Developmental roles of Auxin Binding Protein 1 in Arabidopsis thaliana

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Auxin is a major plant growth regulator, but current models on auxin perception and signaling cannot explain the whole plethora of auxin effects, in particular those associated with rapid responses. A possible candidate for a component of additional auxin perception mechanisms is the AUXIN BINDING PROTEIN 1 (ABP1), whose function in planta remains unclear.

Here we combined expression analysis with gain- and loss-of-function approaches to analyze the role of ABP1 in plant development. ABP1 shows a broad expression and overlapping with, but not regulated by, transcriptional auxin response activity. Furthermore, ABP1 activity is not essential for the transcriptional auxin signaling. Genetic in planta analysis revealed that abp1 loss-of-function mutants show largely normal development with minor defects in bolting. On the other hand, ABP1 gain-of-function alleles show a broad range of growth and developmental defects, including root and hypocotyl growth and bending, lateral root and leaf development, bolting, as well as response to heat stress. At the cellular level, ABP1 gain-of-function leads to impaired auxin effect on PIN polar distribution and affects BFA-sensitive PIN intracellular aggregation.

The gain-of-function analysis suggests a broad, but still mechanistically unclear involvement of ABP1 in plant development, possibly masked in abp1 loss-of-function mutants by a functional redundancy.

1. Introduction

The phytohormone auxin is a major coordinator of plant growth that governs a multitude of developmental processes. Its versatility is related to its differential distribution within plant tissues and the ability of cellular auxin concentrations to determine various cell fate decisions. The establishment of these morphogenic auxin gradients and local auxin maxima is achieved by a combination of local auxin biosynthesis [1] and synergetic, directional cell-to-cell polar auxin transport [2].

AUXIN concentration affects cellular processes, mainly through a modulation of transcription. A broad range of auxin-responsive transcriptional regulators remodel the transcriptome of cells through tissue specific expression and thus trigger complex developmental changes [3]. On this transcriptional level, auxin controls processes such as embryogenesis, vascular tissues formation and organogenesis of the shoot apex or maintenance of the root apical meristem [4].

Nevertheless, some cellular auxin effects occur too fast to be a result of transcriptome remodeling and/or they were shown not to require...
functional transcription or de novo protein synthesis. Auxin triggers rapid hyperpolarization of the plasma membrane leading to protoplast swelling [5,6], induces calcium ion and proton fluxes across the plasma membrane and therefore alkalizes the apoplastic space [7,8], and inhibits clathrin-mediated endocytic trafficking processes [9,10].

The auxin signal is transduced via several mechanisms [11,12]. The canonical pathway is mediated by a nuclear-localized co-receptor complex comprising the TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOX (TIR1/AFB) F-box proteins and the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors. Here, auxin promotes the interaction of TIR1/AFBs with Aux/IAAs that results in ubiquitin-dependent degradation of the Aux/IAA proteins. Aux/IAA proteins act as transcriptional repressors of AUXIN RESPONSE FACTORS (ARFs) transcription factors and thus their degradation activates auxin-responsive transcription [13].

Notably, recent findings suggest that TIR1/AFB signaling mediates both rapid transcriptional as well as even faster non-transcriptional auxin effects on growth. In shoots, auxin via the TIR1/AFB pathway induces fast apoplastic acidification and growth promotion by a rapid transcriptional regulation of SMALL AUXIN UPREGULATED (SAUR) genes [14,15]. In contrast, auxin-mediated growth inhibition in roots occurs within 30 s and does not require de novo protein synthesis but is still strictly dependent on the TIR1/AFB pathway [16]. Furthermore, the auxin-mediated fast depolarization of the plasma membrane and Ca²⁺ uptake were demonstrably linked with the TIR1/AFB signaling pathway [17].

Recently, two additional non-canonical auxin-sensing mechanisms were described. Auxin has been shown to bind directly to the atypical ARF ARF3/ETTIN to modulate chromatin states and interaction with other transcriptional regulators during gynoecium development [18,19]. The other mechanism involves TRANSMEMBRANE KINASE 1 (TMK1), a member of the plasma membrane-localized TMK receptor-like kinase family [20]. It was shown that auxin triggers cleavage of TMK1’s intracellular kinase domain and its consequent translocation to the nucleus. There, the TMK1 kinase domain binds, phosphorylates and thus stabilizes two non-canonical Aux/IAAs, IAA32 and IAA34. Via this alternative transcriptional pathway, auxin regulates apical hook development [21]. TMK1 also regulates lateral root organogenesis and auxin biosynthesis by other cellular mechanisms [22,23]. Importantly, while the canonical TIR1/AFB receptors sense auxin predominantly in the nucleus, the TMKs located in the plasma membrane may perceive auxin from the apoplast by an unknown perception mechanism.

The accumulating developmental roles of TMKs in conjunction with their plasma membrane localization stir up a decades-lasting debate on the existence of a cell-surface auxin receptor. In the past, the best candidate appeared to be AUXIN BINDING PROTEIN 1 (ABP1). This evolutionarily conserved 22-kDa glycoprotein [24,25] has been shown to bind auxin at apoplastic pH 5.5 [26–28] and although it predominantly localizes to the endoplasmic reticulum (ER), a small fraction has been proposed to reside in the apoplast [29]. ABP1 has been proposed to be mainly associated with rapid non-transcriptional auxin-mediated processes, but the genetic analysis has been hampered by the lack of viable loss-of-function mutants. It has also remained unclear how apoplastic ABP1 could transduce the auxin signal into the cell and therefore the requirement of a plasma membrane-localized docking partner was hypothesized. Later, ABP1 was found to interact with TMK1 in an auxin-dependent manner. It was proposed that ABP1 and TMK1 form an auxin-sensing complex at the plasma membrane that activates downstream cellular processes via small GTPases ROP2 and ROP6 and their effector proteins RIC1 and RIC4 [30–32]. Based on the phenotypes of ABP1 gain-of-function mutants, the weak abp1-5 allele and conditional abp1 knock-down lines [33] ABP1 was proposed to play a role in clathrin-mediated endocytosis [10,34,35], growth-correlating microtubule re-orientation [36], cell wall remodeling [37] or interdigitated growth of leaf pavement cells [30,36]. All these proposed roles were called into question by the failure to complement the alleged embryo lethal abp1 phenotypes, by the coding sequence of ABP1 [35] and by the identification of new abp1 knock-out alleles with no obvious morphological phenotypes [38]. These discrepancies were clarified by proofs that the original abp1 embryo lethal phenotypes were caused by disruption of a neighboring gene rather than ABP1 itself [39,40]. Furthermore, the abp1-5 line carries many additional mutations [41] and the conditional knock-down lines, despite independently targeting either ABP1 mRNA or protein [33], also have other targets [42]. Thus, with much of the previously used genetic material called into question and with only superficial phenotype analysis of the more recent, verified knock-out lines [38], the developmental and physiological roles of ABP1 still remain largely unclear.

Here we used the verified gain- and loss-of-function mutant lines in Arabidopsis to (re)evaluate the role of ABP1 in cellular processes, physiological responses and plant development.

2. Materials and methods

2.1. Plant material

Wild-type Col-0 (NASC, The Nottingham Arabidopsis Stock Centre; http://www.arabidopsis.info, N1092) was used as a control line. Previously published Arabidopsis thaliana lines were used in this study: ABP1g::GUS [43]; abp1-C1, abp1-TD1 and background Col-0 used for generating abp1-C1 by CRISPR (in text mentioned as WT for abp1-C1) [38]; DR5rev::GFP [44]; 35S::ABP1-GFP [10]. The following Arabidopsis thaliana lines were generated in this study: DR5rev::GFP;abp1-C1 and DR5rev::GFP;abp1-TD1. DR5rev::GFP was introduced into both abp1 mutant backgrounds by genetic crossing. The ABP1::ABP1;abp1-TD1 line was generated by introducing the ABP1::ABP1 construct into the abp1-TD1 background and the ABP1::GFP;abp1-C1 line was generated by introducing the ABP1::GFP;ABP1 construct into the abp1-C1 background using Agrobacterium-mediated transformation [45]. All transgenic lines and mutants used in this study are listed in Supplemental Table 1. All primers used for genotyping are listed in Supplemental Table 2.

2.2. Vector construction

All plasmids were constructed by the Gateway cloning technology (www.invitrogen.com). Previously generated constructs pDONR221-ABP1cDNA and pDONR221-ABP1cDNA-M2X containing cDNA sequence of ABP1 [35] were used to construct the final plasmids 35S::ABP1 and 35S::ABP1-M2X by recombination into the p2GW7 destination vector. ABP1::ABP1 was constructed as follows: the 3.0 kb promoter, genomic coding region and 0.6 kb of 3’ untranslated region for ABP1 was amplified and inserted into a pDONR-Zeo destination vector. ABP1::GFP-ABP1 was constructed using a 1585 bp promoter fragment [43] and a N-terminal GFP fusion directly after the N-terminal signal peptide. The GFP insertion was flanked at the 5’ end by a PKAPKA linker (tested for cleavage using the SignalNH-5.0 server) and at the 3’ end by a PKAPAPKPA linker. The ABP1 fragments were amplified from genomic DNA using primer pairs 1 and 2 (promoter, signal peptide and 5’ linker), 3 and 4 (GFP and 3’ linker) and 5 and 6 (gABP1 gene body including 3’ UTR). All three fragments were fused in a single overlap PCR reaction and cloned into the pDONR221 entry vector. The resulting construct was cloned into the pKGW0 destination vector and sequenced. All primers used in this study are listed in Supplemental Table 2. All plasmids used in this study are listed in Supplemental Table 3.

2.3. Growth conditions

Seeds were chlorine gas sterilized or sterilized with 70 % EtOH, sown on plates with ½ Murashige and Skoog (MS) medium supplemented with
1 % (w/v) sucrose and 0.8 % (w/v) Phytoagar (pH 5.9) and stratified for 2 days at 4 °C. For experiments using Arabidopsis seedlings, the seedlings were grown on plates at 21 °C under a long-day photoperiod (16 h light/8 h dark) for the required time period. For experiments performed in soil, in vitro grown seedlings were transferred to soil and grown under a long-day photoperiod (16 h light/8 h dark) at 21 °C and 40 % relative humidity for the required time period. The light sources used were Philips GreenPower light emitting diode production modules in a deep red, far red, blue combination with a photon density of 140.4 μmol/m²/s ± 3 %. For phyllotaxis measurement experiment, plants in soil were cultivated in growth chambers at 22 °C and 40 % relative humidity. Plants were kept under short day conditions (8 h light/16 h dark) for 28 days and then transferred to long day conditions (16 h light/8 h dark). Plants were always grown together within the growth chamber and with randomized positions within each tray in order to minimize the effect of environmental fluctuations. For etiolated growth, the plated and stratified seeds were exposed to light for 8 h and further covered with aluminum foil to cultivate them in the dark at 21 °C for 4 days (the shoot gravity response experiment) or in the dark chamber at 24 °C for 5 days (the etiolated growth experiment).

2.4. Histochemical GUS staining

6-d-old light-grown seedlings or 3-d-old etiolated seedlings of ABP1::GUS were stained in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 % X-Gluc sodium salt (Duchefa, 7240-90-6), 2 mM K$_{3}$[Fe(CN)$_{6}$], 2 mM K$_{4}$[Fe(CN)$_{6}$]$_{3}$, and 0.05 % Triton X-100 for 2 h (light-grown seedlings) or 1 h (etiolated seedlings) at 37 °C. Further, samples were incubated overnight in 80 % (v/v) ethanol at room temperature. Tissue clearing was conducted as previously described [46]. DIC microscopy for analysis of GUS staining was performed using an Olympus BX53 microscope equipped with 10x and 20x air objectives and a DP26 CCD camera. For treatment, 5-d-old seedlings of ABP1::GUS were transferred to ½ MS media supplemented with DMSO (mock) for 3.5 h, 25 μM L-Kynurenine (Sigma-Aldrich, 2922-83-0) for 3.5 h and 25 μM L-Kynurenine for 2 h followed by 300 nM IAA (Duchefa, 87-51-4) for 1.5 h. Subsequently, GUS staining and DIC microscopy were performed as described above.

2.5. Quantitative real-time PCR

After treatment with 100 nM IAA, seedlings were sampled in 4 biological replicates at different times (10, 5 min, 30 min, 1 h, 2 h and 4 h). Total RNA was prepared from max100 mg of shoots/roots of 5-d-old seedlings with the RNeasy Plant Mini Kit (Qiagen, 74904) according to the manufacturer’s instructions. cDNA was synthesized from 2 μg of total RNA using the Quant iT Nova Reverse Transcription Kit (Qiagen, 205410). For the mutant expression analyses, 3 biological replicates of full seedlings were used. All samples were pipetted in 3 technical replicates in a 384 well plate using an automated JANUS Workstation (PerkinElmer). According to the manufacturer’s instructions, 5 μL reaction volume contained 2.5 μL Luna® Universal qPCR mastermix (NEB, M3003S). RT-qPCR analyses were performed using the Real-time PCR Roche Lightcycler 480 and the expression of PP2AAt (At1G13320) or EF1α (At5G60390) was used as a reference [47]. For ABP1, 5 different primer pairs were evaluated and one representative graph is included in the manuscript. The primers used for the presented analysis are listed in Supplemental Table 2.

2.6. Confocal laser scanning microscopy and image analysis

Confocal laser scanning microscopy for analysis of fluorescence intensity was performed on a Zeiss LSM800 microscope assisted with Zeiss Zen 2011 software. Images were acquired with 20x/0.8 NA air (DR5 evaluation) or 40x/1.2 NA water immersion objectives (immunostaining).

2.7. DR5-GFP intensity measurement

5-d-old seedlings were transferred from soil ½ MS media to plates supplemented with either DMSO or 1 μM IAA for 3 h and imaged using confocal microscopy. The fluorescence intensity of GFP (excitation wavelength: 488 nm) was measured in ImageJ

2.8. Microfluidic vRootchip

A microfluidic chip, vRootchip was used to analyze root tip growth in real-time. The manufacturing of the chip, sample preparation procedure, and data analysis of root tip growth was performed as described previously [16] and according to Li and Verstraeten et al. (unpublished). vRoot-chip was used with 10 nM IAA treatment in ½ MS and 0.1 % sucrose. For imaging, the vertical confocal microscopy setup was used as described previously [16, 48] and according to Li and Verstraeten et al. (unpublished). The 10 nM IAA solution was supplemented with the cell-impermeable fluorescent dye Tetracylthiophenoxide–Dextran [16].

2.9. Protoplast assay

Protoplasts from 3-d-old Arabidopsis root suspension culture were isolated and transformed as previously described [49]. Plasmids were prepared with an E.Z.N.A. Plasmid Maxi Kit I (Omega Bio-Tek, D6922-02). Protoplasts were co-transfected with 6 μg of 35S::ABP1 or 35S::ABP1-M2X, 2.5 μg of DR5::LUC [50] and 2.5 μg of 35S::Renilla [51]. As a control, protoplasts co-transfected with DR5::LUC and 35S::Renilla were used. The protoplasts were incubated with either 100 nM NAA (Sigma-Aldrich, 86-87-3) for 16 h or without treatment for 12 h followed by 100 nM NAA for 4 h in the dark at room temperature. The corresponding amount of DMSO was used as mock treatment. Chemiluminescence measurement was performed with the Dual-Luciferase Reporter Assay System kit (Promega, E1910) using a Spectrophotometer BioTek SynergyH1 plate reader and Gen 5 software (both BioTek).

2.10. Root length analysis

Plates with 4- and 7-d-old seedlings were scanned using an Epson Perfection V370 Photo flatbed scanner and the root length was measured using ImageJ.

2.11. Root gravitropic assay

For measurements of root gravitropic curvature kinetics, 4-d-old seedlings were placed on plates with ½ MS and rotated 90° and roots were imaged using a vertically placed flatbed scanner (Epson Perfection V370 Photo). Multiple plates were held in place on the scanner by a custom-made holder. Max. 12 ROIs of the seedlings were automatically imaged with a resolution of 1200 dpi in 10 min time intervals using an AutoIt script for 8 h. In ImageJ, the time-lapse movies of the seedlings were manually cropped and registered (stabilized) using the Fiji plugin “StackReg” in “Rigid body” mode.

2.12. Root growth (RG)-tracker

We developed a custom MATLAB application named RG-tracker (https://research-explorer.app.ist.ac.at/librecat/record/8294) with a graphical user interface that allows entirely automated root growth analysis and tracking of the root tips. Root tips were segmented based on the pixel classification workflow of Ilastik [52], which only requires manual retraining in case the imaging conditions change drastically. For each point in time, the positions of the root tips were determined by segmenting the tip-probability output, performing particle size filtering and calculating the center of mass. The root tips were then tracked over time by solving the linear assignment problem using the Hungarian...
algorithm (Munkres). The tracking algorithm can deal with gaps in the root tip detection and both the gap closing and the maximum linking distance can be specified in the GUI. Completed tracks are filtered by minimum track length, duration and maximum growth speed to remove miss-detections and then presented to the user as an overlay of raw data, tip segmentation and tip tracks. At this point the user can exclude additional tracks from further analysis and export the overlay of the tracks and the root time-lapse. The x/y coordinates of each root tip, growth speed, direction of growth, growth angle and root length are then calculated for each point in time and exported for further analysis. All experiment specific parameters such as the segmentation threshold, particle size, and track filters can be saved and together with the segmentation project file form the complete data set to clearly recapitulate the data analysis.

2.13. Lateral root analysis

For the analysis of lateral root primordia, samples of 6-d-old seedling were collected and the tissue was cleared as previously described [46]. To visualize the lateral root primordia DIC microscopy was performed using an Olympus BX53 microscope equipped with a 20x air objective. The lateral root primordia were staged according to Malam and Benfey (1997) [46]. For analysis of emerged lateral roots, 4-d-old seedlings were transferred from ½ MS plates to plates supplemented with 500 nM NAA or DMSO. After 3 days, the plates were scanned using an Epson Perfection V370 Photo flatbed scanner and the pictures were analyzed using ImageJ.

2.14. Etiolated hypocotyl growth

To analyze the growth of etiolated hypocotyls, the seedlings were recorded at 12 h intervals for 120 h in a dark chamber equipped with an infrared light source (880 nm LED; Velleman, Belgium) and a spectrum-enhanced camera (EOS5035 Canon Rebel Xti, 400DH) with built-in clear wideband-multicoated filter and standard accessories (Canon) and operated by the EOS utility software. The hypocotyl length was measured using ImageJ.

2.15. Etiolated hypocotyl bending

To determine hypocotyl gravitropism, the 3-d-old dark grown seedlings were rotated 90°. The plates were scanned using an Epson Perfection V370 Photo flatbed scanner and the hypocotyl bending angle was measured after gravity stimulation in 6 h intervals for 24 h using ImageJ.

2.16. Rosette size analysis

Seeds were germinated and grown on horizontally placed plates for 12 days, scanned using an Epson Perfection V370 Photo flatbed scanner and the rosette size was measured manually in ImageJ.

2.17. Vasculature development analysis

10-d-old cotyledons were collected and the tissue was cleared as follows: 2 days incubation in 70 % ethanol with a subsequent incubation in 4 % HCl, 20 % methanol solution at 65 °C for maximum 15 min, followed by an incubation in 7 % NaOH, 60 % ethanol solution at RT for another 15 min. The cotyledons were then re-hydrated in a series of decreasing ethanol concentrations (60 %, 40 %, 20 % and 10 %) for 1 h in each concentration at RT. Before mounting the cotyledons in 50 % glycerol onto microscopy slides they were incubated for 1 h in 25 % glycerol, 5 % ethanol solution at RT. Imaging was done using an Olympus BX53 microscope equipped with a 4x air objective.

2.18. Hypocotyl growth under high temperature

Seeds were germinated and grown on ½ MS plates with or without 10 g/L sucrose under 28 °C, continuous light for 7 days. The plates were scanned using an Epson Perfection V370 Photo flatbed scanner and the hypocotyl length was measured using ImageJ.

2.19. Hyperosmotic stress assay

4-d-old seedlings were transferred on media supplemented with either 200 mM mannitol or 100 mM NaCl for 4 days. The plates were scanned using an Epson Perfection V370 Photo flatbed scanner and the root extension was measured using ImageJ.

2.20. UV laser ablation and periclinal division analysis

3-d-old seedlings were transferred from solid ½ MS medium to plates containing 10 μM propidium iodide (Sigma-Aldrich, 25535-16-4) supplemented with 1 μM NAA or DMSO. The subsequent sample preparation, UV laser ablation, imaging and periclinal cell division analysis was performed as described previously [53].

2.21. Bolting time, leaf number and branch number analysis

Seeds were suspended in 0.1 % agarose and spread out in soil. The number of plants, bolted and with the primary inflorescence stem grown 1 cm, was recorded every day. The number of rosette and cauline leaves was counted when the first flower of each plant bloomed. The rosette branch was referred to the branch directly attached to the rosette, while the cauline branch was defined as the branch on the primary stem. The number of cauline branches and rosette branches was counted 21 days after sowing.

2.22. Phyllotaxis and internode length measurement

Analyses of 25 plants per genotype were performed when the last flowers had appeared. Angles and internode lengths between two subsequent siliques were measured starting from the lowest one. For each individual of each genotype, the variance of the divergence angles was computed, and individual variances of divergence angles were compared between genotypes using a non-parametric Kruskal–Wallis test in R (version 3.5.1, r-project.org), since their values were not normally distributed.

2.23. Immunostaining

Immunostaining was performed with 3 to 4-d-old seedlings as previously described [54]. The primary antibodies used were rabbit anti-PIN1 [9] diluted 1:1000 (v/v) and rabbit anti-PIN2 [55] diluted 1:1000 (v/v). The secondary antibody used was sheep anti-rabbit conjugated with Cy3 (Sigma-Aldrich, C2306) diluted 1:600 (v/v).

2.24. PIN lateralization

3to 4-d-old seedlings were treated either with 10 μM NAA or DMSO as a control for 4 h in liquid ½ MS medium. Subsequently, immunostaining using PIN1 and PIN2 antibodies was performed. Samples were imaged using confocal microscopy. The fluorescence intensity of Cy3 (excitation wavelength: 548 nm) was measured using ImageJ.

2.25. BFA treatment

4-d-old seedlings were incubated in liquid ½ MS medium at a final concentration of 25 μM BFA (Sigma-Aldrich, 20350-15-6) for 1 h. For BFA/NAA treatment the seedlings were pre-treated with 5 μM NAA for 30 min followed by co-treatment with 25 μM BFA and 5 μM NAA for 1 h.
As control, seedlings were incubated in liquid ½ MS medium supplemented with DMSO substituting NAA. Subsequently, immunostaining using PIN1 and PIN2 antibodies was performed. Samples were imaged using confocal microscopy and the fluorescence signal of Cy3 (excitation wavelength: 548 nm) was detected. BFA body formation was scored from 0 (no BFA body formation) to 3 (maximal BFA body formation) for each image, reflecting both the number of cells with BFA bodies as well as size and number of BFA bodies per cell. To avoid cognitive bias, all images were encoded prior to analysis.

2.26. Global transcriptome data analysis

Tissue-specific expression pattern and expression following different perturbations were obtained using Genevestigator (www.genevestigator.com) and were based on the ‘AT_AFFY_ATH1-0’ dataset.

2.27. Statistical analysis

If not mentioned differently, all data were analyzed using Student’s t tests with p-value (*, P < 0.05; ** P < 0.01; *** P < 0.001) in the software Prism v8.3.0 (GraphPad).

2.28. Accession numbers

Sequences data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: ABP1 (AT4G02980); PP2AA3 (At1G13320); EF1a (At5G60390).

3. Results

3.1. ABP1 expression and regulation by auxin

To obtain indications regarding the developmental processes and conditions in which ABP1 might play a role, we analyzed the ABP1 expression pattern. The analysis of publicly available global transcriptome data in GENEVESTIGATOR® [56] suggested that ABP1 is expressed constitutively in different tissues during development. ABP1 transcription appears to be the highest in rosette leaves and roots, whilst lowest in pollen (Fig. S1A-B). In seedlings, ABP1 is expressed in cotyledons, hypocotyls and root tips as well as in lateral roots. Global transcriptomics data following different perturbations suggested that ABP1 expression is elevated in response to heat and slightly decreased following biotic stress (Fig. S1C).

To obtain more detailed insight into the ABP1 expression pattern and confirm the global transcriptome analysis-based notions, we used an ABP1::GUS line to report ABP1 promoter activity in vivo. GUS staining of

Fig. 1. ABP1 expression and regulation by auxin.

(A–H) ABP1::GUS expression pattern. (A) 6-d-old cotyledon with inset detail of stomata, scale bar =100 μm. (B) shoot with hydathods of 6-d-old seedling, scale bar =50 μm. (C) shoot-root junction of 6-d-old seedling, scale bar =50 μm. (D) apical hook of 3-d-old etiolated seedling, scale bar =100 μm. (E) root tip of 6-d-old seedling, scale bar =100 μm. (F–H) lateral root primordia of 6-d-old seedling in IV, V and emerged stage respectively, scale bar =20 μm.

(I) Representative pictures of ABP1::GUS expression pattern in 5-d-old seedlings after treatment with DMSO (mock) for 3.5 h, 25 μM L-Kynurenine for 3.5 h and 25 μM L-Kynurenine for 2 h followed by 300 nM IAA for 1.5 h. For each treatment, at least 15 seedlings were evaluated. The experiment was repeated 2 times with similar results. Scale bar =20 μm.

(J) Quantitative Real-time PCR of ABP1 expression in roots and shoots of 5-d-old Col-0 seedlings after treatment with DMSO (mock), and 5 min, 60 min, 120 min and 240 min of 100 nM IAA treatments. Expression of ABP1 is normalized on expression of PP2A housekeeping gene. Experiment was repeated 3 times with similar result.
6-d-old seedlings confirmed the \textit{ABP1} expression in cotyledons in which we detected stronger \textit{ABP1} promoter activity in hydathodes and stomata (Fig. 1A-B). In both light- and dark-grown hypocotyls, the \textit{ABP1} promoter activity was very low (Fig. 1C-D). Further, we confirmed \textit{ABP1} expression in the primary root, particularly in the root tip (Fig. 1E) and during different stages of lateral root development (Fig. 1F-H). We observed that \textit{ABP1} expression pattern in hydathodes, root tip and lateral roots largely overlaps with that of \textit{DR5} reporters for transcriptional auxin response [43,44,50,57–59].

Therefore, we tested whether auxin regulates \textit{ABP1} promoter activity and transcription. We employed L-Kynurenine, an inhibitor of auxin biosynthesis [60], to decrease auxin levels in the \textit{ABP1::GUS} seedlings. We tested both, the effect of L-Kynurenine treatment alone or with subsequent auxin treatment, to study the effect of exogenously applied auxin. Overall, we detected no obvious changes in GUS reporter activity either after L-Kynurenine or after L-Kynurenine followed by auxin treatments (Fig. 1I).

To additionally verify these observations, we examined the auxin effect on \textit{ABP1} transcription using real-time quantitative PCR (RT-qPCR). We performed RT-qPCR with roots and shoots of 5-d-old wild-type seedlings after auxin treatment. Consistent to what we observed with the \textit{ABP1::GUS} transgenic line, auxin treatment did not strongly affect \textit{ABP1} transcription (Fig. 1J).

These results show that \textit{ABP1} expression overlaps with auxin

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\textbf{Fig. 2. Involvement of \textit{ABP1} in TIR1/AFB-mediated auxin responses.}

(A–B) \textit{DR5rev::GFP} expression pattern in 5-d-old seedlings of wild-type Col-0, \textit{abp1-C1} and \textit{abp1-TD1} mutants with DMSO (A) or with 1 \textmu M IAA (B) treatment for 3 h. Arrowheads point to DR5 signal expanded to lateral root cap. Scale bar =30 \textmu m.

(C) Representative picture of \textit{DR5rev::GFP} expression in Col-0 with highlighted region that was quantified. Scale bar =50 \textmu m. Quantification of \textit{DR5rev::GFP} signal in root tips of 5-d-old Col-0, \textit{abp1-C1} and \textit{abp1-TD1} seedlings with DMSO (A) or with 1 \textmu M IAA (B) treatment for 3 h. For each genotype per treatment, at least 15 seedlings were measured. The pooled result of 2 independent experiments is presented. For box plot, box defines the first and third quartiles, and the central lines in the box represent the median. Whiskers, from minimum to maximum. Asterisks indicate significant differences according to Student’s t-tests (****, \(P < 0.0001\)).

(D) Root growth rate of \textit{abp1-C1} (upper graph) and \textit{abp1-TD1} (lower graph) compared to Col-0 measured in the vRootchip with repetitive 10 nM IAA treatment (magenta). \(n = 5, 6\) for Col-0 and \textit{abp1-C1}, respectively. \(n = 5\) for \textit{abp1-TD1}; \(n = 3, 2\) for Col-0 from 0–102 min and 102–236 min, respectively. Error bars denote standard deviation. The experiment was repeated 2 times with similar results.

(E) Activity of \textit{DR5::LUC} reporter in response to \textit{ABP1} and \textit{ABP1-M2X} overexpression after mock (DMSO) and 100 nM IAA treatment in protoplasts. The values presented were calculated as a ratio between \textit{DR5::LUC} enzymatic activity and internal control \textit{Renilla::LUC} enzymatic activity and were further normalized on mock treatment values. Error bars denote standard error. The statistical difference was tested by Student’s t-test. The experiment was repeated 2 times with similar results.
response maxima during seedling development, but that ABP1 promoter activity and ABP1 transcription are not significantly regulated by auxin.

3.2. Involvement of ABP1 in TIR1/AFB-mediated auxin responses

Considering that ABP1::GUS expression pattern largely overlaps with that of DR5 reporters for transcriptional auxin response [43,44,50,57–59], we investigated whether ABP1 function is in any way linked to the transcriptional auxin signaling downstream of TIR1/AFB receptors [12,61]. First, we introduced DR5rev::GFP reporter into abp1 loss-of-function mutants (abp1-C1 and abp1-TD1). In the abp1 mutant backgrounds, DR5rev::GFP expression pattern in the root tip was not visibly altered and showed the typical maximum in the columella cells and quiescent center [57,58,62] (Fig. 2A). After auxin treatment, the DR5rev::GFP signals in abp1 mutants expanded to the lateral root cap and stele to the same extent as in the control (Fig. 2B). Quantification of the DR5 signal without and with auxin treatment in the root tips did not reveal any differences between the control and abp1 mutants (Fig. 2C). Taken together, these results show that the DR5 auxin response reporter’s readout does not depend on a functional ABP1.

Recently it was demonstrated, that the TIR1/AFB pathway is required for a rapid non-transcriptional auxin response [16]. We used this experimental system to investigate TIR1/AFB-mediated non-transcriptional auxin effects on root growth in the mutant lines. Evaluation of root growth on the vertical imaging set-up with high spatio-temporal resolution [16,48] revealed a comparable auxin sensitivity of root growth on the vertical imaging set-up with high transcriptional auxin effects on root growth in the mutant lines. Evaluating this experimental system to investigate TIR1/AFB-mediated non-transcriptional auxin effects on root growth in the mutant lines.

Next, we tested the effect of ABP1 gain-of-function on TIR1/AFB-mediated transcriptional auxin signaling by performing a transient expression assay in Arabidopsis protoplasts. We derived protoplasts from root cell culture, co-transfected them with DR5:LUC reporter together with either 3SS::ABP1 or 3SS::ABP1-M2X carrying a mutation in the auxin-binding site [38] and measured the DR5::LUC signal with and without auxin. The DR5-driven luciferase activity increased after both short (4 h) and long (16 h) term auxin treatment, however neither ABP1 nor ABP1-M2X overexpression had any significant influence on this induction (Fig. 2E).

These results do not support a strict requirement of ABP1 function in the canonical, TIR1/AFB-mediated auxin signaling pathway.

3.3. Role of ABP1 in primary root growth and root gravity response

Since ABP1 is expressed in the primary root and root tip (Fig. 1C, E) and auxin is a major regulator of root growth [16,63–65], we analyzed whether abp1 loss-of-function or the overexpression influences primary root growth. We used two independent loss-of-function mutant lines, abp1-C1 and abp1-TD1 and a line expressing ABP1-GFP under the control of the ubiquitous 3SS promoter (ABP1-GFP3SS) (Fig. S2). Visually, roots of all tested lines developed normally (Fig. 3A). We measured the root length of 4- and 7-d-old seedlings and found that the root growth of abp1 mutants was comparable to WT, while roots of ABP1-GFP3SS were shorter.

Fig. 3. Role of ABP1 in primary root growth and root gravity response.
(A) Representative images of 4- (upper panel) and 7-d-old (lower panel) Col-0, abp1-C1, abp1-TD1, and ABP1-GFP3SS seedlings. Scale bar = 5 mm. The boxplot shows the root length of 4- and 7-d-old Col-0, abp1-C1, abp1-TD1, and ABP1-GFP3SS seedlings. For each genotype, at least 15 roots were measured. For box plot, box defines the first and third quartiles, and the central lines in the box represent the median. Whiskers, from minimum to maximum. Asterisks indicate significant differences according to Student’s t tests (*, P < 0.05; ****, P < 0.0001). The experiment was repeated 2 times with similar results.
(B) Representative images of 4-d-old Col-0, abp1-C1, abp1-TD1, and ABP1-GFP3SS seedlings after 8 h gravistimulation by 90° reorientation. Scale bar = 1 cm. Arrow indicates gravity direction. Kinetics of root bending during 8 h of gravity stimulus for Col-0, abp1-C1, abp1-TD1, and ABP1-GFP3SS. For each line at least 15 roots were measured. Error bars denote standard deviation. Asterisks indicate significant differences according to Student’s t tests (****, P < 0.0001). The experiment was repeated 2 times with similar results.
Asymmetric auxin distribution is involved in gravitropism, an important plant adaptive process manifested by shoot and root bending \[66-69\]. In order to describe a role of \textit{ABP1} during root bending, we gravistimulated (90° reorientation) roots of 4-d-old \textit{abp1} and \textit{ABP1-GFP}\textsuperscript{OE} seedlings for 8 h and measured the root bending kinetics. We observed that \textit{abp1} mutants showed a normal root gravitropic response while the roots of \textit{ABP1-GFP}\textsuperscript{OE} bent significantly slower (Fig. 3 B). In summary, the \textit{abp1} loss-of-function mutants do not have any impact on either root growth or root bending, whereas gain-of-function leads to slower root growth and root bending.

### 3.4. Role of \textit{ABP1} during lateral root development

As \textit{ABP1} is expressed during lateral root development (Fig. 1F–H), and auxin promotes lateral root initiation and formation \[59\], we analyzed lateral root development in 6-d-old \textit{abp1} and \textit{ABP1-GFP}\textsuperscript{OE} seedlings. The analysis revealed that both \textit{abp1} mutants and \textit{ABP1-GFP}\textsuperscript{OE} developed a comparable number of lateral root primordia (Fig. 4A). In addition, we could not find any differences in the frequency of individual primordial stages (Fig. 4B).

To test the auxin effect on lateral root emergence, we transferred 4-d-old seedlings to media supplemented with auxin and 3 days later we counted the density of emerged lateral roots. We observed that the density of emerged lateral roots was comparable between \textit{abp1} mutants and WT, while \textit{ABP1-GFP}\textsuperscript{OE} developed less lateral roots (Fig. 4C).

Together, the results presented above demonstrate that both \textit{abp1} loss-of-function mutants do not have any impact on lateral root development, but that \textit{ABP1} overexpression leads to impaired auxin-induced lateral root development.

### 3.5. Role of \textit{ABP1} in etiolated growth and shoot gravity response

Auxin is required for a sustained rapid hypocotyl-elongation of seedlings. We counted and scored all lateral root primordia stages. The analysis revealed that both \textit{abp1} mutants and \textit{ABP1-GFP}\textsuperscript{OE} developed a comparable number of lateral root primordia (Fig. 4A). In addition, we could not find any differences in the frequency of individual primordial stages (Fig. 4B).
plants grown in darkness [70–72]. The auxin-induced growth of etiolated hypocotyl segments is not altered in abp1 loss-of-function mutants [15]. To complement these observations in intact plants, we analyzed growth of etiolated hypocotyls for both abp1 loss- and gain-of-function lines and measured the hypocotyl length of the dark-grown seedlings every twelve hours (Fig. 5A). Initially, the hypocotyls of all tested lines elongated at the same speed. Later, starting 36 h after germination, etiolated hypocotyls of ABP1-GFPOE elongated faster and they were significantly longer than the control 120 h after germination. On the other hand, etiolated hypocotyls of both abp1 mutant alleles elongated comparably to the controls.

The gravitropic response of the hypocotyl is also regulated by auxin [67–69]. To investigate a possible function of ABP1 in hypocotyl gravitropism, we gravistimulated 3-d-old etiolated hypocotyls and measured the bending angle after 6, 18 and 24 h. The analysis revealed that the ABP1-GFPOE hypocotyls bend significantly less than WT (Fig. 5B). The difference was noticeable already 6 h after gravistimulation. Notably, both abp1 mutants showed a similar tendency towards slower bending, albeit not significant.

In summary, these observations unveiled that abp1 loss-of-function alleles do not show defects in etiolated hypocotyl growth and gravitropic responses, whereas gain-of-function of ABP1 leads to increased elongation and defective gravity-mediated hypocotyl bending.

3.6. Role of ABP1 in leaf development and vasculature formation

In cotyledons, auxin and its directional transport act as a positional cue for vasculature vein formation [73,74] and also regulate leaf shape and serration [75]. We analyzed whether ABP1 plays a role in the young rosette growth and development as well as in cotyledon vasculature formation. Macroscopically, neither abp1 mutants nor ABP1-GFPOE showed any defects in cotyledon development (Fig. 6A). We measured the size of young rosettes consisting of both cotyledons and primary leaves. We found that ABP1-GFPOE had slightly bigger rosettes (Fig. 6A).

The vasculature of cotyledons typically consists of four formed closed loops (Fig. 6B). We scored the number and the completeness of these loops in abp1 mutants and ABP1-GFPOE. We observed a normal vasculature pattern in both abp1 mutants, but ABP1-GFPOE showed irregularities at higher frequency than WT (Fig. 6B). The most striking difference in ABP1-GFPOE were fewer loops (22 % in WT and 46 % in ABP1-GFPOE) and loops that were opened at their upper end, which is almost never seen in WT (2 % in WT and 6.5 % in ABP1-GFPOE).

The results show that, whilst abp1 loss-of-function has no impact on leaves growth and venation, ABP1 gain-of-function affects vasculature formation.

3.7. Role of ABP1 during stress

Abiotic stresses, such as salinity and osmotic stress, induce changes in turgor pressure and in polar auxin transport [76–78] and thus lead to root growth inhibition. On the other hand, an increase of auxin biosynthesis results in higher salt tolerance [79,80]. The regulation of ABP1 transcription by various stresses such as heat (Fig. S1) prompted us to test the requirement of ABP1 to adapt to stress.

We challenged abp1 mutants with osmotic stress using mannitol or sodium chloride treatments to assess the involvement of ABP1 in stress...
responses. Overall, following the treatments, root growth and lateral root formation of WT and abp1 mutants were inhibited (Fig. 7A–C). In addition, no obvious differences in root growth inhibition were observed after mannitol or sodium chloride treatment between the tested lines (Fig. 7B–C).

High temperature promotes auxin biosynthesis, thereby leading to rapid hypocotyl growth [70]. To address a potential role of ABP1 in auxin-mediated rapid hypocotyl growth in response to high temperature and the presence of sugar, we characterized hypocotyl elongation of abp1 and ABP1-GFPOE seedlings grown under high temperature (28 °C), on media supplemented with or without sucrose. When grown in high temperature (28 °C) on the medium with sucrose, ABP1-GFPOE exhibited longer hypocotyls compared to WT, whereas the hypocotyl length of abp1 mutants was comparable to that of WT plants (Fig. 7D). At high temperature (28 °C), but in absence, of sucrose the hypocotyl elongation of ABP1-GFPOE line was less inhibited than in WT (Fig. 7E).

To test whether ABP1 plays a role in wound healing responses, we performed a targeted cell ablation in the root tips of abp1-TD1 and ABP1-GFPOE lines [53,81]. After cell ablation, the numbers of initiating periclinal cell divisions in abp1-TD1 and ABP1-GFPOE were similar to that in WT (Fig. S3).

Taken together, the results show that the root growth of abp1 loss-of-function mutants is not influenced differently by salt stress and high temperature. ABP1 gain-of-function seedlings show increased hypocotyl growth when grown at high temperature.
3.8. Role of ABP1 in rosette leaves and inflorescence development

The establishment of auxin maxima in the shoot apical meristem (SAM) and directed basipetal polar auxin transport are crucial for overall shoot development [59, 82–86]. ABP1 is expressed in both SAM and rosette leaves (Fig. S1B), therefore we investigated its possible function in shoot development.

First, we characterized leaf development. Visually, the size and shape of rosette leaves in abp1 mutants and ABP1-GFP OE plants were comparable to that of WT plants. We quantified the rosette leaves number at the stage when the first flower of each individual plant bloomed. We observed that the abp1-TD1 mutant developed slightly more, whereas the ABP1-GFP OE line developed significantly less rosette leaves in comparison to WT (Fig. 8 A). However, the results for the abp1-TD1 mutant line were variable between the experimental repetitions. We found no difference in the number of cauline leaves for any of the analyzed lines (Fig. S4A).

Further, we studied the function of ABP1 during bolting. We measured the length of the first internode of abp1 mutants and ABP1-GFP OE plants and we recorded the timing to reach 1 cm. Compared to WT, both abp1 mutants and ABP1-GFP OE line bolt earlier, at 21st and 22nd day after sowing versus 23rd day in WT (Fig. 8B).

To determine whether ABP1 is involved in phyllotaxis establishment, we measured the sequence of divergence angles between siliques in abp1 mutants. Visually, abp1 mutants developed normal inflorescence stems (Fig. 8C). WT plants typically exhibit a spiral phyllotaxis that leads to a distribution of the consecutive organs on the stem with a divergence angle close to 137.5° [87]. Our analysis revealed that the distribution of divergence angles in abp1 mutants was not altered (Fig. 8D–F). We also analyzed the internode length between the siliques and counted the number of rosette and cauline branches of abp1 mutant and ABP1-GFP OE plants. However, we did not detect any differences (Fig. S4B–D).

The results show that overexpression of ABP1 affects the number of rosette leaves and that both ABP1 loss- and gain-of-function accelerate bolting.

3.9. Role of ABP1 in auxin-mediated PIN polarization and BFA-visualized PIN trafficking

The formation of organized vasculature requires coordinated cell polarization. The canalization hypothesis proposes that auxin acts as a polarizing cue in this process [88] and that auxin feed-back on PIN polarity, together with constitutive PIN endocytic trafficking are important features in this process [54, 89, 90]. Since overexpression of ABP1 results in defects in vascular tissue formation (Fig. 6B), we tested whether abp1 loss- or gain-of-function alleles show defects in these

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**Fig. 7. Role of ABP1 during stress.**

(A) Representative images of 8-d-old Col-0, abp1-C1, abp1-TD1 and ABP1-GFP OE seedlings grown for 4 days on control media or on media supplemented with either 200 mM mannitol or 100 mM NaCl. Scale bar = 5 mm.

(B–C) Quantification of the root growth inhibition of Col-0, abp1-C1 and abp1-TD1 seedlings after treatment with 200 mM mannitol (B) or 100 mM NaCl (C). For each genotype, at least 10 roots were measured per experiment. The experiment was repeated 3 times with the similar results and the pooled values are presented. The statistical significance was tested by Wilcoxon test.

(D) Quantification of the hypocotyl length of 7-d-old Col-0, abp1-C1, abp1-TD1 and ABP1-GFP OE seedlings grown under continuous light, higher temperature (28 °C).

For each genotype and experiment, at least 25 hypocotyls were analyzed. Error bars denote standard error. Asterisks indicate significant differences according to Student’s t tests (****, P < 0.0001). The experiment was repeated 3 times with the similar results.

(E) Quantification of the hypocotyl growth inhibition sucrose of Col-0, abp1-C1, abp1-TD1 and ABP1-GFP OE seedlings grown under continuous light, higher temperature (28 °C) and in absence of sucrose. For each genotype, at least 10 roots were measured per experiment. Asterisks indicate significant differences according to Student’s t tests (****, P < 0.0001). The experiment was repeated 3 times with the similar results and the pooled values are presented.
Fig. 8. Role of ABP1 in rosette leaves and inflorescence development.

(A) Boxplot showing the number of rosette leaves of Col-0, abp1-C1, abp1-TD1 and ABP1-GFPOE plants. For each genotype per experiment, at least 10 rosettes were scored when the first flower bloomed on each single plant. For box plot, box defines the first and third quartiles, and the central lines in the box represent the median. Whiskers, from minimum to maximum. Asterisks indicate significant differences according to Student’s t tests (*, P < 0.05; ****, P < 0.0001). The experiment was repeated 3 times with the similar result.

(B) Quantification of bolting time of Col-0, abp1-C1, abp1-TD1, ABP1-GFPOE, WT for abp1-C1, and ABP1::ABP1;abp1-TD1. The graph shows number of plants with inflorescence stem ≥ 1 cm for the given day in percentage. For each genotype per experiment, at least 20 plants were scored. The experiment was at this given setup repeated 2 times with the similar result, and additionally 2 times for Col-0, abp1-C1, abp1-TD1, ABP1-GFPOE with the similar result.

(C) Representative pictures of the inflorescence stem of Col-0, abp1-C1, and abp1-TD1. Scale bar = 1 cm.

(D–F) Distribution of divergence angles between the siliques in Col-0, abp1-C1, and abp1-TD1. For each genotype divergence angles of 25 individual plants were measured.
processes. To evaluate the effect of auxin on PIN polarity, we analyzed the repolarization of PIN1 from the basal to the inner lateral side in root endodermis cells and the repolarization of PIN2 from the basal to the outer lateral side in root cortex cells [54] following auxin treatment in abp1 mutants and ABP1-GFPOE. Anti-PIN1 and anti-PIN2 immunolocalization revealed that PIN1 and PIN2 repolarization was not altered in abp1 mutants, while overexpression of ABP1 led to reduced or no repolarization of PIN1 and PIN2 respectively (Fig. 9A–B).

Further, we used the trafficking inhibitor Brefeldin A (BFA) to indirectly visualize PIN intracellular trafficking [91]. BFA treatment results in PIN internal aggregation manifested as BFA-body formation and this effect is decreased when BFA is used together with auxin [9]. The anti-PIN1 immunostaining in roots after BFA treatment showed that the intracellular aggregation of PIN1 was similar to that of WT in both abp1 mutants (Fig. 9C) and [92]. In ABP1-GFPOE we observed repeatedly that BFA affected PIN1 intracellular aggregation more severely (BFA bodies were more pronounced) (Fig. 9C). Anti-PIN1 immunostaining after auxin and BFA co-treatment confirmed that auxin inhibited BFA-body formation. Comparison of the abp1 mutants with the corresponding complemented lines did not reveal any consistent changes in the auxin effect on BFA-induced PIN1 aggregation, whereas ABP1-GFPOE showed again slightly more BFA-induced PIN1 aggregation even in presence of auxin (Fig. 9C). The analysis of the BFA effect on PIN2 intracellular aggregation revealed no consistent and reproducible differences in BFA-body formation between WT, abp1 mutants and ABP1-GFPOE (Fig. 9D). Accordingly, auxin and BFA co-treatment led to a comparable and variable decrease of PIN2 intracellular aggregation in WT, abp1 mutants and ABP1-GFPOE (Fig. 9D).

Taken together, the ABP1 overexpression interferes with auxin-induced PIN repolarization and slightly affects BFA-induced, constitutive PIN1 but not PIN2 trafficking, while mutation in ABP1 does not show altered auxin feed-back on PIN polarity or constitutive PIN recycling.

4. Discussion

ABP1 has been identified in maize decades ago based on its potential ability to bind auxin [93,94]. Nonetheless, the developmental roles and cellular functions of ABP1 remain unclear due to problems with some of the genetic material [35,39,40,42] and due to the lack of obvious developmental defects after superficial analyzes of the verified knock-out lines [38].

Here, we assessed the function of ABP1 in various developmental processes and (re)evaluated its role in cellular processes related to trafficking and polar distribution of PIN auxin transporters.

4.1. ABP1 is not essential for or regulated by TIR1/AFB-mediated auxin responses

ABP1 promoter activity has been reported to overlap, to some extent, with that of the transcriptional DRS auxin reporter during early seedling development [43]. Our analysis revealed a similar overlap in hydathodes, root tips and lateral root primordia as well as in older seedlings. The activity of the ABP1 promoter at places with high auxin response suggested either that auxin might regulate the transcription of ABP1 or that ABP1 is somehow linked to TIR1/AFB-mediated transcriptional auxin signaling.

Indeed, ABP1 was previously identified among early auxin-regulated genes. ABP1 transcription was upregulated by auxin in a dose dependent manner within 30 min in 19-d-old WT seedlings [95]. Our observations in 5-d-old WT roots and shoots did not reveal any changes in ABP1 expression following auxin treatment. These contradictory findings suggest that a potential auxin effect on ABP1 transcription could be tissue- and/or developmental stage-dependent.

Also, the connection between ABP1 and TIR1/AFB-mediated auxin signaling was previously investigated. Downregulation of the ABP1 activity was shown to affect transcription of auxin-responsive genes [33,95,96], to regulate Aux/IAA homeostasis and thus negatively impact on the SCFTIR1/AFB pathway [97]. However, these observations are inconclusive due to the potential off-targets in the conditional knock-down lines [42] and the inactivation of ABP1 did not have any significant effects on the DRS auxin response reporter activity [33]. In the verified abp1 knock-out lines it was reported that auxin-regulated gene expression is unchanged [38] and our analysis in these lines and following ABP1 overexpression in protoplasts did also not reveal any changes in DRS reporter activity. Furthermore, abp1 knock-out lines also showed normal TIR1/AFB-mediated non-transcriptional auxin effect on root growth. Overall, these observations suggest that ABP1 is not directly involved in the TIR1/AFB-mediated auxin response.

4.2. ABP1 loss-of-function mutants show minor defects in development

The initial analysis of CRISPR and T-DNA insertion abp1 knock-out mutants did not reveal any obvious defects during development under normal conditions leading to a conclusion that ABP1 is not required for Arabidopsis development [38]. We analyzed different auxin-related phenotypes of the corresponding abp1 knock-out mutants in more detail. We observed that both abp1 alleles exhibited normal root growth, etiolated hypocotyl, root and shoot gravitropic responses, lateral root and leaf development, including venation and phyllotaxis. Notably, both abp1 mutant alleles bolted earlier compared to the control lines. Accelerated bolting in abp1 mutants might be caused by changes in auxin levels caused by either impaired biosynthesis, auxin transport or eventually a change in auxin sensitivity. Nonetheless, it is unclear why such changes are not reflected also in other developmental processes regulated by auxin.

4.3. ABP1 gain-of-function lines show a plethora of auxin-related phenotypes

ABP1 overexpression has been shown previously to cause several postembryonic developmental defects [5,10,35,98,99]. Similarly, our analysis of a stable line expressing 35S::ABP1-GFP revealed that ABP1 gain-of-function leads to developmental changes. Seedlings over-expressing ABP1 have reduced root length, impaired auxin-induced lateral root development, enhanced elongation of both high temperature- and dark-grown hypocotyls, reduced root and shoot gravitropic response, defective vasculature development, increased size of young rosettes but decreased number of rosette leaves. Additionally, similar to the abp1 mutants, ABP1 overexpressors also bolted earlier. At the cellular level, we confirmed the previous observations [10,35] that the ABP1 gain-of-function affects the BFA-sensitive PIN endocytic trafficking and newly showed that they also impair auxin effects on PIN polar distribution in root cells.

All aforementioned processes, which were found defective in ABP1 gain-of-function mutants are linked to auxin regulation. It is therefore conceivable that, in line with the importance of the auxin binding pocket for the ABP1 function [35], ABP1 plays so far a mechanistically unclear role in auxin perception and signaling.

4.4. Potential role and functional mechanism of Arabidopsis ABP1

Arabidopsis ABP1 was identified based on the orthology with ABP1 previously found in maize [98,100]. Auxin-binding properties of maize ABP1 are well characterized. Several biochemical studies along with the structural analysis of the ABP1-auxin co-crystal revealed that maize ABP1 binds auxin with the highest affinity at apoplastic pH 5.5, while binding at pH 7.2 corresponding to the ER lumen where the majority of protein is localized, is much lower [26–29,94,101]. In contrast, the auxin-binding properties of Arabidopsis ABP1 have not been characterized yet. Based on the high homology with the maize protein, it is assumed that Arabidopsis ABP1 binds auxin in a similar manner. This
Fig. 9. Role of ABP1 in auxin-mediated PIN polarization and BFA-visualized PIN trafficking.

(A) Representative pictures of PIN1 immunolocalization in root meristem of 4-d-old Col-0, abp1-C1, abp1-TD1 and ABP1-GFP\textsuperscript{OE} after mock (upper panel) and 4 h 10μM NAA treatment (lower panel). Scale bar =5 μm. The letters indicate an appropriate cell file - S (stele), En (endodermis), C (cortex). Arrow heads point to basal/lateral PIN1 localization in endodermis. The quantitative evaluation shows mean ratio of PIN1 lateral-to-basal signal intensity ratio in endodermis cells of Col-0, abp1-C1, abp1-TD1, ABP1::ABP1;abp1-TD1 and ABP1-GFP\textsuperscript{OE}. Error bars denote standard error. Asterisks indicate significant differences according to Student’s t tests (***, P < 0.0001). The experiment was repeated 3 times, one representative experiment is presented. 

(B) Representative pictures of PIN2 immunolocalization in root meristem of 4-d-old Col-0, abp1-C1, abp1-TD1 and ABP1-GFP\textsuperscript{OE} after mock (upper panel) and 4 h 10μM NAA treatment (lower panel). Scale bar =5 μm. The letters indicate an appropriate cell file – Ep (epidermis), C (cortex). Arrow heads point to basal/lateral PIN2 localization in cortex. The quantitative evaluation shows mean ratio of PIN2 lateral-to-basal signal intensity ratio in cortex cells of Col-0, abp1-C1, abp1-TD1 and ABP1-GFP\textsuperscript{OE}. Error bars denote standard error. Asterisks indicate significant differences according to Student’s t tests (***, P < 0.0001). The experiment was repeated 3 times, one representative experiment is presented. 

(C) Representative pictures of PIN1 immunolocalization in primary root stele of 4-d-old Col-0, abp1-C1, abp1-TD1 and ABP1-GFP\textsuperscript{OE} after 1 h 25 μM BFA treatment (upper panel) and after 30 min 5 μM NAA pre-treatment followed by 1 h 25 μM BFA and 5 μM NAA co-treatment (lower panel). Arrow heads point to affected cells. Scale bar ~20 μm. The quantitative evaluation shows the scoring of an overall count of formed BFA bodies in Col-0, abp1-C1, abp1-TD1, ABP1::GFP-ABP1;abp1-C1, ABP1::ABP1;abp1-TD1 and ABP1-GFP\textsuperscript{OE}. At least 8 roots were scored for each genotype and experiment. The pooled result of 3 independent experiments is presented. 

(D) Representative pictures of PIN2 immunolocalization in primary root epidermis of 4-d-old Col-0, abp1-C1, abp1-TD1 and ABP1-GFP\textsuperscript{OE} after 1 h 25 μM BFA treatment (upper panel) and after 30 min 5 μM NAA pre-treatment followed by 1 h 25 μM BFA and 5 μM NAA co-treatment (lower panel). Arrow heads point to affected cells. Scale bar ~20 μm. The quantitative evaluation shows the scoring of an overall count of formed BFA bodies in Col-0, abp1-C1, abp1-TD1, ABP1::GFP-ABP1;abp1-C1, ABP1::ABP1;abp1-TD1 and ABP1-GFP\textsuperscript{OE}. At least 8 roots were scored for each genotype and experiment. The pooled result of 3 independent experiments is presented.
statement is supported by the finding that the auxin-binding pocket of Arabidopsis ABP1 is important for its gain-of-function cellular and developmental roles [35]. The ABP1 binding optimum at pH 5.5 would imply that ABP1 is functional in the apoplast, further supported by auxin-dependent interaction between ABP1 and the plasma membrane-localized receptor-like kinase TMK1 [30,31]. TMK1 belongs to a four-member TMK receptor-like kinase family, that function redundantly and multiple mutants show severe reduction in organ size and substantial growth retardation [20]. Both TMK1 and TMK4 play roles in auxin-mediated developmental processes and in the control of local auxin biosynthesis [22,23,30]. Importantly, TMK1 mediates auxin signaling that regulates differential growth of the apical hook [31]. However, the mechanism of how TMK1 perceives auxin remains elusive.

The function of ABP1 as a part of the auxin perception machinery contributing towards TMK-based downstream signaling, is a tempting hypothesis consistent with a rather broad spectrum of auxin-related growth defects. But it is not supported by the rather mild phenotypic defects in the abp1 loss-of-function mutants, especially considering that ABP1 is a single copy gene in Arabidopsis [100]. On the other hand, ABP1 is evolutionarily conserved and ubiquitous in vascular plants [102], suggesting that it has an important and conserved function. Structurally ABP1 belongs to an ancient group of germin and germin-like proteins that have a highly conserved tertiary structure despite low similarity in primary sequence among the members [28,103]. Therefore, it is possible that some other proteins from the germin family are functionally redundant with ABP1, thus masking the effect of the abp1 mutation. Nonetheless, to identify and characterize functional homologues within this large family will be a challenging task. An alternative explanation for the weak developmental defects in abp1 loss-of-function mutants is that ABP1 plays an important role in specific processes that provide competitive advantage in nature but are not easily manifested under laboratory conditions.

5. Conclusions

In conclusion, our detailed phenotypic analysis of both ABP1 gain- and loss-of-function lines provides new insights into the developmental role of ABP1. Despite the overlap of ABP1 expression pattern with auxin response maxima during seedling development, none of our observations supports a direct involvement of ABP1 in the TIR1/AFB-mediated transcriptional auxin response. abp1 knock-out mutants show only mild phenotypic defects, whereas ABP1 overexpression generates a broad range of potentially auxin-related phenotypes. The previously described strong and related defects in conditional abp1 knock-down lines let us hypothesize that the discrepancy between the effects of loss- and gain-of-function is due to the action of unknown germin family proteins that are functionally redundant with ABP1.

Author contribution

Z.G., Mi.Ga., M.P., X.Z., Ma.Gl., L.L., J.M., I.V., H.H., M.C., M.Z., L.H., M.F., T.V. and J.F. designed and conducted experiments and analyzed data. G.B. and Z.P. helped with performing experiments. R.H. and I.V. developed RG-tracker. Z.G., I.H. and T.X. contributed with the generation of the genetic material. Z.G., X.Z., Mi.Ga. and J.F. wrote the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110750.

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