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

Lijuan Han
Hebei Normal University

Shuyan Xia
Hebei Normal University

Jiawei Xu
Hebei Normal University

Ruojia Zhu
Hebei Normal University

Zhonglin Shang (✉ shangzhonglin@hebtu.edu.cn)
Hebei Normal University

Erfang Kang
Hebei Normal University

Research Article

Keywords: extracellular ATP, annexin, auxin, seedling growth, Arabidopsis thaliana

DOI: https://doi.org/10.21203/rs.3.rs-841741/v1

License:  This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
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,
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 \textit{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 \textit{atann1}, \textit{atann2} and \textit{atann4} seedlings responded to eATP in same manner as Col-0, which showed strongly suppressed and markedly bent growth, while the response of \textit{atann3} seedlings was significantly weaker than that of wild type (Fig. 2A). Data analysis showed that, compared to Col-0, eATP-treated \textit{atann3} seedlings were much longer and less curved. Compared with the control, \textit{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, \textit{atann1/atann2} and \textit{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 (\textit{atann1/atann2/atann4}), either growing under light or in darkness, responded to 0.5 mM ATP as sensitively as the wildtype (Fig.S3).

\textbf{AtANN3 is involved in eATP-induced auxin accumulation and distribution}

To clarify the role of AtANN3 in eATP regulated seedling growth, \textit{Dr5-GUS} and \textit{Dr5-GFP} fusion genes were transformed into \textit{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 \textit{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 \textit{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 \textit{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 attann3 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% phytal gel) 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% phytage 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% phytal gel) 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**

**Funding**

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

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**
Data availability

Not applicable.

References

1. Cao Y, Tanaka K, Nguyen CT, Stacey G. Extracellular ATP is a central signaling molecule in plant stress responses. *Curr Opin Plant Biol* 2014, 20:82–87. https://doi.org/10.1016/j.pbi.2014.04.009

2. Roux SJ, Steinebrunner I. Extracellular ATP: an unexpected role as a signaler in plants. *Trends Plant Sci* 2007, 12(11):522–527. https://doi.org/10.1016/j.tplants.2007.09.003

3. Tanaka K, Gilroy S, Jones AM, Stacey G. Extracellular ATP signaling in plants. *Trends Cell Biol* 2010, 20(10):601–608. https://doi.org/10.1016/j.tcb.2010.07.005

4. Pietrowska-Borek M, Dobrogojski J, Sobieszczuk-Nowicka E, Borek S. New insight into plant signaling: extracellular ATP and uncommon nucleotides. *Cells* 2020, 9(2):345. https://doi.org/10.3390/cells9020345

5. Kim SY, Sivaguru M, Stacey G. Extracellular ATP in plants. Visualization, localization, and analysis of physiological significance in growth and signaling. *Plant Physiol* 2006, 142(3):984–992. https://doi.org/10.1104/pp.106.085670

6. Yang X, Wang B, Farris B, Clark G, Roux SJ. Modulation of root skewing in Arabidopsis by apyrases and extracellular ATP. *Plant Cell Physiol* 2015, 56(11):2197–2206. https://doi.org/10.1093/pcp/pcv134

7. Zhu R, Dong X, Hao W, Gao W, Zhang W, Xia S, Liu T, Shang Z. Heterotrimeric G protein-regulated Ca\(^{2+}\) influx and PIN2 asymmetric distribution Are involved in Arabidopsis thaliana roots’ avoidance response to extracellular ATP. *Front Plant Sci* 2017, 8:1522. https://doi.org/10.3389/fpls.2017.01522

8. Zhu R, Dong X, Xue Y, Xu J, Zhang A, Feng M, Zhao Q, Xia S, Yin Y, He S et al. Redox-Responsive Transcription Factor 1 (RRFT1) is involved in extracellular ATP-regulated Arabidopsis thaliana seedling growth. *Plant Cell Physiol* 2020, 61(4):685–698. https://doi.org/10.1093/pcp/pcaa014

9. Tonon C, Cecilia Terrile M, Jose Iglesias M, Lamattina L, Casalongue C. Extracellular ATP, nitric oxide and superoxide act coordinately to regulate hypocotyl growth in etiolated Arabidopsis seedlings. *J Plant Physiol* 2010, 167(7):540–546. https://doi.org/10.1016/j.jplph.2009.11.002

10. Wu Y, Qin B, Feng K, Yan R, Kang E, Liu T, Shang Z. Extracellular ATP promoted pollen germination and tube growth of Nicotiana tabacum through promoting K\(^{+}\) and Ca\(^{2+}\) absorption. *Plant Reprod* 2018, 31(4):399–410. https://doi.org/10.1007/s00497-018-0341-6

11. Reichler SA, Torres J, Rivera AL, Cintolesi VA, Clark G, Roux SJ. Intersection of two signalling pathways: extracellular nucleotides regulate pollen germination and pollen tube growth via nitric oxide. *J Exp Bot* 2009, 60(7):2129–2138. https://doi.org/10.1093/jxb/erp091
12. Chen D, Cao Y, Li H, Kim D, Ahsan N, Thelen J, Stacey G. Extracellular ATP elicits DORN1-mediated RBOHD phosphorylation to regulate stomatal aperture. *Nat Commun* 2017, 8(1):2265. https://doi.org/10.1038/s41467-017-02340-3

13. Clark G, Fraley D, Steinebrunner I, Cervantes A, Onyirimba J, Liu A, Torres J, Tang W, Kim J, Roux SJ. Extracellular nucleotides and apyrases regulate stomatal aperture in Arabidopsis. *Plant Physiol* 2011, 156(4):1740–1753. https://doi.org/10.1104/pp.111.174466

14. Hao LH, Wang WX, Chen C, Wang YF, Liu T, Li X, Shang ZL. Extracellular ATP promotes stomatal opening of Arabidopsis thaliana through heterotrimeric G protein alpha subunit and reactive oxygen species. *Mol Plant* 2012, 5(4):852–864. https://doi.org/10.1093/mp/ssr095

15. Tang W, Brady SR, Sun Y, Muday GK, Roux SJ. Extracellular ATP inhibits root gravitropism at concentrations that inhibit polar auxin transport. *Plant Physiol* 2003, 131(1):147–154. https://doi.org/10.1104/pp.013672

16. Chen D, Hao F, Mu H, Ahsan N, Thelen JJ, Stacey G. S-acylation of P2K1 mediates extracellular ATP-induced immune signaling in Arabidopsis. *Nat Commun* 2021, 12(1):2750. https://doi.org/10.1038/s41467-021-22854-1

17. Choi J, Tanaka K, Liang Y, Cao Y, Lee SY, Stacey G. Extracellular ATP, a danger signal, is recognized by DORN1 in Arabidopsis. *Biochem J* 2014, 463(3):429–437. https://doi.org/10.1042/BJ20140666

18. Deng S, Sun J, Zhao R, Ding M, Zhang Y, Sun Y, Wang W, Tan Y, Liu D, Ma X et al. Populus euphratica APYRASE2 enhances cold tolerance by modulating vesicular trafficking and extracellular ATP in Arabidopsis plants. *Plant Physiol* 2015, 169(1):530–548. https://doi.org/10.1104/pp.15.00581

19. Jewell JB, Sowders JM, He R, Willis MA, Gang DR, Tanaka K. Extracellular ATP shapes a defense-related transcriptome both independently and along with other defense signaling pathways. *Plant Physiol* 2019, 179(3):1144–1158. https://doi.org/10.1104/pp.18.01301

20. Kumar S, Tripathi D, Okubara PA, Tanaka K. Purinoceptor P2K1/DORN1 enhances plant resistance against a soilborne fungal pathogen, Rhizoctonia solani. *Front Plant Sci* 2020, 11:572920. https://doi.org/10.3389/fpls.2020.572920

21. Pham AQ, Cho SH, Nguyen CT, Stacey G. Arabidopsis lectin receptor kinase P2K2 is a second plant receptor for extracellular ATP and contributes to innate immunity. *Plant Physiol* 2020, 183(3):1364–1375. https://doi.org/10.1104/pp.19.01265

22. Tripathi D, Tanaka K. A crosstalk between extracellular ATP and jasmonate signaling pathways for plant defense. *Plant Signal Behav* 2018:e1432229. https://doi.org/10.1080/15592324.2018.1432229

23. Clark G, Roux SJ. Apyrases, extracellular ATP and the regulation of growth. *Curr Opin Plant Biol* 2011, 14(6):700–706. https://doi.org/10.1016/j.pbi.2011.07.013

24. Lim MH, Wu J, Yao J, Gallardo IF, Dugger JW, Webb LJ, Huang J, Salmi ML, Song J, Clark G et al. Apyrase suppression raises extracellular ATP levels and induces gene expression and cell wall changes characteristic of stress responses. *Plant Physiol* 2014, 164(4):2054–2067. https://doi.org/10.1104/pp.113.233429
25. Choi J, Tanaka K, Cao Y, Qi Y, Qiu J, Liang Y, Lee SY, Stacey G. Identification of a plant receptor for extracellular ATP. *Science* 2014, 343(6168):290–294. https://doi.org/10.1126/science.343.6168.290

26. Weerasinghe RR, Swanson SJ, Okada SF, Garrett MB, Kim SY, Stacey G, Boucher RC, Gilroy S, Jones AM. Touch induces ATP release in Arabidopsis roots that is modulated by the heterotrimeric G-protein complex. *FEBS Lett* 2009, 583(15):2521–2526. https://doi.org/10.1016/j.febslet.2009.07.007

27. Demidchik V, Shang Z, Shin R, Thompson E, Rubio L, Laohavisit A, Mortimer JC, Chivasa S, Slabas AR, Glover BJ *et al.* Plant extracellular ATP signalling by plasma membrane NADPH oxidase and Ca^{2+} channels. *Plant J* 2009, 58(6):903–913. https://doi.org/10.1111/j.1365-313X.2009.03830.x

28. Shang Z, Laohavisit A, Davies JM. Extracellular ATP activates an Arabidopsis plasma membrane Ca^{2+}-permeable conductance. *Plant Signal Behav* 2009, 4(10):989–991

29. Demidchik V, Shang Z, Shin R, Colaco R, Laohavisit A, Shabala S, Davies JM. Receptor-like activity evoked by extracellular ADP in Arabidopsis root epidermal plasma membrane. *Plant Physiol* 2011, 156(3):1375–1385. https://doi.org/10.1104/pp.111.174722

30. Wang LM, Stacey G, Leblanc-Fournier N, Legue V, Moulia B, Davies JM. Early extracellular ATP signaling in Arabidopsis root epidermis: a multi-conductance process. *Front Plant Sci* 2019, 10:1064. https://doi.org/10.3389/fpls.2019.01064

31. Wang LM, Wilkins KA, Davies JM. Arabidopsis DORN1 extracellular ATP receptor; activation of plasma membrane K^{+}-and Ca^{2+}-permeable conductances. *New Phytol* 2018, 218(4):1301–1304. https://doi.org/10.1111/nph.15111

32. Clark G, Roux SJ. Role of Ca^{2+} in mediating plant responses to extracellular ATP and ADP. *Int J Mol Sci* 2018, 19(11):3590. https://doi.org/10.3390/ijms19113590

33. Song CJ, Steinebrunner I, Wang X, Stout SC, Roux SJ. Extracellular ATP induces the accumulation of superoxide via NADPH oxidases in Arabidopsis. *Plant Physiol* 2006, 140(4):1222–1232. https://doi.org/10.1104/pp.105.073072

34. Wu SJ, Wu JY. Extracellular ATP-induced NO production and its dependence on membrane Ca^{2+} flux in Salvia miltiorrhiza hairy roots. *J Exp Bot* 2008, 59(14):4007–4016. https://doi.org/10.1093/jxb/erm242

35. Chivasa S, Tome DF, Hamilton JM, Slabas AR. Proteomic analysis of extracellular ATP-regulated proteins identifies ATP synthase beta-subunit as a novel plant cell death regulator. *Mol Cell Proteomics* 2011, 10(3):M110.003905. https://doi.org/10.1074/mcp.M110.003905

36. Konopka-Postupolska D, Clark G. Annexins as overlooked regulators of membrane trafficking in plant cells. *Int J Mol Sci* 2017, 18(4):863. https://doi.org/10.3390/ijms18040863

37. Davies JM. Annexin-mediated calcium signalling in plants. *Plants (Basel)* 2014, 3(1):128–140. https://doi.org/10.3390/plants3010128

38. Clark GB, Morgan RO, Fernandez MP, Roux SJ. Evolutionary adaptation of plant annexins has diversified their molecular structures, interactions and functional roles. *New Phytol* 2012, 196(3):695–712. https://doi.org/10.1111/j.1469-8137.2012.04308.x
39. Chu P, Chen H, Zhou Y, Li Y, Ding Y, Jiang L, Tsang EW, Wu K, Huang S. Proteomic and functional analyses of Nelumbo nucifera annexins involved in seed thermotolerance and germination vigor. *Planta* 2012, 235(6):1271–1288. https://doi.org/10.1007/s00425-011-1573-y

40. Cantero A, Barathakur S, Bushart TJ, Chou S, Morgan RO, Fernandez MP, Clark GB, Roux SJ. Expression profiling of the Arabidopsis annexin gene family during germination, de-etiolation and abiotic stress. *Plant Physiol Biochem* 2006, 44(1):13–24. https://doi.org/10.1016/j.plaphy.2006.02.002

41. Clark GB, Lee D, Dauwalder M, Roux SJ. Immunolocalization and histochemical evidence for the association of two different Arabidopsis annexins with secretion during early seedling growth and development. *Planta* 2005, 220(4):621–631. https://doi.org/10.1007/s00425-004-1374-7

42. Lichocka M, Rymaszewski W, Morgiewicz K, Barymow-Filoniuk I, Chlebowski A, Sobczak M, Samuel MA, Schmelzer E, Krzymowska M, Hennig J. Nucleus- and plastid-targeted annexin 5 promotes reproductive development in Arabidopsis and is essential for pollen and embryo formation. *BMC Plant Biol* 2018, 18(1):183. https://doi.org/10.1186/s12870-018-1405-3

43. Zhu J, Wu X, Yuan S, Qian D, Nan Q, An L, Xiang Y. Annexin5 plays a vital role in Arabidopsis pollen development via Ca\(^{2+}\)-dependent membrane trafficking. *PLoS One* 2014, 9(7):e102407. https://doi.org/10.1371/journal.pone.0102407

44. Jami SK, Clark GB, Ayele BT, Ashe P, Kirti PB. Genome-wide comparative analysis of annexin superfamily in plants. *PLoS One* 2012, 7(11):e47801. https://doi.org/10.1371/journal.pone.0047801

45. He F, Gao C, Guo G, Liu J, Gao Y, Pan R, Guan Y, Hu J. Maize annexin genes ZmANN33 and ZmANN35 encode proteins that function in cell membrane recovery during seed germination. *J Exp Bot* 2019, 70(4):1183–1195. https://doi.org/10.1093/jxb/ery452

46. Qiao B, Zhang Q, Liu D, Wang H, Yin J, Wang R, He M, Cui M, Shang Z, Wang D *et al*. A calcium-binding protein, rice annexin OsANN1, enhances heat stress tolerance by modulating the production of H2O2. *J Exp Bot* 2015, 66(19):5853–5866. https://doi.org/10.1093/jxb/erv294

47. Zhang Y, Wang Q, Zhang X, Liu X, Wang P, Hou Y. Cloning and characterization of an annexin gene from Cynanchum komarovii that enhances tolerance to drought and Fusarium oxysporum in transgenic cotton. *J Plant Biol* 2011, 54(5):303–313. https://doi.org/10.1007/s12374-011-9167-6

48. Yadav D, Ahmed I, Shukla P, Boyidi P, Kirti PB. Overexpression of Arabidopsis AnnAt8 alleviates abiotic stress in transgenic Arabidopsis and tobacco. *Plants (Basel)* 2016, 5(2):18. https://doi.org/10.3390/plants5020018

49. Li X, Zhang Q, Yang X, Han J, Zhu Z. OsANN3, a calcium-dependent lipid binding annexin is a positive regulator of ABA-dependent stress tolerance in rice. *Plant Sci* 2019, 284:212–220. https://doi.org/10.1016/j.plantsci.2019.04.019

50. Laohavisit A, Mortimer JC, Demidchik V, Coxon KM, Stancombe MA, Macpherson N, Brownlee C, Hofmann A, Webb AAR, Miedema H *et al*. Zea mays annexins modulate cytosolic free Ca\(^{2+}\) and generate a Ca\(^{2+}\)-permeable conductance. *Plant Cell* 2009, 21(2):479–493. https://doi.org/10.1105/tpc.108.059550
51. Laohavisit A, Shang ZL, Rubio L, Cuin TA, Very AA, Wang AH, Mortimer JC, Macpherson N, Coxon KM, Battey NH et al. Arabidopsis Annexin1 mediates the radical-activated plasma membrane Ca\(^{2+}\)- and K\(^{+}\)-permeable conductance in root cells. *Plant Cell* 2012, 24(4):1522–1533. https://doi.org/10.1105/tpc.112.097881

52. Kodavali PK, Skowronek K, Koszela-Piotrowska I, Strzelecka-Kiliszek A, Pawlowski K, Pikula S. Structural and functional characterization of annexin 1 from Medicago truncatula. *Plant Physiol Biochem* 2013, 73:56–62. https://doi.org/10.1016/j.plaphy.2013.08.010

53. Mu C, Zhou L. Phosphatase GhDsPTP3a interacts with annexin protein GhANN8b to reversely regulate salt tolerance in cotton (Gossypium spp.). 2019, 223(4):1856–1872. https://doi.org/10.1111/nph.15850

54. Gorecka KM, Konopka-Postupolska D, Hennig J, Buchet R, Pikula S. Peroxidase activity of annexin 1 from Arabidopsis thaliana. *Biochem Biophys Res Commun* 2005, 336(3):868–875. https://doi.org/10.1016/j.bbrc.2005.08.181

55. Dalal A, Kumar A, Yadav D, Gudla T, Viehhauser A, Dietz KJ, Kirti PB. Alleviation of methyl viologen-mediated oxidative stress by Brassica juncea annexin-3 in transgenic Arabidopsis. *Plant Sci* 2014, 219–220:9–18. https://doi.org/10.1016/j.plantsci.2013.12.016

56. Konopka-Postupolska D. Annexins: putative linkers in dynamic membrane-cytoskeleton interactions in plant cells. *Protoplasma* 2007, 230(3–4):203–215. https://doi.org/10.1007/s00709-006-0234-7

57. Scheuring D, Viotti C, Krüger F, Künzl F, Sturm S, Bubeck J, Hillmer S, Frigerio L, Robinson DG, Piml P et al. Multivesicular bodies mature from the trans-Golgi network/early endosome in Arabidopsis. *Plant Cell* 2011, 23(9):3463–3481. https://doi.org/10.1105/tpc.111.086918

58. Jami SK, Clark GB, Turlapati SA, Handley C, Roux SJ, Kirti PB. Ectopic expression of an annexin from Brassica juncea confers tolerance to abiotic and biotic stress treatments in transgenic tobacco. *Plant Physiol Biochem* 2008, 46(12):1019–1030. https://doi.org/10.1016/j.plaphy.2008.07.006

59. Huh SM, Noh EK, Kim HG, Jeon BW, Bae K, Hu HC, Kwak JM, Park OK. Arabidopsis annexins AnnAt1 and AnnAt4 interact with each other and regulate drought and salt stress responses. *Plant Cell Physiol* 2010, 51(9):1499–1514. https://doi.org/10.1093/pcp/pcq111

60. Zhang Y, Friml J. Auxin guides roots to avoid obstacles during gravitropic growth. *New Phytol* 2020, 225(3):1049–1052. https://doi.org/10.1111/nph.16203

61. Han H, Adamowski M, Qi L, Alotaibi SS, Friml J. PIN-mediated polar auxin transport regulations in plant tropic responses. *New Phytol* 2021(ePub). https://doi.org/10.1111/nph.17617

62. Lee HJ, Kim HS, Park JM, Cho HS, Jeon JH. PIN-mediated polar auxin transport facilitates root-obstacle avoidance. *New Phytol* 2020, 225(3):1285–1296. https://doi.org/10.1111/nph.16076

63. Zhou JJ, Luo J. The PIN-FORMED auxin efflux carriers in plants. *Int J Mol Sci* 2018, 19(9):2759. https://doi.org/10.3390/ijms19092759

64. Adamowski M, Friml J. PIN-dependent auxin transport: action, regulation, and evolution. *Plant Cell* 2015, 27(1):20–32. https://doi.org/10.1105/tpc.114.134874
65. Narasimhan M, Gallei M, Tan S, Johnson A, Verstraeten I, Li L, Rodriguez L, Han H, Himschoot E, Wang R et al. Systematic analysis of specific and nonspecific auxin effects on endocytosis and trafficking. *Plant Physiol* 2021, 186(2):1122–1142. https://doi.org/10.1093/plphys/kiab134

66. Barbosa ICR, Hammes UZ, Schwechheimer C. Activation and polarity control of PIN-FORMED auxin transporters by phosphorylation. *Trends Plant Sci* 2018, 23(6):523–538. https://doi.org/10.1016/j.tplants.2018.03.009

67. Tan S, Luschnig C, Friml J. Pho-view of auxin: reversible protein phosphorylation in auxin biosynthesis, transport and signaling. *Mol Plant* 2021, 14(1):151–165. https://doi.org/10.1016/j.molp.2020.11.004

68. Zhang L, Ma J, Liu H, Yi Q, Wang Y, Xing J, Zhang P, Ji S, Li M, Li J et al. SNARE proteins VAMP721 and VAMP722 mediate the post-Golgi trafficking required for auxin-mediated development in Arabidopsis. *Plant J* 2021(ePub). https://doi.org/10.1111/tpj.15450

69. Ding Z, Galvan-Ampudia CS, Demarsy E, Langowski L, Kleine-Vehn J, Fan Y, Morita MT, Tasaka M, Fankhauser C, Offringa R et al. Light-mediated polarization of the PIN3 auxin transporter for the phototropic response in Arabidopsis. *Nat Cell Biol* 2011, 13(4):447–452. https://doi.org/10.1038/ncb2208

70. Rakusova H, Gallego-Bartolome J, Vanstraelen M, Robert HS, Alabadi D, Blazquez MA, Benkova E, Friml J. Polarization of PIN3-dependent auxin transport for hypocotyl gravitropic response in Arabidopsis thaliana. *Plant J* 2011, 67(5):817–826. https://doi.org/10.1111/j.1365-313X.2011.04636.x

71. Kleine-Vehn J, Ding Z, Jones AR, Tasaka M, Morita MT, Friml J. Gravity-induced PIN transcytosis for polarization of auxin fluxes in gravity-sensing root cells. *Proc Natl Acad Sci U S A* 2010, 107(51):22344–22349. https://doi.org/10.1073/pnas.1013145107

72. Narasimhan M, Johnson A, Prizak R, Kaufmann WA, Tan S, Casillas-Perez B, Friml J. Evolutionarily unique mechanistic framework of clathrin-mediated endocytosis in plants. *Elife* 2020, 9:e52067. https://doi.org/10.7554/eLife.52067

73. Ganguly A, Sasayama D, Cho HT. Regulation of the polarity of protein trafficking by phosphorylation. *Mol Cells* 2012, 33(5):423–430. https://doi.org/10.1007/s10059-012-0039-9

74. Liu X, Wu J, Clark G, Lundy S, Lim M, Arnold D, Chan J, Tang W, Muday GK, Gardner G et al. Role for apyrases in polar auxin transport in Arabidopsis. *Plant Physiol* 2012, 160(4):1985–1995. https://doi.org/10.1104/pp.112.202887

75. Ma L, Ye J, Yang Y, Lin H, Yue L, Luo J, Long Y, Fu H, Liu X, Zhang Y et al. The SOS2-SCaBP8 complex generates and fine-tunes an AtANN4-dependent calcium signature under salt stress. *Dev Cell* 2019, 48(5):697–709. https://doi.org/10.1016/j.devcel.2019.02.010

76. Ahmed I, Yadav D, Shukla P, Vineeth TV, Sharma PC, Kirti PB. Constitutive expression of Brassica juncea annexin, AnnBj2 confers salt tolerance and glucose and ABA insensitivity in mustard transgenic plants. *Plant Sci* 2017, 265:12–28. https://doi.org/10.1016/j.plantsci.2017.09.010
77. Laohavisit A, Richards SL, Shabala L, Chen C, Colaço RD, Swarbreck SM, Shaw E, Dark A, Shabala S, Shang Z et al. Salinity-induced calcium signaling and root adaptation in Arabidopsis require the calcium regulatory protein annexin1. *Plant Physiol* 2013, 163(1):253–262. https://doi.org/10.1104/pp.113.217810

78. Lin X, Li R, Zhou Y, Tang F, Wang Y, Lu X, Wang S, Yao Y, Liu J, Hu X et al. Overexpression of cassava MeAnn2 enhances the salt and IAA tolerance of transgenic Arabidopsis. *Plants (Basel)* 2021, 10(5):941. https://doi.org/10.3390/plants10050941

79. Zhang Y, Xiao G, Wang X, Zhang X, Friml J. Evolution of fast root gravitropism in seed plants. *Nat Commun* 2019, 10(1):3480. https://doi.org/10.1038/s41467-019-11471-8

80. Kleine-Vehn J, Leitner J, Zwiewka M, Sauer M, Abas L, Luschnig C, Friml J. Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. *Proc Natl Acad Sci U S A* 2008, 105(46):17812–17817. https://doi.org/10.1073/pnas.0808073105

81. Wan Y, Jasik J, Wang L, Hao H, Volkmann D, Menzel D, Mancuso S, Baluska F, Lin J. The signal transducer NPH3 integrates the phototropin1 photosensor with PIN2-based polar auxin transport in Arabidopsis root phototropism. *Plant Cell* 2012, 24(2):551–565. https://doi.org/10.1105/tpc.111.094284

82. Zwiewka M, Nodzynski T, Robert S, Vanneste S, Friml J. Osmotic stress modulates the balance between exocytosis and clathrin-mediated endocytosis in Arabidopsis thaliana. *Mol Plant* 2015, 8(8):1175–1187. https://doi.org/10.1016/j.molp.2015.03.007

83. Wang C, Yan X, Chen Q, Jiang N, Fu W, Ma B, Liu J, Li C, Bednarek SY, Pan J. Clathrin light chains regulate clathrin-mediated trafficking, auxin signaling, and development in Arabidopsis. *Plant Cell* 2013, 25(2):499–516. https://doi.org/10.1105/tpc.112.108373

84. Galvan-Ampudia CS, Julkowska MM, Darwish E, Gandullo J, Korver RA, Brunoud G, Haring MA, Munnik T, Vernoux T, Testerink C. Halotropism is a response of plant roots to avoid a saline environment. *Curr Biol* 2013, 23(20):2044–2050. https://doi.org/10.1016/j.cub.2013.08.042

85. Zhang Y, Yu Q, Jiang N, Yan X, Wang C, Wang Q, Liu J, Zhu M, Bednarek SY, Xu J et al. Clathrin regulates blue light-triggered lateral auxin distribution and hypocotyl phototropism in Arabidopsis. *Plant Cell Environ* 2017, 40(1):165–176. https://doi.org/10.1111/pce.12854

86. Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jurgens G. Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. *Nature* 2003, 426(6963):147–153. https://doi.org/10.1038/nature02085

**Figures**
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
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 2
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.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Hanetal.BMCPBsupplementfigure.docx