the stele cells (Fig. 3A). In untreated *atann3* seedlings, GUS was located in root tip cells and some stele cells, with an abundance was similar to the wild type. After ATP treatment, GUS accumulated in root tip cells, but its abundance and the extent of its distribution were both lower than wild type, demonstrating that *atann3* seedlings show a weakened response to eATP (Fig. 3A). In etiolated Col-0 hypocotyls, GUS abundance increased after ATP treatment, and a marked asymmetric distribution appeared at the hypocotyl curve, with GUS abundance higher in the outer-side cells than in the inner-side cells. In ATP-treated *atann3* etiolated seedlings, GUS accumulation and asymmetric distribution were not detected, indicating that the eATP-induced effect was impaired (Fig. 3B).

DR5-GFP fluorescence detection results showed that, in eATP-treated Col-0 root tips of green seedlings, DR5-GFP fluorescence intensity increased in the QC, stele, and epidermal cells, and a marked asymmetric distribution in epidermal cells appeared (Fig. 4A). At the root curve, fluorescence intensity in the inner side cells was much stronger than that in the outer side cells ($p < 0.05$) (Fig. 4C). In ATP-treated *atann3* green seedlings, DR5-GFP fluorescence intensity increased and an asymmetric distribution in epidermal cells was not detected. Fluorescence intensity in the outer- and inner-side cells was not significantly different (Fig. 4A, C). In ATP-treated Col-0 etiolated seedlings, DR5-GFP fluorescence accumulated in hypocotyl cells and an asymmetric fluorescence distribution was detected (Fig. 4B). At the bending area, fluorescence intensity in the outer-side cells was significantly higher than in the inner-side cells ($p < 0.01$) (Fig. 4D). In eATP-treated *atann3* seedlings, neither fluorescence accumulation nor asymmetric distribution were detected, and fluorescence intensity was not different before and after ATP treatment ($p > 0.05$) (Fig. 4B, D).

AtANN3 is involved in eATP-induced auxin transporter accumulation and distribution

To verify the role of AtANN3 in eATP-regulated auxin transport, we used PIN2-GFP and PIN3-GFP transformed wild type and *atann3* mutant and measured the effect of eATP on the abundance and distribution of the two auxin transporters in seedlings.

After ATP stimulation, fluorescence intensity of PIN2-GFP in Col-0 root tip cells slightly decreased, and a marked asymmetric distribution appeared (Fig. 5A). At the bending area, fluorescence intensity in the inner-side epidermal cells was significantly greater than in the outer-side cells. Data analysis showed that the fluorescence intensity ratio (inner-side/outer-side) significantly increased after ATP stimulation ($p < 0.05$) (Fig. 5C). Conversely, in *atann3* roots, ATP treatment led to a remarkable decrease in PIN2-GFP fluorescence intensity and an asymmetric distribution of PIN2-GFP was not detected (Fig. 5A). Data analysis showed that the fluorescence intensity ratio did not significantly change after ATP stimulation ($p > 0.05$) (Fig. 5C). Examination of PIN2-GFP in etiolated hypocotyls showed that, after ATP stimulation, fluorescence intensity and distribution of PIN2-GFP did not change, either in Col-0 or in *atann3* seedlings (Fig. 5B, D). These results indicate that PIN2 is involved in eATP-regulated root growth but is unlikely to be involved in eATP-regulated hypocotyls growth.

Examination of PIN3-GFP in root tip cells of green seedlings showed that fluorescence was mainly located in the QC and stele cells. After ATP stimulation, fluorescence intensity of PIN3-GFP in Col-0 root tip cells markedly decreased, either in QC or in stele cells (Fig. 6A). In *atann3* seedlings, ATP treatment led to a significant decrease in PIN3-GFP fluorescence intensity as well. The loss of PIN3-GFP fluorescence intensity was not significantly different between Col-0 and *atann3* seedlings (Fig. 6C). In etiolated hypocotyls of Col-0, PIN3-GFP fluorescence was enriched in epidermal, cortex, and stele cells. After ATP treatment, fluorescence intensity slightly decreased in epidermal and cortex cells, and was unchanged in stele cells. At the bending area of curved hypocotyls, fluorescence intensity in the outer-side epidermal and cortex cells was significantly stronger than in the inner-side cells (Fig. 6B). Data analysis showed that

the fluorescence intensity ratio (outer-side/inner-side) significantly increased after ATP stimulation ($p < 0.05$) (Fig. 6C). Conversely, in hypocotyls of etiolated *atann3* seedlings, PIN3-GFP fluorescent intensity did not change after ATP treatment and an asymmetric distribution of PIN3-GFP was not detected (Fig. 6B). Data analysis showed that the fluorescence intensity ratio did not significantly change after ATP stimulation ($p > 0.05$) (Fig. 6D). These results indicate that PIN3 is involved in eATP-regulated hypocotyl growth but is unlikely to be involved in eATP-regulated root growth.

Discussion

In eATP-regulated plant growth and development, eATP-regulated root and hypocotyl growth were intensively investigated. It has been revealed that high concentrations of eATP suppressed root elongation rate, led root bending, skewing or curling growth [5, 6, 15, 26, 74]. In our previous work, it is revealed that main root of *Arabidopsis thaliana* responded to millimolar ATP as decreased growth rate and markedly bending (which is termed as “eATP avoidance”), confirming that plant cells regard high concentrations of eATP as a “danger signal” [7, 8]. eATP addition also effectively changed hypocotyl growth, altered the growth rate and direction of etiolated hypocotyls, the physiological relevance of eATP stimulated bending growth of hypocotyls remains unclear [8, 9]. eATP addition lead suppressed elongation rate and bending of growing hypocotyls, similarly to ethylene stimulated “triple response”, suggesting ethylene signaling may possibly be involved in eATP induced response [8].

The responses of roots and hypocotyls to eATP can be used as readouts for identifying components involved in eATP signal transduction. In untreated medium, the growth rate and direction of seedlings of 4 mutants were similar to those of wild type controls, indicating that loss-of-function of individual annexin did not significantly affect seedling growth. After ATP addition, mutants of AtANN1, AtANN2 and AtANN4 responded to eATP to the same degree as wild type controls, indicating that these three annexins may be not involved in the eATP response. Conversely, *atann3* seedlings exhibited a weaker response to eATP in terms of root and hypocotyl growth, suggesting that AtANN3 is involved in eATP-regulated seedling growth. Among the 8 annexin members in *Arabidopsis thaliana*, to our knowledge, there is no evidence that the annexins participate in eATP-regulated growth of vegetative organs. The role of AtANN3 in plant growth and development is unknown, although it is implicated in vesicular formation and fusion with vacuoles in root cells [57]. Here, we present the first evidence for a role of AtANN3 in regulating seedling growth.

AtANN1 is involved in eATP-induced ROS accumulation and Ca^{2+} influx [51]. AtANN4 mediates eATP-stimulated Ca^{2+} influx when expressed in *Xenopus* oocytes [75]. Ca^{2+} participates in eATP-avoidance of root tips by using Ca^{2+} chelators. AtANN1 and AtANN4 are involved in eATP-stimulated Ca^{2+} influx [28, 30]. eATP-stimulated Ca^{2+} signaling is involved in eATP regulated seedling growth [7]. However, as the supplementary figures showed, AtANN1, AtANN2 and AtANN4 were not required for eATP-regulated root or hypocotyl growth, for unknown reasons that need further investigation.

eATP stimulation leads to asymmetric auxin transport and accumulation in root cells. Here, we further show that asymmetric distribution of auxin also occurs in eATP-treated etiolated hypocotyls, which leads to bending growth of hypocotyls. Since root cells are very sensitive to auxin, auxin that accumulates in root cells will suppress elongation of the epidermal cells. Conversely, in hypocotyl cells, which are less sensitive to auxin than root cells, accumulation of auxin promotes cell elongation. It had been reported that eATP stimulates asymmetric auxin distribution in Arabidopsis seedlings [6, 74]. In our previous work, we found that RRTF1, an ethylene responsive transcription factor, is involved in eATP-induced asymmetric distribution of auxin [8]. Our finding that AtANN3 is involved in eATP-regulated auxin distribution is useful for understanding the mechanism of eATP-stimulated auxin re-distribution in vegetative organs.

Gene expression of some annexins has been revealed to be regulated by plant hormones, e.g. ABA, ethylene, or auxin, suggesting that annexins may be involved in plant hormone-regulated growth, development, and stress responses [36, 49, 76, 77]. However, annexin-regulated accumulation or transport of plant hormone has rarely been reported. An annexin in cassava, MeANN2, which is similar to AtANN1, is involved in stress tolerance via regulating auxin signaling [78]. Our results provide direct evidence for the involvement of AtANN3 in auxin accumulation and transport regulation in response to an extracellular signal molecule.

Auxin efflux transporters, especially PINs, play essential roles in polar auxin transport and asymmetric distribution-induced physiological responses. In response to stimuli, the abundance and distribution of PINs change dynamically. Vesicle transport, including endocytosis and exocytosis, are involved in protein location and re-location from one part of the cell to another. In *Arabidopsis thaliana*, PIN2 and PIN3 are involved in root and hypocotyl's tropic responses. During root's phototropism or gravitropism, PIN2 mediates auxin asymmetric distribution of auxin in root tip cells, especially in the elongation zone [67, 79]. PIN3 is the main mediator of lateral auxin flow during hypocotyl's phototropism or gravitropism [61]. Unidirectional blue light stimulates the movement of PIN3 from irradiated side to the shade side of etiolated hypocotyl epidermal cells [69]. During hypocotyl's gravitropism, PIN3-mediated auxin directional flux and accumulation in lower side cells result in negative gravitropic bending growth [70]. Consistent with these reports, it was revealed in our previous work that PIN2 is involved in eATP-induced root bending, while PIN3 is involved in eATP-induced hypocotyl bending [8]. The results here further confirm the role of two PINs in eATP regulated growth of vegetative organs.

Subcellular trafficking is involved in PIN2 redistribution and subsequent auxin asymmetric distribution, which lead bending of roots away from light or salinity [80–84]. PIN3 redistribution results from subcellular trafficking as well [70, 71, 85]. AtANN3 is involved in regulating vesicle transport from the Golgi apparatus to vacuoles [57]. ATP-stimulated asymmetric distribution of auxin and auxin transporters in root tip cells exposed to eATP was absent in *atann3* mutants, suggesting that AtANN3-regulated PINs transport and asymmetric auxin distribution might be an important event in eATP signal transduction. In a preliminary experiment, rough data showed that eATP stimulation led decrease of PIN2-GFP fluorescent intensity in root tip cells, the vesicle-like tiny bodies' abundance seemly asymmetric in Col-0 seedlings, i.e. there were more vesicles in the inner-side cells than the outer-side cells at the root curve. Such an

asymmetric distribution was not detected in *atann3* seedlings (Fig.S4). The result provides some clues for verifying the mechanism of AtANN3-mediated auxin transporter recycling, however, much more investigation is needed before we can draw a conclusion.

Methods

Plant materials

Arabidopsis thaliana L. wild type (Col-0) and null mutants of *Annexins* (including *atann1*, *atann2*, *atann3* and *atann4*) were used. *Annexins* null mutant seeds were a gift from Dr. Julia Davies, Department of Plant Science, University of Cambridge, UK. All seeds were genotyped to confirm the homozygous mutation of corresponding gene. Double- or triple-null mutants were screened from offspring obtained by hybridization of these mutants.

Root or hypocotyl growth measurement

Seeds were surface-sterilized with 70% ethanol for 2 min followed by 5% sodium hypochlorite for 5 min. After two washes with sterilized water, seeds were sown on the surface of solid 1/2 MS medium (containing 0.8% phytagel) in square culture dishes. The culture dishes were stored at 4°C for 2 days and then were vertically cultured at 22°C and 130 μmol·m^{-2}·s^{-1} illumination with a 16/8 light/dark cycle.

To investigate the response of roots to eATP, seedlings were grown on a combined medium made according to the protocol of Zhu et al. (2017). A solution of 1/2 MS salt and 0.8% phytagel was sterilized and poured into 10×10 cm square culture dishes, with each dish containing 50 mL liquid medium. After the medium solidified, the medium was cut with a sterilized blade along the midline of the culture dish, and half of the medium was removed. The interspace was then re-filled with 25 mL sterilized 1/2 MS medium containing ATP. ATP was dissolved in 1/2 MS solution to make a stock solution and the pH was adjusted to 6.0 with Tris. The stock solution was filtered with a sterilized filter (SLGP033RB, 0.22 μm, Millipore, USA) and mixed with sterilized 1/2 MS medium which was cooled down to 50°C and poured into the interspace in the culture dish. After solidification, the refilled medium was level with the original medium. The concentration of ATP in the medium was set according to Zhu et al. (2017). Seedlings which were growing in 1/2 MS medium for 4 days were transplanted onto the untreated part of the combined medium, with the root tip toward the refilled medium and 0.3 ~ 0.5 cm from the border between media. The culture dishes were placed vertically, with the untreated part on top and the refilled medium at the bottom so that the root will grow downward toward the refilled part where it will encounter ATP in the medium.

To detect the hypocotyl growth of etiolated seedlings, surface-sterilized seeds were sown on the surface of solid 1/2 MS medium (containing 0.8% phytagel) with ATP added in square culture dishes. The culture dishes were stored at 4°C for 2 days and then were vertically cultured at 22°C, 130 μmol·m^{-2}·s^{-1} illumination, and a 16/8 h light/dark ratio for 24 h. Thereafter, culture dishes were coated with tin foil and placed vertically for further seedling culture at 22°C.

To measure root or hypocotyl length and curvature, photos of seedlings were captured using an optical scanner and then analyzed using Image J software. In each experiment, at least 30 seedlings were measured, and the mean was calculated from 3 replicates. Data were statistically analyzed using Sigmaplot software. The significance of differences between control and treatment groups was determined by Student's *t*-test.

Histochemical GUS staining

GUS transformants were grown on ATP-containing 1/2 MS medium and cultured under light for root staining or in darkness for hypocotyl staining. Seedlings were collected after ATP treatment and incubated at 37°C in GUS staining solution consisting of 1 mM X-Gluc, 50 mM PBS, 10 mM EDTA, 0.1% Triton X-100, and 0.5 mM potassium ferricyanide. After a period of time, the stained seedlings were transferred into absolute ethanol to decolorize them until the tissues became totally transparent. Images were captured using a stereomicroscope (Olympus SZX16, Japan).

Confocal laser scanning microscopy (CLSM)

Published *DR5-GFP*, *PIN2-GFP*, *PIN3-GFP* transgenic lines [86] (seeds were purchased from Arabidopsis Biological Resource Center, Ohio State University) were hybridized with *atann3* and expression of the transformed genes was detected by PCR. Seeds were germinated on solid 1/2 MS medium, and 4-d-old seedlings were transplanted onto ATP-containing medium and cultured further. Materials were collected after a period of time (see detail in the corresponding figures). Seedlings were placed onto the stage of a microscope equipped with a laser confocal scanning system (LSM 710, Zeiss). The excitation and emission wavelengths were 488 nm and 515 nm, respectively. Images were processed with Confocal Assistant software and edited with Adobe Photoshop 7.0.

To measure fluorescence intensity in root tip cells, a region of interest 0.5 mm long in the root tip was delineated. To obtain the ratio of fluorescence intensity, cells on the left-side and right-side of the root or hypocotyl were delineated as area of interest. Fluorescence intensity in root cells was measured with Image J software and then averaged. The significance of differences between control and treatment groups was determined by Student's *t*-test.

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability

Not applicable.

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Figures

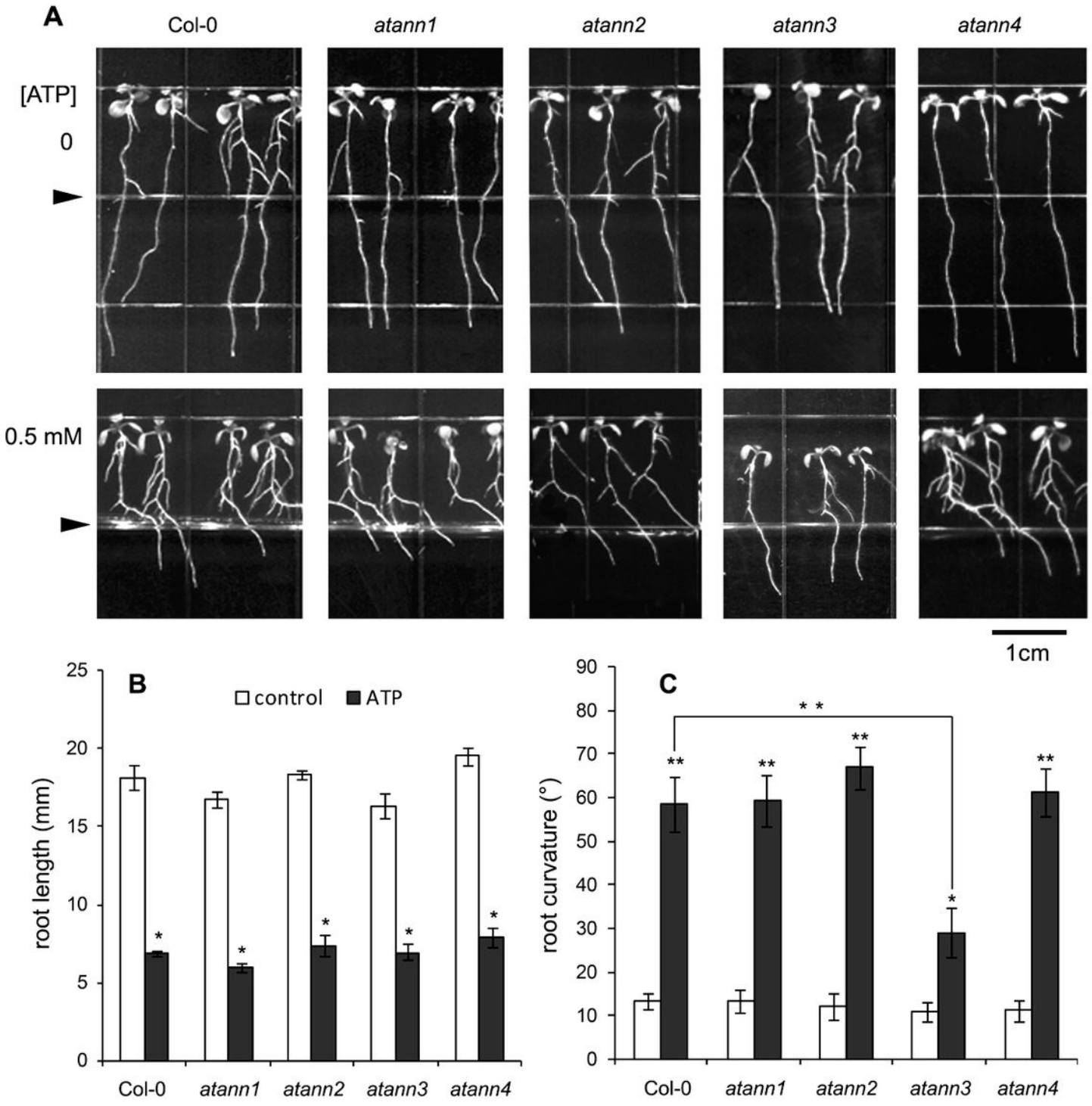

Figure 1

AtANN3 is involved in the eATP avoidance response of Arabidopsis thaliana roots. Four days old seedlings were transplanted onto combined medium and cultured under light for 5 more days. The concentration of ATP in the lower compartment was 0.5 mM. The triangles mark the border between the two media. Figure A shows the photograph of growing seedlings. The scale bar is shown in the lower-right corner. Figure B and C show the root length (B) and root curvature (C) of seedlings. In each

experiment, at least 30 seedlings were measured and data from at least three replicates were combined to obtain mean ± SD. Student's t-test p-values: * p<0.05, ** p<0.01.

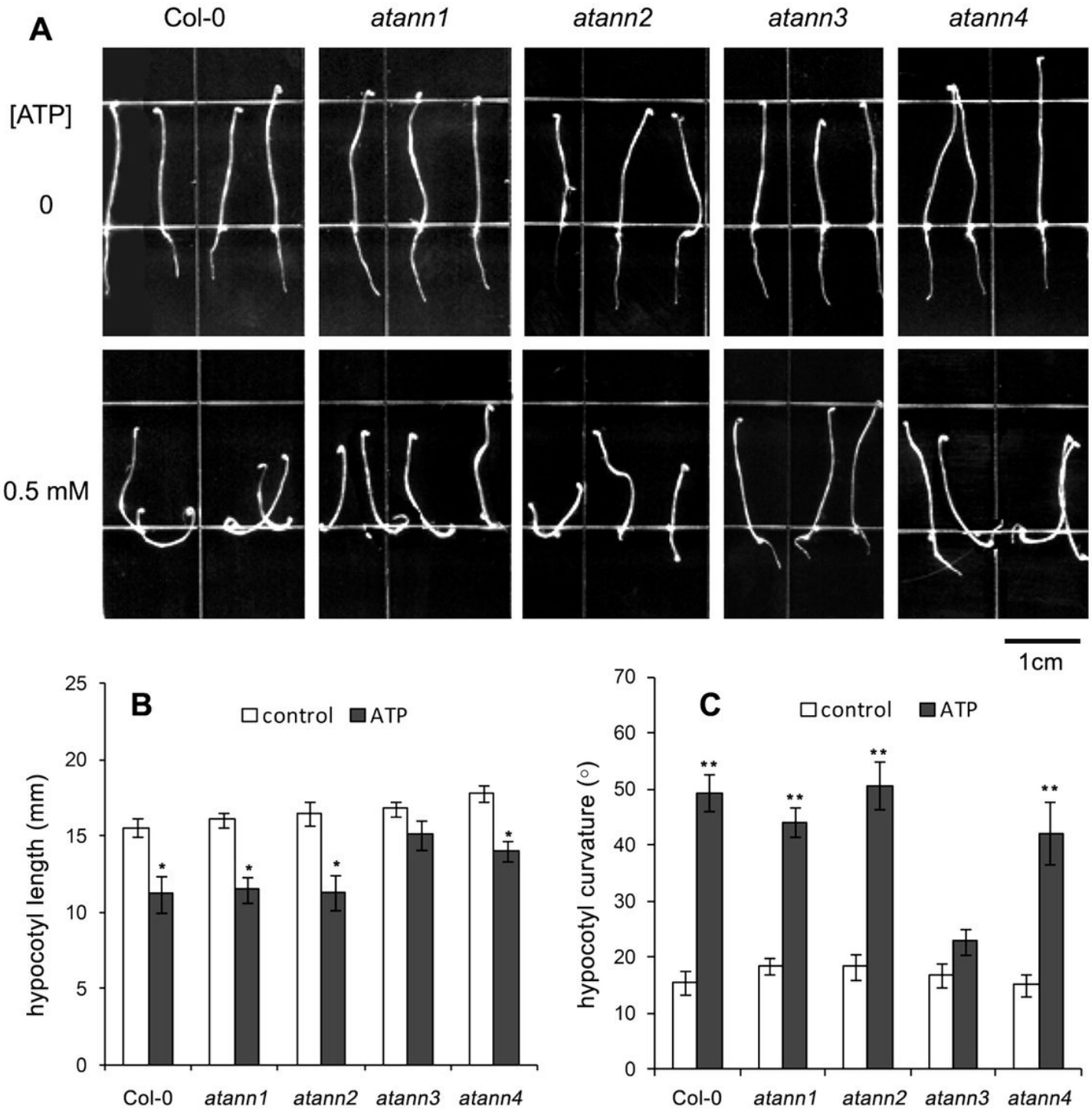

Figure 2

AtANN3 is involved in eATP-regulated hypocotyl growth of Arabidopsis thaliana. Arabidopsis seeds were sown and cultured on 0.5 mM ATP-containing medium in the dark for 5 days. Figure A shows the photograph of growing seedlings. The scale bar is shown in the lower-right corner. Figure B and C note the hypocotyl length (B) and curvature (C) of seedlings. In each experiment, at least 30 seedlings were

measured and data from at least three replicates were combined to obtain mean ± SD. Student's t-test p-values: * $p<0.05$, ** $p<0.01$.

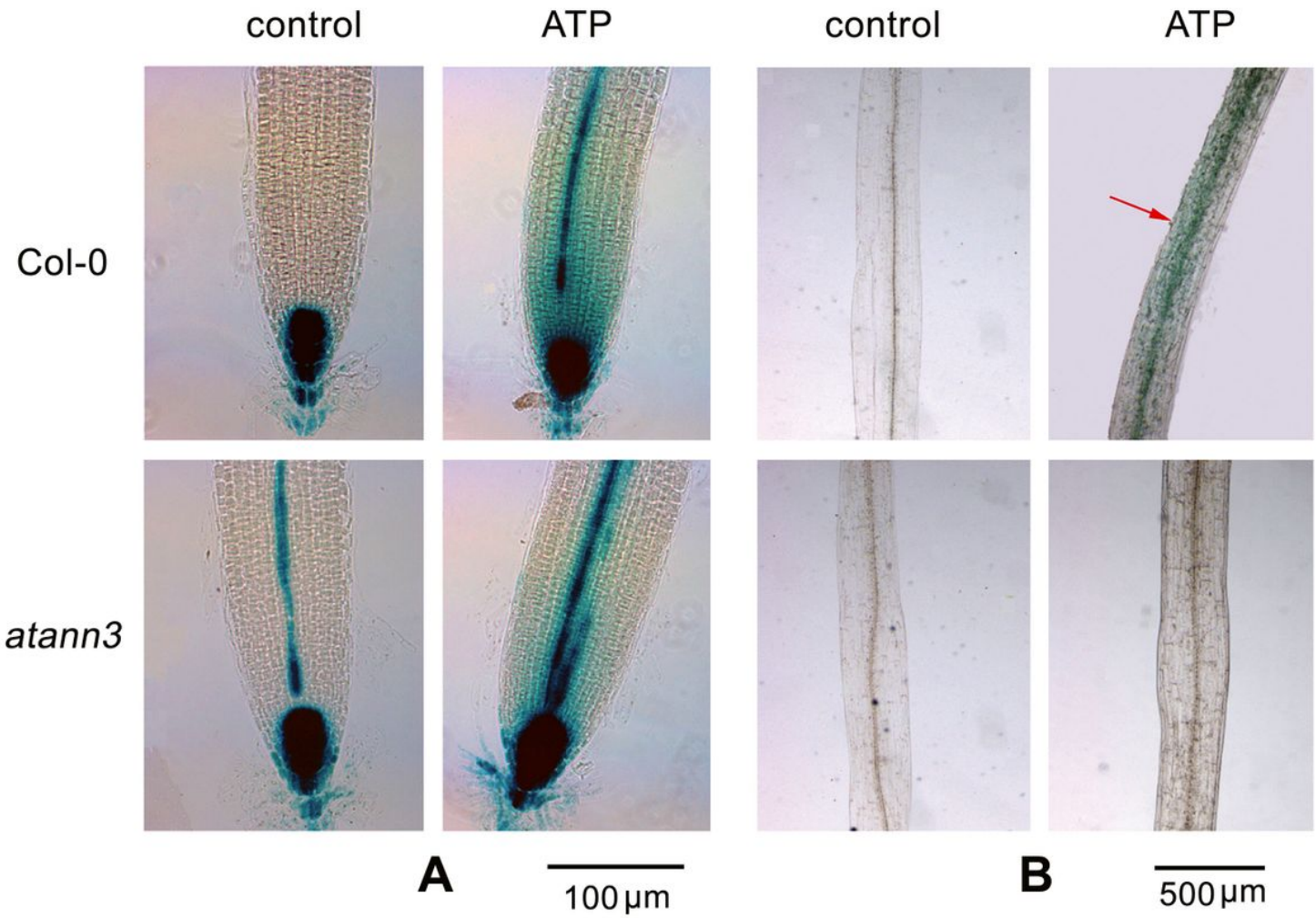

Figure 3

AtANN3 is involved in eATP-induced Dr5-GUS accumulation and distribution. Seedlings of DR5-GUS transgenic lines were transplanted onto 0.5 mM ATP-containing medium and cultured for 2 more days. DR5-GUS expression was measured by GUS staining. Figure A and B show root tip (A) and hypocotyl section (B) of seedling after ATP treatment. The scale bar is shown in the lower-right corner of each figure. The red arrow marks the asymmetric localization of Dr5-GUS in hypocotyl.

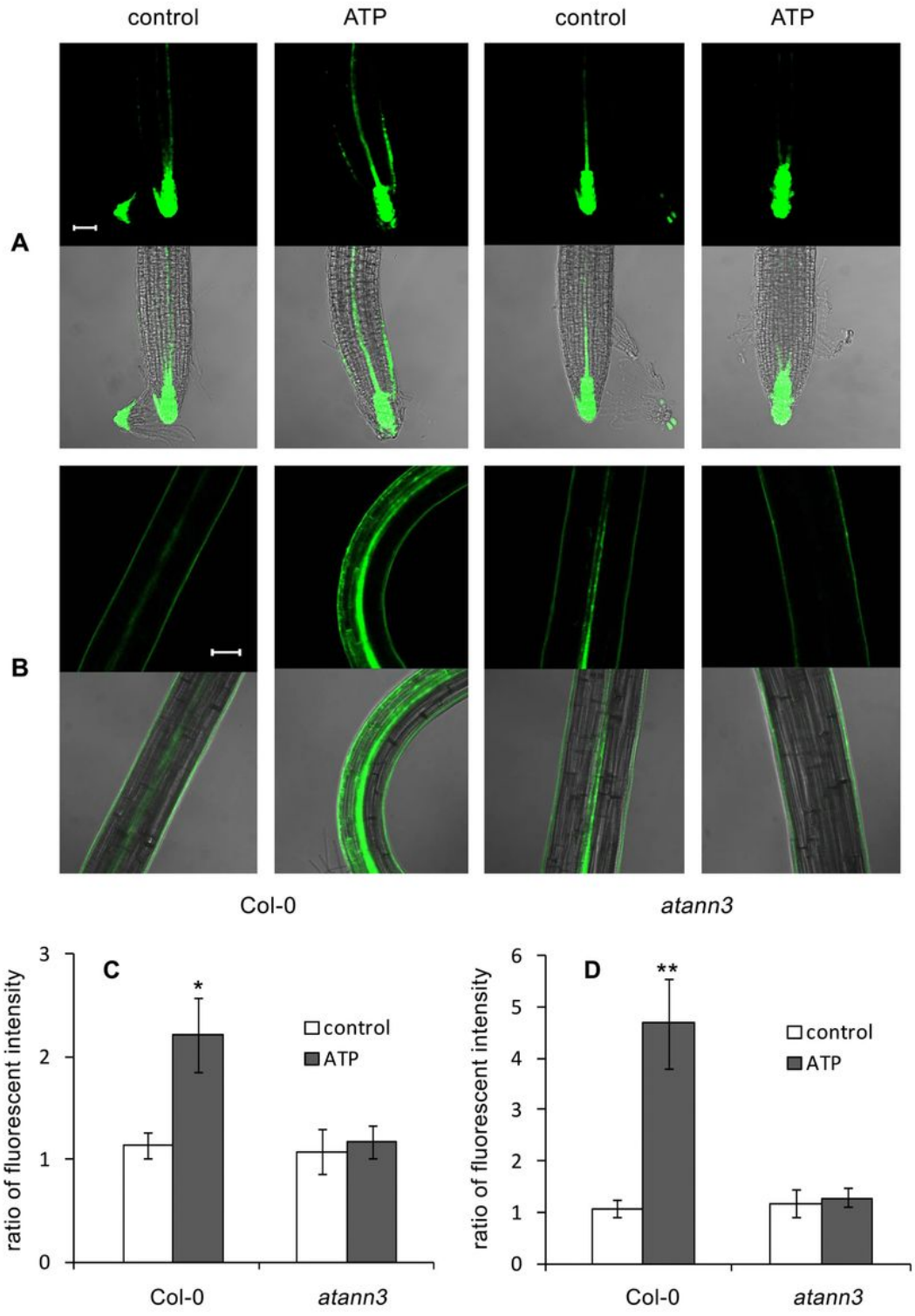

Figure 4

AtANN3 is involved in eATP-induced Dr5-GFP accumulation and distribution. Seedlings of DR5-GFP transgenic lines were transplanted onto 0.5 mM ATP-containing medium and cultured for 2 more days. Figure A and B show image of root tip (A) and hypocotyl section (B) of seedling after ATP treatment. The scale bar (=50 μm) is shown in the left-most image. C. Ratio of fluorescence intensity in inner-side/outer-side cells at the root curve. D. Ratio of fluorescence intensity in outer-side/inner-side cells at the hypocotyl

curve. In each experiment, at least 15 samples were measured and data from at least three replicates were combined to obtain mean±SD. Student's t-test p-values: *p<0.05, **p<0.01.

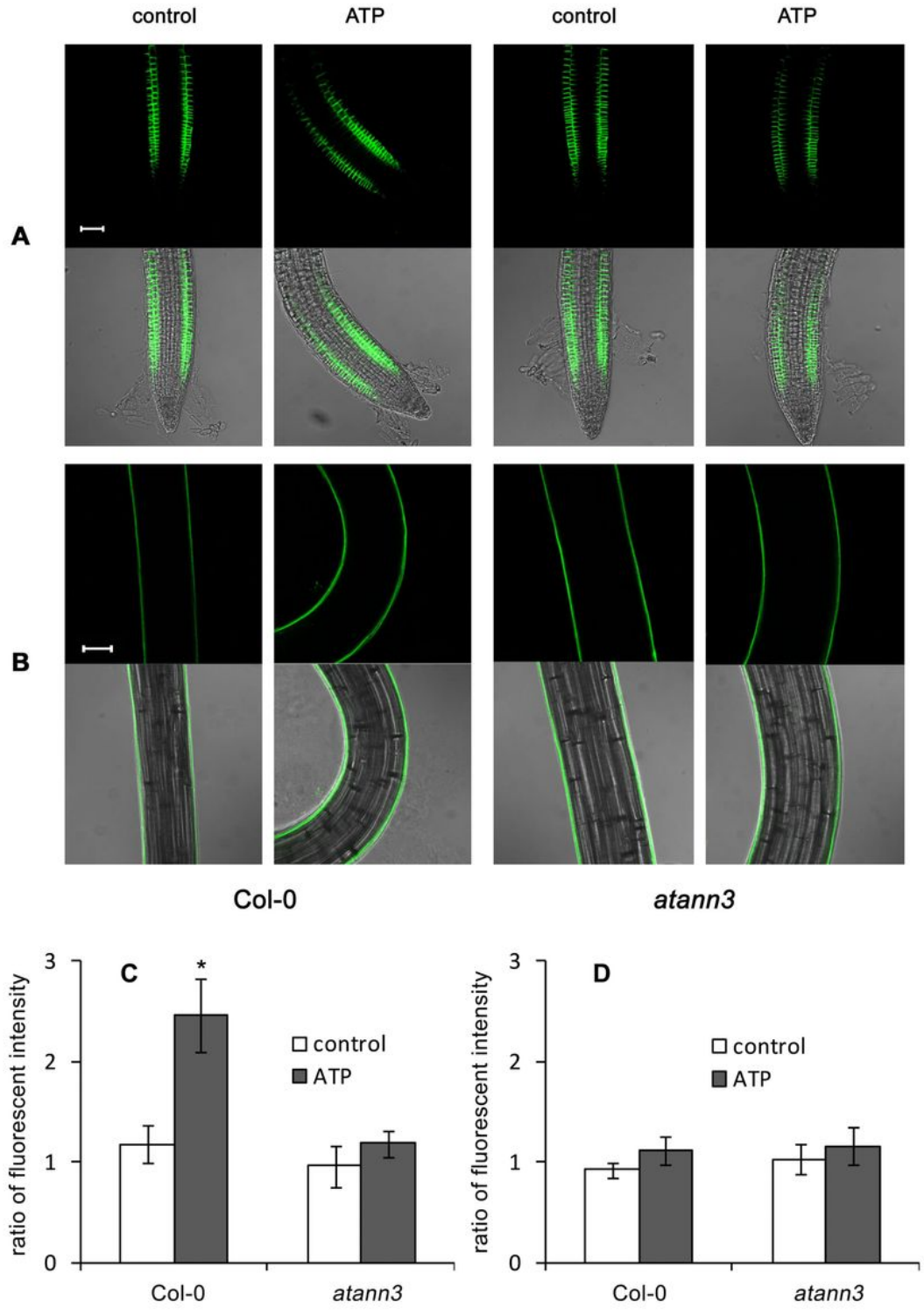

Figure 5

AtANN3 is involved in eATP-induced PIN2-GFP distribution. Seedlings of PIN2-GFP transgenic lines were transplanted onto 0.5 mM ATP-containing medium and cultured for 2 more days. Figure A and B show image of root tip (A) and hypocotyl section (B) of seedling after ATP treatment. The scale bar (=50 μm) is

shown in the left-most image. C. Ratio of fluorescence intensity in inner-side/outer-side cells at the root curve. D. Ratio of fluorescence intensity in outer-side/inner-side cells at the hypocotyl curve. In each experiment, at least 15 samples were measured and data from at least three replicates were combined to obtain mean ± SD. Student's t-test p- values: * p<0.05, ** p<0.01.

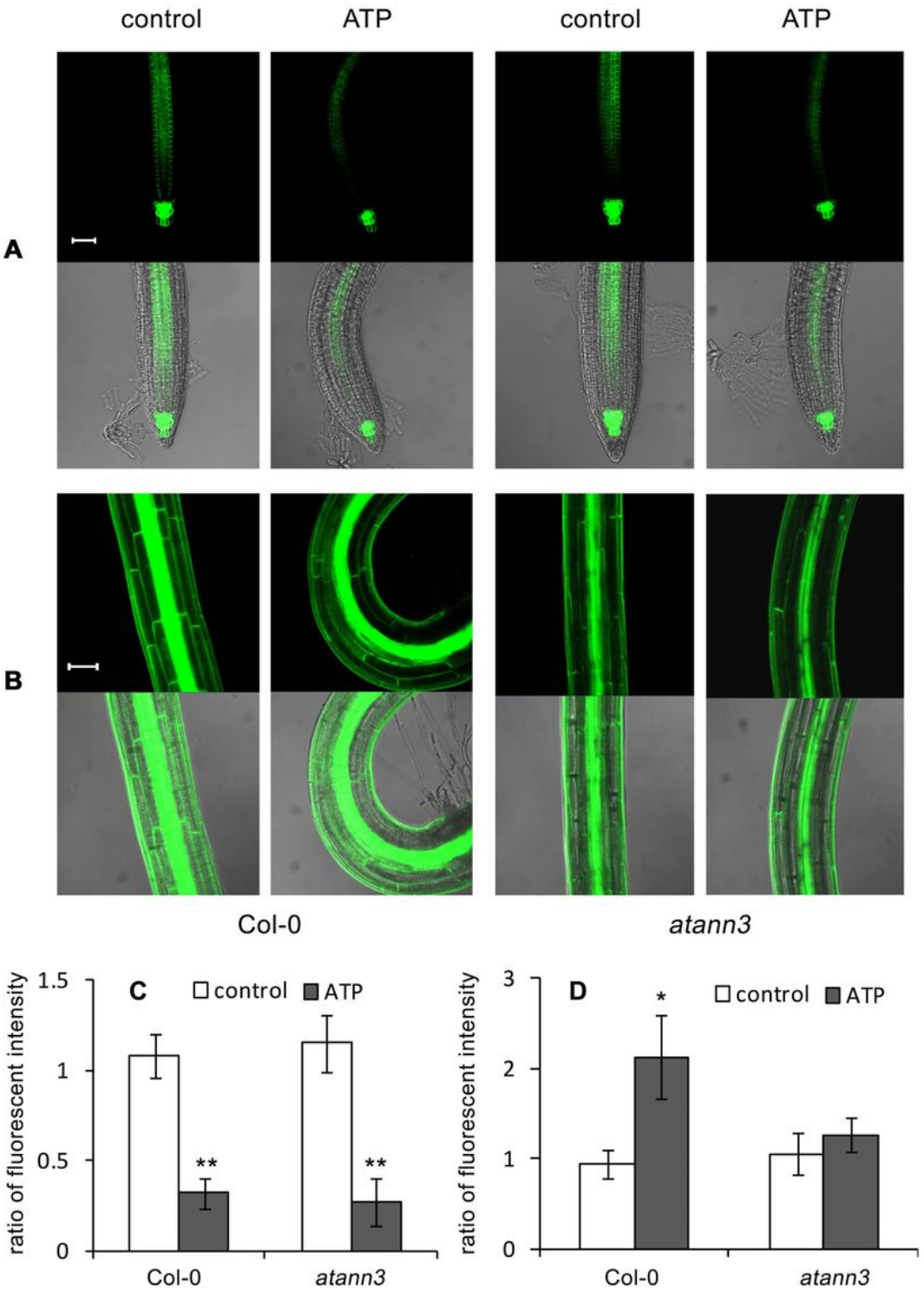

Figure 6

AtANN3 is involved in eATP-induced PIN3-GFP distribution. Seedlings of PIN3-GFP transgenic lines were transplanted onto 0.5 mM ATP-containing medium and cultured for 2 more days. Figure A and B show image of root tip (A) and hypocotyl section (B) of seedling after ATP treatment. The scale bar (=50 μm) is shown in the left-most image. C. Ratio of fluorescence intensity in root cells after/before ATP treatment. D. Ratio of fluorescence intensity in outer-side/inner-side cells at the hypocotyl curve. In each experiment, at least 15 samples were measured and data from at least three replicates were combined to obtain mean ± SD. Student's t-test p-values: * $p<0.05$, ** $p<0.01$.

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