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

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Research Article

Keywords: Extracellular ATP, Annexin, Auxin, Seedling growth, *Arabidopsis thaliana*

Posted Date: October 12th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-841741/v2>

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Abstract

Extracellular ATP (eATP) exists in the apoplast and plays multiple roles in growth, development, and stress responses. eATP has been revealed to suppresses growth rate and alters growth orientation of root and hypocotyl of *Arabidopsis thaliana* seedlings by affecting auxin transport in these organs. However, the mechanism of eATP-stimulated auxin distribution 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 mutants of *AtANN3* did not respond to eATP sensitively, the role of *AtANN3* in eATP regulated auxin transport was intensively investigated. Firstly, the inhibitory effect of eATP on root or hypocotyl elongation was weakened or impaired in *AtANN3* null mutants (*atann3-1* and *atann3-2*). Meanwhile, single-, double- or triple-null mutant of *AtANN1*, *AtANN2* or *AtANN4* responded to eATP in same manner and degree with Col-0. The distribution of DR5-GUS and DR5-GFP indicated that eATP-induced asymmetric distribution of auxin in root tip or hypocotyl cells, which appeared in wild type controls, were lacking in *atann3-1* seedlings. Further, eATP-induced asymmetric distribution of PIN2-GFP in root tip cells or PIN3-GFP in hypocotyl cells were reduced in *atann3-1* seedlings. Based on these results, we suggest that *AtANN3* may be involved in eATP-regulated seedling growth through regulating auxin transport in vegetative organs.

Key Message

Lost-of-function mutant of *AtANN3*, an annexin in *Arabidopsis thaliana*, extracellular ATP- regulated seedling growth, asymmetric auxin distribution in roots and hypocotyls were decreased or impaired.

Introduction

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 (Cao et al. 2014; Roux and Steinebrunner 2007; Tanaka et al. 2010; Pietrowska-Borek et al. 2020). During plant growth and development, eATP is involved in maintaining cell viability, regulating growth rate and direction of some vegetative organs (roots and hypocotyls) (Kim et al. 2006; Yang et al. 2015; Zhu et al. 2017; Zhu et al. 2020; Tonon et al. 2010), and regulating reproductive processes (Wu et al. 2018; Reichler et al. 2009). eATP is also involved in regulating stomatal movement and gravitropism (Chen et al. 2017; Clark et al. 2011; Hao et al. 2012; Tang et al. 2003). 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” (Cao et al. 2014; Chen et al. 2021; Choi et al. 2014b; Deng et al. 2015; Jewell et al. 2019; Kumar et al. 2020; Pham et al. 2020; Tripathi and Tanaka 2018). As the main eATP hydrolyzing enzyme,

extracellular apyrase is involved in terminating signal transduction and maintaining eATP level (Clark and Roux 2011; Lim et al. 2014).

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 (Choi et al. 2014a; Pham et al. 2020). 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 (Hao et al. 2012; Zhu et al. 2017; Weerasinghe et al. 2009), NADPH oxidase (Chen et al. 2017; Demidchik et al. 2009; Hao et al. 2012), and ion channels (Demidchik et al. 2009; Shang et al. 2009; Demidchik et al. 2011; Wang et al. 2019; Wang et al. 2018) 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 (Demidchik et al. 2009; Clark and Roux 2018; Hao et al. 2012; Zhu et al. 2017; Song et al. 2006; Tonon et al. 2010; Wu and Wu 2008). After eATP stimulation, altered gene expression and protein synthesis had been observed and proposed to change plant growth & development in response to environmental signals (Chivasa et al. 2011; Zhu et al. 2020; Choi et al. 2014a).

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 (Konopka-Postupolska and Clark 2017; Davies 2014; Clark et al. 2012). Annexins are involved in seed germination and early seedling growth (Chu et al. 2012; Cantero et al. 2006; Clark et al. 2005), the transition from the vegetative to the reproductive phase (Lichocka et al. 2018), pollen germination and tube growth (Zhu et al. 2014), 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 (Cantero et al. 2006; Jami et al. 2012; He et al. 2019; Chu et al. 2012; Qiao et al. 2015; Zhang et al. 2011; Yadav et al. 2016; Li et al. 2019).

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 (Laohavisit et al. 2009; Laohavisit et al. 2012; Kodavali et al. 2013; Mu and Zhou 2019); 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 (Gorecka et al. 2005; Dalal et al. 2014). 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 (Clark et al. 2005; Konopka-Postupolska 2007; Scheuring et al. 2011; Zhu et al. 2014). Annexins have also been observed in the nucleus where they are thought to regulate gene expression (Jami et al. 2008; Huh et al. 2010).

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 (Zhang and Friml 2020; Han et al. 2021). Auxin transporters, especially PIN-FORMED transporters (PINs), play key roles in polar auxin transport (Han et al. 2021; Lee et al. 2020; Zhou and Luo 2018). 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 (Adamowski and Friml 2015; Narasimhan et al. 2021; Barbosa et al. 2018; Tan et al. 2021; Zhang et al. 2021). Small G protein- or clathrin-mediated vesicle trafficking is involved in PIN trafficking during photo- and gravity-tropic bending growth (Ding et al. 2011; Rakusova et al. 2011; Kleine-Vehn et al. 2010; Narasimhan et al. 2020). Most recently, two SNARE proteins were reported to be involved in auxin regulated seedling growth via regulating subcellular trafficking of auxin transporters (Zhang et al. 2021). 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 (Ganguly et al. 2012; Barbosa et al. 2018; Tan et al. 2021). 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 (Han et al. 2021; Zhang and Friml 2020; Lee et al. 2020; Adamowski and Friml 2015).

eATP regulates auxin 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 (Liu et al. 2012; Zhu et al. 2017; Zhu et al. 2020). However, the mechanism underlying eATP-regulated PINs abundance and re-location, which in turn alters auxin 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 distribution in seedlings of annexin-null mutants. Since two *AtANN3* null mutant lines responded to eATP significantly differently from wildtype, the role of *AtANN3* was intensively investigated.

Materials And Methods

Plant materials

Arabidopsis thaliana L. wild type (Col-0) and null mutants of *Annexins*, including *atann1* (salk_132169), *atann2* (salk_054223), *atann3-1* (salk_082344), *atann3-2* (salk_130101C) and *atann4* (salk_019725), were used. *Annexins* null mutant seeds were ordered from ABRC (Arabidopsis Biological Resource Center, the Ohio State University, USA). 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% phytigel) in square culture dishes. The culture dishes were stored at 4°C for 2 days and then were vertically cultured at 22°C and $130 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 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. [7]. A solution of 1/2 MS salt and 0.8% phytigel 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.*[7]. 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% phytigel) 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, $130 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 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. The angle between the direction of bending growth and the vertical direction was measured as the curvature. 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 (Friml et al. 2003) (Seeds were a gift from Dr. Jiří Friml in Institute of Science and Technology Austria, Austria.) were hybridized with *atann3-1* 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 4.0 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.

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 [7–8]. To verify the role of annexins in eATP signaling, the response of annexin-null mutant seedlings (*atann1*, *atann2*, *atann3-1* 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). By contrast, the eATP avoidance response of *atann3-1*

seedlings was significantly weaker than Col-0 (Fig. 1A). Although eATP effectively induced root bending growth of *atann3-1* 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 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. 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 *atann3-1* seedlings was significantly weaker than that of wild type (Fig. 2A). Data analysis showed that, compared to Col-0, *atann3-1* hypocotyls were more resistant to eATP in length and curvature (Fig. 2B, C).

To confirm the involvement of AtANN3 in eATP-regulated seedling growth, another *AtANN3* null mutant line (*atann3-2*) was used as material, growth parameters of seedlings which were growing under light or in darkness were measured. Results showed that, seedlings of *atann3-2* responded to 0.5 mM ATP less sensitively than the wildtype. For the seedlings grown under light, *atann3-2* seedlings' root length was significantly longer and root curvature was significantly smaller than that in Col-0. For the seedlings grown in darkness, *atann3-2* seedlings' hypocotyl length was significantly longer and hypocotyl curvature was significantly smaller than that in Col-0 (Fig. S1)

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 as 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 curvature of seedlings grown under light were not significantly different from that in Col-0, and the hypocotyl length and curvature of seedlings grown in darkness were in the same degree with that in Col-0 (Fig. S2, Fig.S3). Seedlings of the triple null mutants (*atann1/atann2/atann4*), either grown under light or in darkness, responded to 0.5 mM ATP as sensitively as the wildtype (Fig.S4).

AtANN3 is involved in eATP-regulated auxin distribution

To clarify the role of AtANN3 in eATP regulated seedling growth, *DR5-GUS* and *DR5-GFP* fusion genes were transformed into *atann3-1*. The transgenic seedlings were transplanted onto ATP-containing medium and then GUS staining and CLSM (Confocal Laser Scanning Microscopy) were used to investigate the effect of eATP on abundance and distribution of GUS or GFP in root and hypocotyl cells.

GUS staining in Col-0 seedlings 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 *atann3-1* 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 *atann3-1* 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 *atann3-1* etiolated seedlings, GUS accumulation or asymmetric distribution was impaired (Fig. 3B).

DR5-GFP fluorescence detection results showed that, in eATP-treated Col-0 root tips of seedlings grown under light, 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 roots of *atann3-1* seedlings grown under light, DR5-GFP fluorescence intensity increased, while the asymmetric distribution of fluorescence did not appear (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 *atann3-1* seedlings, neither intensity increase nor asymmetric distribution of the fluorescence was detected (Fig. 4B, D).

AtANN3 is involved in eATP-induced auxin transporter distribution

To verify the role of AtANN3 in eATP-regulated auxin transport, *PIN2-GFP* or *PIN3-GFP* transformed wild type and *atann3-1* were used to investigate the effect of eATP on abundance and distribution of the two auxin transporters in seedlings.

In seedlings grown under light, 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). By contrast, in *atann3-1* roots, ATP treatment led to a remarkable decrease in PIN2-GFP fluorescence intensity, while asymmetric distribution of PIN2-GFP did not appear (Fig. 5A, C). In hypocotyl of etiolated seedlings, fluorescence in hypocotyl cells was very weak, a thin layer of fluorescence appeared at the surface of hypocotyl, which was unlikely belong to any cell structure. After ATP stimulation, fluorescence intensity and distribution did not change, either in Col-0 or in *atann3-1* seedlings (Fig. 5B, D). These results indicate that PIN2 is involved in eATP-regulated root growth. Since the abundance of PIN2 is very low, it is unlikely involved in eATP-regulated hypocotyl growth.

Examination of PIN3-GFP in root tip cells of seedlings grown under light 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 *atann3-1* 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 *atann3-1* 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). By contrast, in hypocotyls of etiolated *atann3-1* seedlings, PIN3-GFP fluorescent intensity did not change after ATP treatment, and asymmetric distribution of PIN3-GFP did not appear (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 involved in eATP-regulated root growth.

Discussion

In eATP-regulated plant growth and development, 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 (Tang et al. 2003; Kim et al. 2006; Weerasinghe et al. 2009; Liu et al. 2012; Yang et al. 2015). 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” (Zhu et al. 2017; Zhu et al. 2020). 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 (Tonon et al. 2010; Zhu et al. 2020).

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. By contrast, *atann3-1* and *atann3-2* seedlings exhibited 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 (Scheuring et al. 2011). Here, we present novel evidence for a role of AtANN3 in regulating seedling growth.

eATP stimulation leads to asymmetric auxin transport 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. By contrast, 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 (Liu et al. 2012; Yang et al. 2015). 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 (Li et al. 2019; Ahmed et al. 2017; Laohavisit et al. 2013; Konopka-Postupolska and Clark 2017). However, annexin-regulated 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 (Lin et al. 2021). Our results provide direct evidence for the involvement of AtANN3 in regulating auxin transport in response to an extracellular signal.

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, respectively. During root's phototropism or gravitropism, PIN2 mediates asymmetric distribution of auxin in root tip cells, especially in the elongation zone (Zhang et al. 2019; Tan et al. 2021). PIN3 is the main mediator of lateral auxin flow during hypocotyl's phototropism or gravitropism (Han et al. 2021). Unidirectional blue light stimulates the movement of PIN3 from irradiated side to the shaded side of etiolated hypocotyl cells (Ding et al. 2011). During hypocotyl's gravitropism, PIN3-mediated auxin directional flux and accumulation in lower side cells result in negative gravitropic bending growth (Rakusova et al. 2011). 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 (Zhu et al. 2020). 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 (Kleine-Vehn et al. 2008; Wan et al. 2012; Zwiewka et al. 2015; Wang et al. 2013; Galvan-Ampudia et al. 2013). PIN3 redistribution results from subcellular trafficking as well (Zhang et al. 2017; Kleine-Vehn et al. 2010; Rakusova et al. 2011). AtANN3 is involved in regulating vesicle transport from the Golgi apparatus to vacuoles (Scheuring et al. 2011). ATP-stimulated asymmetric distribution of auxin and auxin transporters in root tip cells exposed to eATP was absent in *atann3-1* 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 *atann3-1* seedlings (Fig.S5). The result provides a clue for verifying the possible mechanism of AtANN3-mediated auxin transporter recycling.

Declarations

Funding

This work was funded by National Natural Science Foundation of China (grant number 31370319, 31871409, 31800233).

Acknowledgement

We thank Dr. Julia Davies in Department of Plant Science, University of Cambridge, UK. for providing seeds of some mutants.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author contribution:

Z.S. and E.K. conceived and designed research, and finished writing the article; J.X., L.H., and S.X. conducted experiments; R.Z contribute to data analysis. All authors read and approved the manuscript.

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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 $\pm$ 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 $\pm$ 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 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 μ m) 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 only in root cells. 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 μ m) 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 $\pm$ SD. Student's *t*-test *p*- values: * *p*<0.05, ** *p*<0.01.

Figure 6

AtANN3 is involved in eATP-induced PIN3-GFP distribution only in etiolated hypocotyls. 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 μ m) 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 $\pm$ SD. Student's *t*-test *p*-values: * *p*<0.05, ** *p*<0.01.

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