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RESEARCH PAPER

Auxin-binding pocket of ABP1 is crucial for its gain-of-function cellular and developmental roles

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Abstract

The plant hormone auxin is a key regulator of plant growth and development. Auxin levels are sensed and interpreted by distinct receptor systems that activate a broad range of cellular responses. The Auxin-Binding Protein1 (ABP1) that has been identified based on its ability to bind auxin with high affinity is a prime candidate for the extracellular receptor responsible for mediating a range of auxin effects, in particular, the fast non-transcriptional ones. Contradictory genetic studies suggested prominent or no importance of ABP1 in many developmental processes. However, how crucial the role of auxin binding to ABP1 is for its functions has not been addressed. Here, we show that the auxin-binding pocket of ABP1 is essential for its gain-of-function cellular and developmental roles. In total, 16 different abp1 mutants were prepared that possessed substitutions in the metal core or in the hydrophobic amino acids of the auxin-binding pocket as well as neutral mutations. Their analysis revealed that an intact auxin-binding pocket is a prerequisite for ABP1 to activate downstream components of the ABP1 signalling pathway, such as Rho of Plants (ROPs) and to mediate the clathrin association with membranes for endocytosis regulation. *In planta* analyses demonstrated the importance of the auxin binding pocket for all known ABP1-mediated postembryonic developmental processes, including morphology of leaf epidermal cells, root growth and root meristem activity, and vascular tissue differentiation. Taken together, these findings suggest that auxin binding to ABP1 is central to its function, supporting the role of ABP1 as auxin receptor.

Key words: Auxin; ABP1; Auxin binding; Site-directed mutagenesis; PIN proteins.

Introduction

The plant hormone auxin, a key regulator of plant growth and development, controls fundamental cellular processes, such as cell division, expansion and differentiation, but its overall role in plant development is still not fully understood (Bennett and Leyser, 2014). Cellular auxin levels have been shown to be perceived by multiple auxin receptor/coreceptor systems, one of which is the well characterized nucleus-localized S-PHASE KINASE-ASSOCIATED PROTEIN1–CULLIN1–F-BOX

Abbreviations: ABP1, Auxin Binding Protein1; BFA, brefeldin A; ER, endoplasmic reticulum; GFP, Green Fluorescent Protein; NAA, 1-naphthaleneacetic acid; PIN, Pin-formed protein; PM, plasma membrane RIC, ROP-interactive CRIB motif-containing protein; RFP, red fluorescent protein.

(SCF) TRANSPORT INHIBITOR RESPONSE1 (TIR1) coreceptor system that mediates auxin-dependent transcription (Ruegger *et al.*, 1998; Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Calderón Villalobos *et al.*, 2012). In contrast, the putative receptor AUXIN-BINDING PROTEIN1 (ABP1) is believed to be associated mainly with fast non-transcriptional auxin effects (Rück *et al.*, 1993; Steffens *et al.*, 2001; Xu *et al.*, 2010; Robert *et al.*, 2010; Chen *et al.*, 2014).

Although ABP1 is mostly localized at the endoplasmic reticulum (ER) (Jones and Herman, 1993), the physiological roles have been characterized for a small ABP1 fraction at the cell surface (Grones and Friml, 2015). This cell surface activity of ABP1 has recently been reinforced by the identification of the plasma membrane (PM)-localized transmembrane receptor-like kinase family (TMK) as a docking station for ABP1 that transmits the signal from the extracellular space to the cytosol (Dai *et al.*, 2013; Xu *et al.*, 2014). Cytosolic downstream components include small Rho of plants (ROP) GTPases and their interacting ROP-interacting CRIB motif-containing (RIC) proteins that can be activated in an auxin-dependent manner, translocating to the PM vicinity as a consequence (Xu et al., 2010, 2014).

Despite the proposed crosstalk between the TIR1 and ABP1 pathways in gene expression regulation (Tromas *et al.*, 2013; Paque *et al.*, 2014), ABP1 typically mediates fast, non-transcriptional effects. Early studies have demonstrated that APB1 is involved in the rapid regulation of the membrane potential and ion fluxes at the PM and that it mediates the auxin-induced cell swelling and cell elongation (Gehring *et al.*, 1998; Steffens *et al.*, 2001; Yamagami *et al.*, 2004). The recently identified cellular roles for ABP1 include cell morphogenesis, cytoskeleton rearrangement (Xu *et al.*, 2010; Chen et al., 2012, 2014; Nagawa *et al.*, 2012), and clathrin-mediated endocytosis (Robert *et al.*, 2010). At the tissue level, ABP1 acts as a coordinator of cell division and expansion (Braun *et al.*, 2008; Tromas *et al.*, 2009). The developmental roles of ABP1 are far less clear. *ABP1* is a single-copy gene in *Arabidopsis thaliana* and two allelic independently identified loss-of-function alleles have been reported to be embryo lethal (Chen *et al.*, 2001; www.seedgenes.org/SeedGeneProfile?geneSymbol=ABP+1). However, two recently reported new *abp1* null alleles are not embryo lethal (Gao *et al.*, 2015). In support of the importance of the ABP1 for different cellular and developmental processes, a number of independent conditional and/or constitutive loss- and gain-of-function alleles show postembryonic developmental defects in processes, such as leaf epidermal cell interdigitation, root meristem maintenance, shoot and root organogenesis, and leaf vascular tissue development (David *et al.*, 2007; Braun *et al.*, 2008; Tromas *et al.*, 2009; Robert *et al.*, 2010; Rakusová *et al.*, 2015). Thus, the issue of the full knockout mutant phenotypes remains unresolved.

Decades of biochemical studies clearly established a high-affinity binding of auxin to ABP1 (Napier and Venis, 1995; Napier *et al.*, 2002) and revealed a protein structure including an auxin-binding pocket (Woo *et al.*, 2002). However, the exact cellular and developmental roles of auxin binding to ABP1 remain to be unravelled.

Here, we addressed this issue by targeted mutagenesis of the auxin-binding pocket. A number of different *abp1* mutants with amino acids substitutions were evaluated for their importance during ABP1-mediated cellular and developmental processes showing that the auxin-binding pocket plays a crucial role for ABP1 function.

Materials and methods

Plant material and growth conditions

Seedlings of *Arabidopsis thaliana* (L.) Heyhn., accession Columbia (Col-0), were vernalized for 2 d in the dark at 4°C and grown on vertical half-strength Murashige and Skoog (0.5 MS) plates containing with 1% sucrose and 0.8% agar at 18°C in a 16 h light/8 h dark photoperiod for 5 d. Liquid 0.5 MS medium was used for all chemical treatments. The transgenic lines and constructs used have been described previously: *35S::ABP1-GFP* construct (Robert *et al.*, 2010), *35S::ABP1* line (Xu *et al.*, 2014), *abp1-1* heterozygous mutant line (Chen *et al.*, 2001), and *35S::PIN1-RFP* construct (Robert *et al.*, 2010).

Drug treatments

Five-day-old seedlings were incubated with the following chemicals: 25 µM BFA dissolved in DMSO (Sigma-Aldrich) for 90 min, 10 µM NAA dissolved in DMSO (Sigma-Aldrich) for 30 min for pretreatments and with 25 µM BFA/10 µM NAA for 90 min for cotreatments, 50 µM PEO-IAA dissolved in DMSO for 30 min (Hayashi *et al.*, 2008) for pretreatments and 25 µM BFA/50 µM PEO-IAA for 90 min for cotreatments, 10 µM auxinole dissolved in DMSO for 30 min (Hayashi *et al.*, 2012) for pretreatments and 25 µM BFA/10 µM auxinole for 90 min for cotreatments, and 10 µM NAA for 4 h. In control treatments, equal amounts of solvent were used.

Construct preparation and transformation

The *35S::ABP1g, shortABP1::ABP1g*, *longABP1::ABP1g*, and *longABP1::ABP1g::ABP1-3'UTR* plasmids were constructed with the Gateway cloning technology (www.invitrogen.com). Genomic fragments of the *ABP1* gene were cloned into the donor vector *pDONR221* and the *shortABP1* promoter (708 bp upstream of ATG) and *longABP1* promoter (1585 bp upstream of ATG; adapted from Klode *et al.*, 2011) were cloned into the *pDONRP4P1r* vector. The *35S::ABP1g* construct was created by recombining *ABP1* in *pDONR221* into *pB7GW2*. The expression clones containing the native ABP1 promoter variants were generated by recombining these fragments into the expression vector *pB7m24GW,3*. *35S::RIC4-RFP* was constructed by recombining the *RIC4* genomic fragment from *pDONR221* into the *p2GWR7* destination vector.

The *ABP1-M1X* and *ABP1-M2X* constructs were made by substituting the modified fragment by classical cloning via *Sac*I and *Pas*I in the *35S::ABP1, shortABP1::ABP1*, *longABP1::ABP1*, *longABP1::ABP1::ABP1-3'UTR*, and *35S::ABP1GFP* vectors. The mutations R59K, L62V, Q83D, T91V, P92L, F186L, W190Y, R59K/L62V, T91V/P92L, F186L/W190Y, V101A, F127L, P138L, and Q193D in ABP1 were done by site-direct mutagenesis PCR with modified primers.

The resulting constructs were transformed into *Arabidopsis* (Col-0) and into *abp1/ABP1* heterozygous plants by floral dipping in *Agrobacterium tumefaciens* liquid cultures. Transformants were selected on phosphinothricin-containing plates. From each construct, at least three independent lines with similar expression levels were chosen and analysed. All primers and prepared fragments used for cloning are summarized in Supplementary Table S1.

Genotyping and qRT-PCR

The ABP1 T-DNA insertion line was genotyped with the right border primer for the Wisconsin T-DNA lines in combination with the ABP1-specific primers (Supplementary Table S1). To test the *ABP1* gene expression level in the T-DNA insertion lines and other transformants, qRT-PCR was performed. Five-day-old seedlings were harvested, RNA extracted, and cDNA synthesized. Of a 1:10 cDNA dilution, 5 µl was used in a 20 µl qRT-PCR reaction containing 1× DyNAmoTM SYBR® Green Mastermix (FINNZYMES). Real-time PCR reactions were run in triplicate. Gene expression was calculated with the 2-ΔΔCT method (Livak and Schmittgen, 2001). Tubulin was used as endogenous control for the relative quantification of the *ABP1* gene expression. Primers used for qRT-PCR are summarized in Supplementary Table S1.

Immunodetection and microscopy

Arabidopsis roots were analysed by immunofluorescence as described in Sauer *et al.* (2006). The anti-PIN1 antibody (1:1000) (Benková *et al.*, 2003), the anti-PIN2 antibody (1:1000) (Abas *et al.*, 2006), and the anti-CHC antibody (1:400) (Agrisera) were used as well as the fluorochrome-conjugated secondary antibodies Alexa488 and the anti-rabbit-Cy3 (1:600) (Dianova). Live-cell microscopy was done on a Zeiss 710 confocal microscope and pictures were analysed by ImageJ (ImageJ; National Institutes of Health; http://rsb.info.nih.gov/ij). BFA bodies and CHC were quantified by measuring the PM and intracellular signal ratio and lateralization by measuring the signal on the basal or apical membranes and by comparing it to the signal on lateral membranes.

Transient transformation of tobacco BY-2 cells

Ten millilitres from 3-day-old liquid cell culture, was harvested on filter paper by vacuum filtration and kept on solid BY-2 medium. The cells were transformed via particle bombardment with a PDS-1000/He biolistic system (Biorad) according to the manufacturer's recommendations. To coat the gold particles with DNA, 2 ml of plasmid DNA (0.05 mg/ml of each to be transformed construct) was added to 6.25 ml of 1.6 mm-diameter gold particles and the suspension was supplemented with 2.5 ml spermidine (0.1 M stock solution) and 6.25 ml $CaCl_2$ (2.5 M stock solution). The particles were pelleted by centrifugation and washed twice with 70% and 100% ethanol. The pellet was suspended in 10 ml of 100% ethanol. Cells were bombarded under a pressure of 1100 psi with the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). After transformation, 1 ml of auxin-free medium (mock) or medium enriched with 10 µM NAA was added to the cells. The plates were sealed with parafilm and kept in the dark for 18 h at 25°C. Samples were imaged via confocal microscopy (Zeiss 710) and analysed as described (Robert *et al.*, 2010). Experiments were done in triplicate for all prepared constructs and each time between 15–20 cells were analysed. Cells were divided into three groups based on the number of intracellular particles containing PIN proteins: 0–3, 'no internalization'; 4–10, 'weak internalization'; >10, 'severe internalization'.

Phenotypical analysis

Root length, hypocotyl length and lateral root density were measured by the Java-based ImageJ application (National Institutes of Health; http://rsb.info.nih.gov/ij). At least 20 seedlings were measured in three independent experiments, giving the same statistically significant results. The statistical significance was evaluated with Student's T-test.

The pavement cell shape from *Arabidopsis* cotyledons was imaged directly on a confocal microscopy (Leica SP2) after the cotyledons had been treated with propidium iodide for visualization of the cell outline. At least 10 cotyledons and 30 cells from each were analysed from each line and the number of lobes and the cell size were measured in ImageJ. The experiment was repeated twice.

RIC4 activation assay

Changes in the RFP-RIC4 localization to the PM were monitored in isolated protoplasts. Protoplasts were isolated from an *Arabidopsis* suspension culture (PSB-L) as described in Goossens and Pauwels (2013). Four micrograms of the *35S::RFP-RIC4* construct, *35S::ABP1GFP,* or particular *abp1* mutant variant was introduced into protoplasts by PEG-mediated transformation (Mathur and Koncz, 1998). Typically, ~70% of the protoplasts were transformed and cells showing both signals were imaged by a Zeiss 710 confocal microscope. Evaluation was done based on the localization of the RIC4-RFP signal, thus dividing cells into two groups with the signal either at the PM or in the cytosol. From each transfection, 10–15 cells showing both signals were evaluated and the experiment was repeated three times.

Results and discussion

Design of mutant ABP1 variants defective in auxin binding pocket

The auxin-binding protein ABP1 was identified decades ago in different plant species (Hertel *et al.*, 1972, Löbler and Klambt, 1985) with subsequent characterization of its structure by crystallization and detection of the auxin-binding pocket (Chen *et al.*, 2001, Woo *et al.*, 2002). Nevertheless, little is known about the significance of the auxin binding for the cellular functions and developmental roles of ABP1.

To address this question, a series of mutations were generated. Single amino acids important for the formation of the auxin-binding pocket (Fig. 1A) were mutated based on the known crystal structure (Woo *et al.*, 2002). Given the conserved stability of the β-barrel fold (Woo *et al.*, 2002) and the selected amino acid substitutions, the overall protein structure would probably not be compromised. As the metal core seems to be most important for the auxin binding (Woo *et al.*, 2002), two disrupting ABP1 variants, ABP1-H59A (ABP1-M1X) and ABP1-H59A/H61A (ABP1-M2X) were prepared (Fig. 1B). The mutation in the ABP1-M1X variant targets the same amino acid as that already described in the *abp1-5* mutant allele (Xu *et al.*, 2010).

Additionally, we mutagenized seven largely hydrophobic amino acids that play a role in the stabilization of the indole or aromatic rings of the auxin molecule. Seven single (R24K, L27V, Q48D, T56V, P57L, F148L and W152Y) and three double (R24K/L27V, T56V/P57L, and F148L/W152Y) mutants (Fig. 1B) were generated to evaluate their role in the ABP1 activity and to test the hypothesis that auxin binding is necessary for the ABP1 activity. As a negative control, four random amino acids outside the binding pocket were chosen and mutation variants were prepared (ABP1-V66A, ABP1-F92L, ABP1-P103L and ABP1-Q155D). These *abp1* mutant variants were then evaluated for their impact on the cellular and developmental roles of ABP1.

Downstream ROP2/RIC4 activation by abp1 auxin binding pocket mutants

Next, we tested the ability of the auxin-binding pocket mutants to activate downstream signalling processes. As the Rho GTPases, ROP2 and ROP6, have been shown to

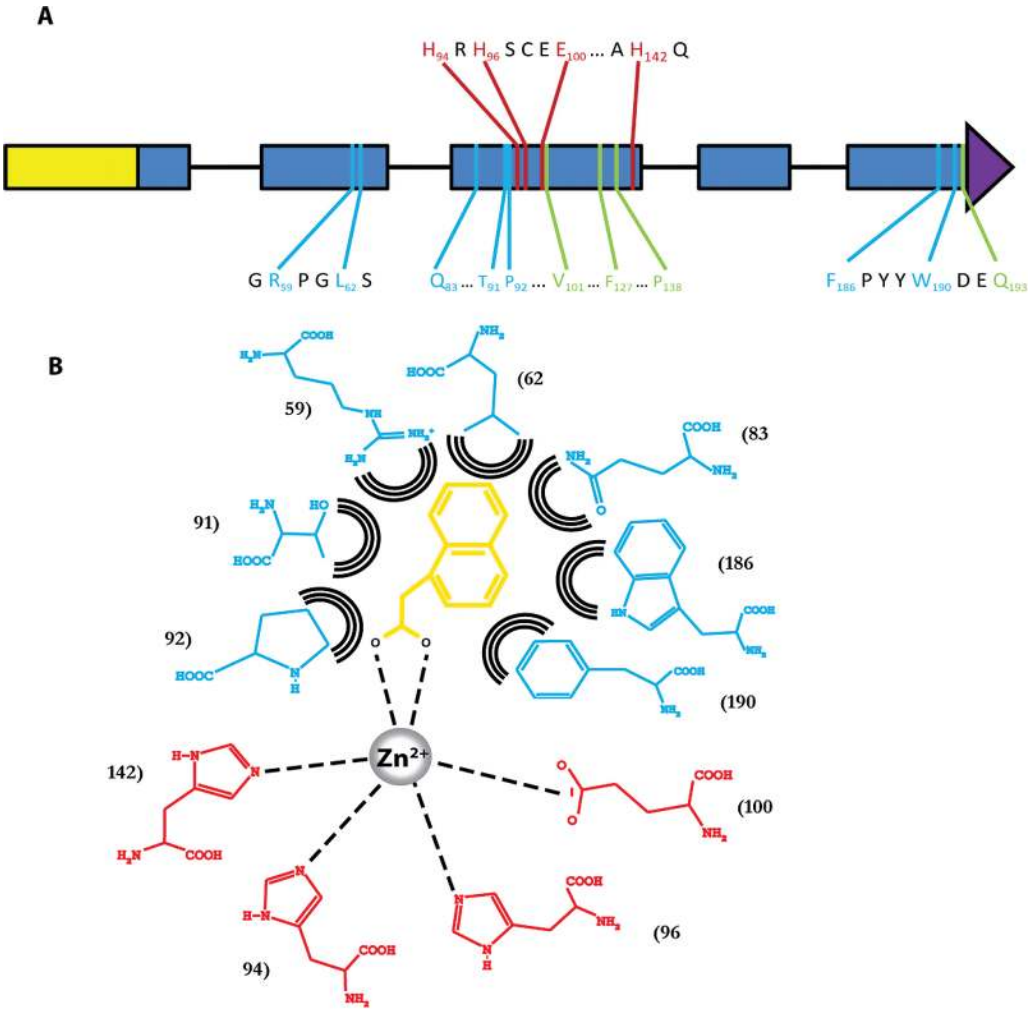

Fig. 1. Structure of the ABP1 auxin-binding pocket. (A) Schematic structure of ABP1 with amino acid positions that participate in the formation of the binding pocket. Residues interacting with the zinc ion are shown in red, hydrophobic residues stabilizing the aromatic ring system of active auxin are shown in blue. Positions of amino acids that were used for preparation of neutral mutations are indicated in green. The N-terminal signal sequence for delivering ABP1 to the apoplast is indicated in yellow and the C-terminal ER retention motif KDEL is indicated in purple. (B) Simplified representation of the interactions between amino acids from the ABP1-binding pocket and the auxin molecule. Numbering is based on the *Arabidopsis* protein sequence.

be activated within a few minutes by the ABP1-dependent auxin signalling (Xu *et al.*, 2010) leading to translocation of the interacting partners, RIC4 and RIC1, from the cytosol to the PM, *Arabidopsis* protoplasts were cotransfected with *35S::RIC4-RFP* and with the corresponding *35S::ABP1-GFP* constructs possessing different mutations. Of the protoplasts transfected with the wild-type ABP1, 90% showed no RIC4 activation, manifested by the RFP signal remaining predominantly intracellular. However, after short auxin treatment (5 min with 1 µM NAA), RIC4 was activated and translocated to the PM (Xu *et al.*, 2010) (Fig. 2A, B, E).

In the *abp1* auxin binding mutant variants, RIC4 activation was highly reduced, particularly in the ABP1-M1X and ABP1-M2X variants, in which up to 45% and 70% of the cells for M1X and M2X, respectively, still had a RIC4 cytosolic localization (Fig. 2C, D, E). In the other *abp1* mutant variants with mutations in hydrophobic amino acids that are responsible for the interaction with indole or aromatic rings of auxin molecules, we observed a slight increase of 20% in the proportion of cells with a cytosolic RIC4 localization, but never as high as in ABP1-M1X or ABP1-M2X (Fig. 2F, G, H). The neutral mutant variants ABP1-V66A, ABP1-F92L, ABP1-P103L, and ABP1-Q155D did not differ in the RIC4 translocation when compared to wild-type ABP1 protein.

Hence, the mutations in the auxin-binding pocket interfere with the ABP1 capability to activate/translocate the RIC4 protein. The most important amino acid residues are those that reside in the metal core. Seven mostly hydrophobic amino acids interacting with indole or aromatic rings of auxin molecules play a rather minor role in this process.

Endocytosis inhibition by abp1 *mutant variants in BY-2 cells*

Regulation of the clathrin-mediated endocytosis is one of the rapid auxin effects ascribed to the ABP1 function (Robert *et al.*, 2010; Čovanová *et al.*, 2013). Auxin, via ABP1, inhibits endocytosis of some PM-residing proteins, including internalization of PIN-FORMED (PIN) auxin efflux carriers (Petrášek *et al.*, 2006). By this mechanism, auxin is supposed

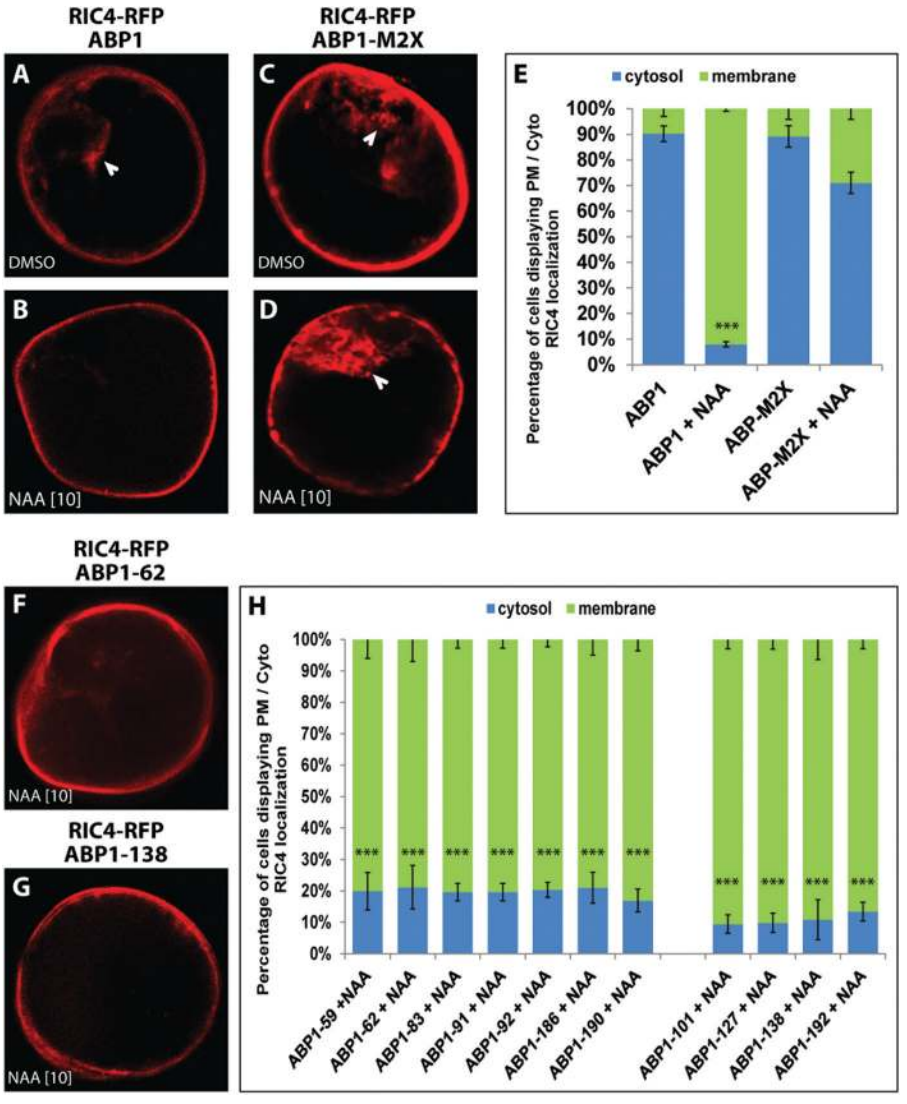

Fig. 2. Activation of RIC4 by *abp1* mutant variants. (A–D) Cotransfection of *35S::RIC4-RFP* (red) with the particular *35S::ABP1-GFP* variant in *Arabidopsis* protoplast cells. (A, C) In the control situation, RIC4-RFP is localized in the cytosol. (B) After auxin treatment, ABP1-GFP activated RIC4-RFP that translocated to the PM. (D) The ABP1-GFP-M2X mutant variant mostly failed to activate RIC4-RFP in the presence of auxin. (E) Percentage of cells showing a cytosolic or PM localization of RIC4-RFP if cotransfected with *35S::ABP-GFP* or *35S::ABP1-GFP-M2X*. Representative pictures of activated RIC4-RFP by the *abp1* mutant variant with mutation in (F) hydrophobic amino acids and with (G) neutral mutation. (H) Percentage of cells showing cytosolic or PM localization of RIC4-RFP if cotransfected with mutant variants containing mutation in hydrophobic amino acids or containing mutation in neutral amino acids outside of binding pocket. Three independent experiments were carried out and at least 15 protoplasts for each were counted. Arrowheads indicate the RIC4-RFP cytosolic localization. ABP1-M2X and ABP1-59, -62, -83, -91, -92, -186, -190 are mutations in the auxin-binding pocket whereas ABP1-101, -127, -138, -192 represent neutral mutations. Student's T-test was calculated for the comparison of number of cells showing cytosolic RIC4 localization between non-treated and treated samples of each construct (*** $P<0.001$). Error bars represent SE.

to regulate its own transport out of cells (Paciorek *et al.*, 2005).

For evaluation of the effect of the *abp1* auxin binding mutations on PIN internalization, we cotransfected Bright Yellow 2 (BY-2) tobacco (*Nicotiana tabacum*) suspension-cultured cells with PIN1 (*35S:PIN1-RFP*) together with the *35S::ABP1-GFP* constructs containing different mutations, and analysed the amount of cells showing PIN1 internalization. Wild-type ABP1 promotes endocytosis of PIN1 from the PM and, consistently, more than 60% of cells had severely internalized PIN1. This process was largely inhibited by addition of auxin (10 µM NAA), when over 80% of cells showed no or very weak internalization (Fig. 3C, D, G). When BY-2 cells were cotransfected with the ABP1-M2X construct, the PIN1-RFP internalization was promoted to the same extent as with wild-type ABP1 construct, however, auxin treatment was completely ineffective in inhibiting PIN1 internalization (Fig. 3E–G). Similarly, we analysed other *abp1* variants such as ABP1-M1X or versions bearing mutations in the hydrophobic residues. All these mutant variants promoted PIN1 internalization but showed decreased sensitivity to the inhibitory auxin effect, albeit not complete auxin resistance as observed for the ABP1-M2X construct (Supplementary Fig. S1A, B, D). In contrast, none of the neutral mutations outside the binding pocket showed changes in the PIN1 internalization rate after auxin treatment when compared to the control (Supplementary Fig. S1A, C, E). Notably,

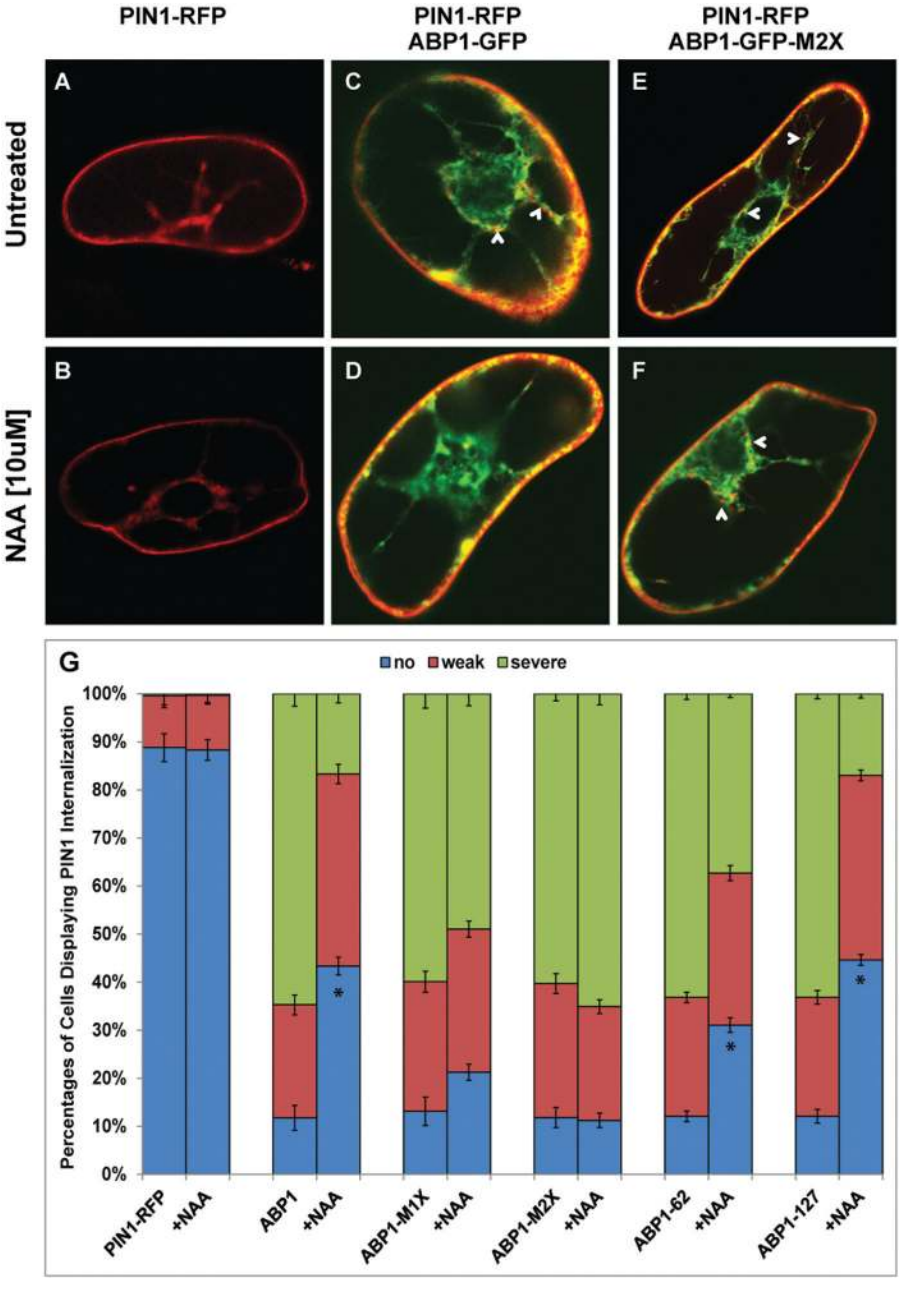

Fig. 3. Inhibition of endocytosis in tobacco BY-2 cells by *abp1* mutant variants. (A, B) Transfection of tobacco BY-2 cells with PIN-RFP cannot be internalized in the presence of auxin without ABP1 and localize to the PM and ER. (C–F) Cotransfection of tobacco BY-2 cells with *35S:PIN1-RFP* (red) and the particular *35S::ABP1-GFP* variant (green). (C) ABP1-GFP-dependent (green) promotion of PIN1-RFP (red) internalization is significantly reduced after auxin treatment (D). (E, F) Internalization of PIN1-RFP cannot be inhibited by auxin in the presence of the ABP1-GFP-M2X mutant variant. Three independent experiments were carried out and at least 20 cells for each were counted. Arrows indicate PIN protein internalization. (G) Percentage of cells displaying severe (green), mild (red), or not detectable (blue) PIN1-RFP internalization. ABP1-M1X, ABP1-M2X and ABP1-62 are mutation in auxin binding pocket whereas ABP1-127 represents a neutral mutation. Student's T-test was calculated for the comparison of number of cells showing no PIN internalization between non-treated and treated samples of each construct (*, $P<0.001$). Error bars represent SE.

only a very small amount of PIN1-RFP was internalized in the absence of the ABP1 cotransfection suggesting the importance of both auxin-bound and auxin-unbound ABP1 for endocytosis (Fig. 3A, B, G).

Altogether, the ABP1 with or without the intact auxin-binding pocket was crucial for PIN internalization but the auxin-mediated inhibitory effect on this process required the intact auxin-binding pocket, the metal core being the most critical part.

Complementation of the abp1 *knockout mutant*

Next we planned to express the *abp1* mutant variants in *Arabidopsis* to complement the *abp1-1* knockout mutant allele, which has been reported to cause slow proliferation, reduced cell elongation, cell division and embryo lethality (Chen *et al.*, 2001). The phenotypes of the *abp1-1* mutant were reported to be rescued by the *35S::ABP1* overexpression construct. We used the same approach and via *Agrobacterium*-mediated transformation we introduced several different constructs

into the *abp1*/ABP1 heterozygous mutant. However, we did not achieve an *abp1-1* complementation with any of our constructs, i.e. *35S::ABP1*, *35S::ABP1-GFP*, *35S::ABP1-M2X or 35S::ABP1-GFP-M2X*, since by genotyping we still confirmed the presence of a functional wild-type *ABP1* allele in all progenies (Supplementary Fig. S2).

In our subsequent attempt, the native ABP1 promoter was used to bring the expression to a level similar to that *in planta*. We used two versions of the native promoter, a long one (1585 bp; adapted from Klode *et al.*, 2011) and a short one (708 bp, until the start codon of the next gene). We screened more than three independent lines with an expression lower than, higher than, or equal to that of the wild-type. None of these variants (with or without a tag or mutation) were able to rescue the mutant phenotypes of the *abp1-1* allele. The same negative result was observed when the native promoter with the genomic ABP1 sequence and the 3′-untranslated region were cloned together in one piece and transformed into the *abp1*/*ABP1* heterozygous line (Supplementary Fig. S2). Taken together, we were unable to complement the *abp1-1* phenotypes with any prepared construct, which precluded us to analyse the importance of auxin-binding pocket for the ABP1 developmental roles *in planta* in the absence of the wild-type *ABP1* allele.

Endocytosis inhibition by abp1 *mutant variants in* Arabidopsis *roots*

To test the effect of auxin-binding mutations on endocytosis in *Arabidopsis*, we introduced all the above-mentioned constructs into the *Arabidopsis* Col-0 background via *Agrobacterium*-mediated transformation. Most experiments were carried out with the *ABP1-M2X* overexpression line because this construct showed the most severe auxin insensitivity in protoplast and BY-2 assays. Three independent lines with expression levels similar to that of *35S::ABP1* (Rakusová *et al.*, 2015) were chosen for the analysis. As a control, the *35S::ABP1* line (Xu *et al.*, 2014; Rakusová *et al.*, 2015) was used with the corresponding *ABP1* expression level with phenotypes similar to the *35S::ABP1-GFP* line (Robert *et al.*, 2010; Xu *et al.*, 2014; Rakusová *et al.*, 2015).

The fungal toxin Brefeldin A (BFA) is a useful tool to investigate PIN trafficking and endocytosis. BFA treatments lead to the accumulation of internalized PIN proteins in pronounced intracellular aggregates—so-called BFA bodies, because BFA inhibits preferentially the recycling of PIN proteins to the PM (Geldner *et al.*, 2001; Kleine-Vehn *et al.*, 2008). PIN protein immunodetection in the wild-type treated with 25 µM BFA led to accumulation of PIN proteins in BFA bodies.

Auxin can inhibit this internalization and stabilizes PIN proteins at the PM (Paciorek *et al.*, 2005) (Fig. 4A, D, G). In *35S::ABP1* roots, the BFA-induced PIN internalization was slightly higher than of the wild-type. This difference was less pronounced as compared to previous work (Robert *et al.*, 2010) because a different quantification method was used. Instead of counting the number of BFA bodies per cell (Robert *et al.*, 2010), the signal at the PM and in the intracellular part was measured. As shown before (Robert *et al.*, 2010), internalization of PINs to BFA bodies was almost completely inhibited by auxin (10 µM NAA) in both the wild-type and the *35S::ABP1* line (Fig. 4B, E, G). In contrast, the inhibitory effect of auxin on the BFA body formation was much less pronounced in the *35S::ABP1-M2X* line, as manifested by the presence of pronounced PIN1-containing BFA bodies (Fig. 4D, F, G).

In addition, we tested PEO-IAA (Hayashi *et al.*, 2008), an auxin analogue that inhibits endocytosis, but does not activate auxin-mediated transcriptional responses (Robert *et al.*, 2010). PIN1 immunodetection in the wild-type and *35S::ABP1* overexpression seedlings cotreated with PEO-IAA and BFA inhibited PIN1 internalization similarly to auxin (Supplementary Fig. S3A, B, G). In contrast, *35S::ABP1-M2X* seedlings showed a persistent presence of BFA bodies after cotreatment with PEO-IAA and BFA (Supplementary Fig. S3C, G). Similar observations were made when seedlings were cotreated with BFA and auxinole (Hayashi *et al.*, 2012), which is an auxin analogue with structure and function similar to those of PEO-IAA (Supplementary Fig. S3D–H). These results revealed that wild-type plants expressing *35S::ABP1-M2X* mutant variant are much less sensitive to the inhibitory effect of auxin and its analogues on PIN internalization.

Clathrin association to the PM and TGN by abp1 *mutant variants in* Arabidopsis *roots*

PIN proteins are internalized by the endocytic machinery that involves the coating protein clathrin (Dhonukshe *et al.*, 2007). ABP1 acts as a positive regulator in clathrin recruitment and association with the PM and thus promotes endocytosis (Robert *et al.*, 2010; Chen *et al.*, 2012; Wang *et al.*, 2013). We investigated the auxin effect on levels of the membrane-associated clathrin heavy chains (CHCs) by using anti-CHC antibodies. In the wild-type, *trans*-Golgi network/early endosomes (TGN/EEs) and PM-associated CHC signals increased after auxin treatment (10 µM NAA). The intensity of the CHC signal at the PM and TGN/EE was 50% higher than that of the mock controls (Fig. 4H, K, N).

Interestingly, the CHC signal at the PM and TGN/EE increased in the *35S::ABP1* overexpression line already without auxin treatment and the increase after the auxin treatment was not as pronounced as in the wild-type (Fig. 4I, L, N). In contrast, in the *ABP1-M2X* overexpression line, the CHC signal did not increase and the line was also resistant to auxin treatments (Fig. 4J, M, N).

Altogether, our results suggest that ABP1 regulates the clathrin heavy chain association with the PM and TGN/EEs and that the ABP1 auxin-binding site is important for this role.

Auxin-mediated PIN polarization by abp1 *mutant variants in* Arabidopsis *roots*

Another prominent cellular auxin effect is the change in PIN polarity (Sauer *et al.*, 2006) that seems to require both TIR1 and ABP1 signalling (Rakusová *et al.*, 2015) as manifested

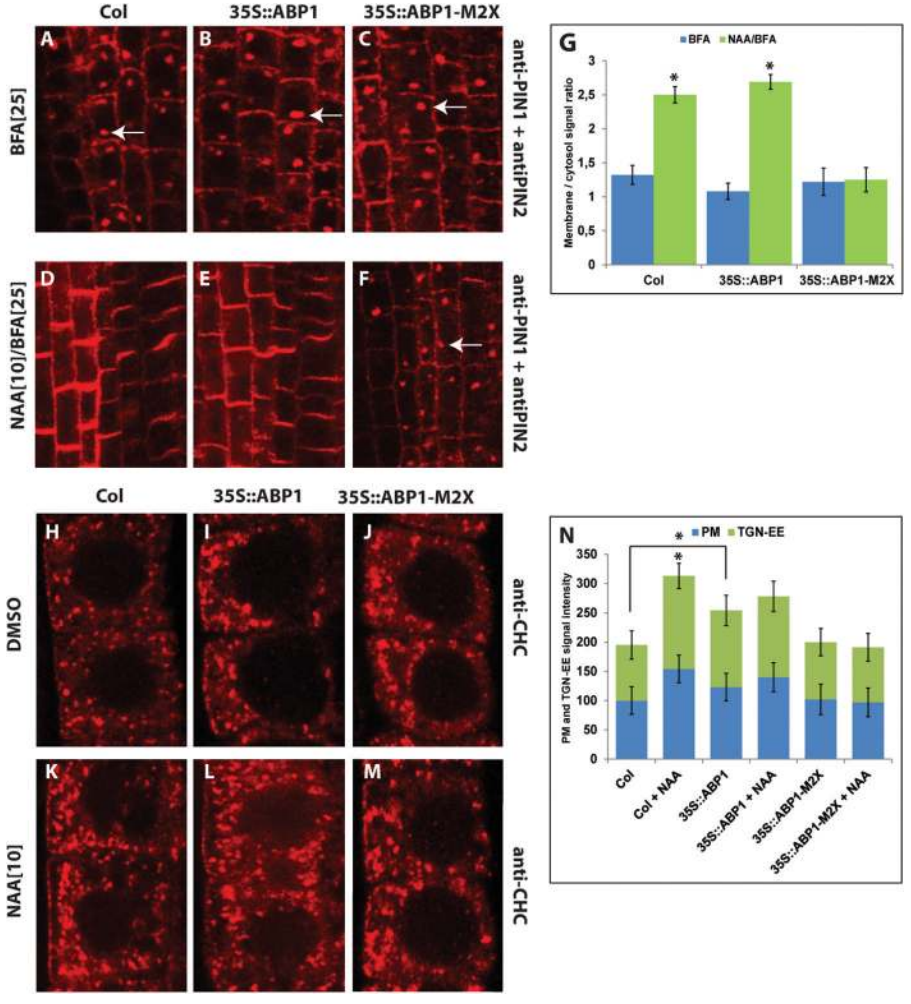

Fig. 4. Inhibition of endocytosis in *Arabidopsis* roots by *abp1* mutant variants. (A–G) BFA-induced internalization of PIN1 and PIN2 leads to the formation of BFA bodies (A–C) that can be inhibited by auxin pretreatment (D, E). (F) In *35S::ABP1-M2X* seedlings, auxin cannot inhibit the BFA body formation. (G) Quantification of the signal ratio at the PM and in the cytosol. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (*, $P<0.01$). Error bars represent SE. (H–N) Auxin effects on the clathrin association with PM and TGN/EE. Increase of the signal in wild-type seedlings after treatment with (K) 10 μM auxin compared to (H) the solvent-treated control. In *35S::ABP1* seedlings, (I) signal increased even without treatment and (L) increased slightly after auxin. *35S::ABP1-M2X* seedlings showed (J) no increase with solvent and (M) resistance toward auxin treatment. (N) Quantification of the signal intensity of clathrin associated with PM or TGN/EE. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (*, $P<0.01$). Error bars represent SE. Three independent experiments were carried out and at least 50 cells for each were counted.

by the basal-to-outer lateral relocation of PIN2 in the cortex and basal-to-inner lateral relocation of PIN1 in the endodermis of *Arabidopsis* roots after auxin treatment (10 μM NAA) (Sauer *et al.*, 2006). In the wild-type, lateralization of PIN proteins occurred after 4 h of auxin treatment; the lateral-to-basal signal ratio of PIN1 in endodermis increased up to 50% and up to 25% for PIN2 in the cortical cell layer (Fig. 5A, D, G, H).

The ABP1 involvement is noticeable by the PIN lateralization in the *ABP1* overexpression line already without any auxin treatment (Rakusová *et al.*, 2015). This phenotype could not be enhanced by additional auxin treatments (Fig. 5B, E, G, H). In contrast, overexpression of *ABP1-M2X* did not cause any PIN lateralization that occurred only after auxin treatment (Fig. 5C, F, G, H), possibly due to the presence of the wild-type *ABP1* allele in the Col-0 background and also due to the involvement of the second auxin signalling pathway SCF^{TIR1} (Sauer *et al.*, 2006). Hence, the auxin-mediated PIN polarization requires ABP1, and the ability of auxin to bind the ABP1 molecule is a crucial part of this regulation.

Morphological phenotypes of plants expressing the auxin binding abp1 *mutants*

It has been proposed that ABP1 takes part in many important developmental processes (Braun *et al.*, 2008; Tromas *et al.*, 2009; Xu et al., 2010, 2014; Chen *et al.*, 2014). Among others, it mediates the auxin-dependent pavement cell interdigitation (Xu *et al.*, 2010). The number of lobes in pavement cells increased up to 25% after auxin treatment (20 nM NAA) in the wild-type or in seedlings of the *35S::ABP1* overexpression line. In contrast, in the *abp1* auxin-binding mutant (ABP1-M2X), the number of lobes in pavement cells did not change even after auxin treatment (Fig. 6A–E).

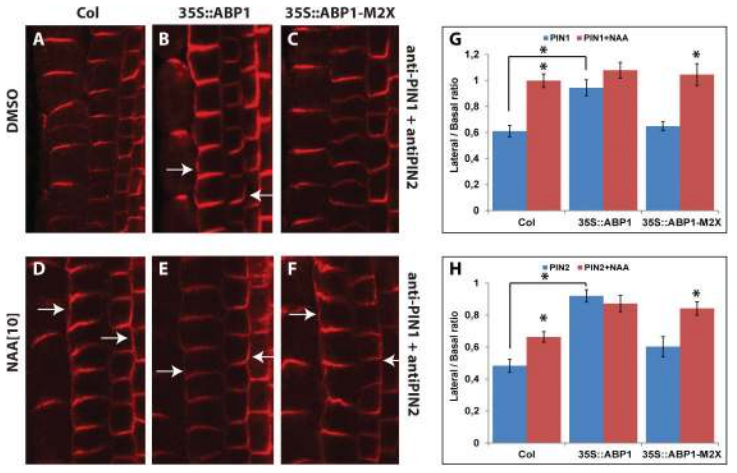

Fig. 5. Auxin-dependent PIN polarization by *abp1* mutant variants. (A–F) Lateralization of PIN1 and PIN2 in *Arabidopsis* roots after 4 h of auxin treatment (10 μM). *35S::ABP1* seedlings exhibited lateralization when treated with (B) solvent when compared to (A) the wild-type and (C) *35S::ABP1-M2X.* Auxin induced lateralization of PIN proteins in (D) the wild-type and (F) *35S::ABP1-M2X* but did not have a pronounced phenotype in (E) *35S::ABP1.* (G, H) Quantification of auxin-dependent lateralization of the (G) PIN1 and (H) PIN2 proteins. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (*, $P<0.01$). Error bars represent SE. Experiments were carried out independently three times and at least 50 cells per experiment were counted.

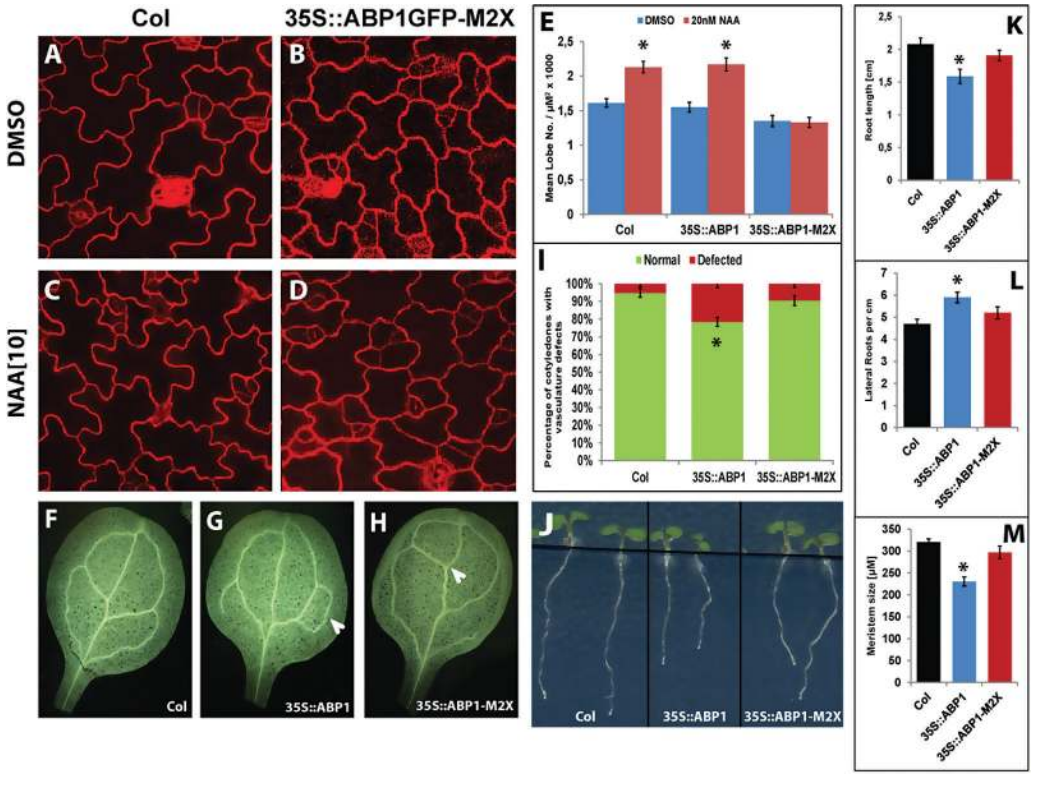

Fig. 6. Morphological defects in lines expressing *abp1* auxin binding mutants. (A–D) Induction of pavement cells interdigitation by auxin treatment (20 nM NAA) appeared in (C) wild-type seedlings compared to (A) the untreated control, but not in (B, D) *35S::ABP1-M2X* seedlings. (E) Quantification of mean lobe number of leaf pavement cells. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (*, $P<0.01$). Error bars represent SE. Experiment was carried out twice, each time 100 cells per line were counted. Error bars represent SE. (F–H) Cotyledon vasculature defects in (F) wild-type, (G) *35S::ABP1* and (H) *35S::ABP1-M2X* seedlings. (I) Quantification of cotyledon number with vasculature defects. Student's T-test was calculated for the comparison to the control (Col-0) (*, $P<0.05$). Error bars represent SE. (J). Quantification of root length (K), lateral roots (L) and meristem size (M). Student's T-test was calculated for the comparison to the control (Col-0) (*, $P<0.001$). Error bars represent SE.

Seedlings overexpressing the wild-type ABP1 had a reduced root length, decreased root meristem size, increased lateral root number, and defects in cotyledon vasculature when compared to the wild-type (Fig. 6F–M). These lines also showed a decrease in apical dominance, resulting in more branching. All these aberrant phenotypes were significantly reduced in the *35S::ABP1-M2X* overexpression lines. Altogether, these observations suggest that the ABP1 auxin-binding pocket is crucial for performing its gain-of-function developmental roles.

Conclusion

ABP1 has been identified repeatedly from different species as a protein that binds auxin with high affinity and has been suggested as a receptor responsible for mediating a broad range of auxin responses (Bargmann and Estelle, 2014; Grones and Friml, 2015). However, the importance of the auxin binding to ABP1 for its functions has not been assessed until now. To provide insights into this question, we prepared a series of different *abp1* variants containing mutations in the metal core or in the seven hydrophobic amino acids in the binding pocket (Woo *et al.*, 2002). In two assays, activation of the downstream ABP1 signalling components ROP2/RIC4 in *Arabidopsis* protoplasts (Xu *et al.*, 2010) and inhibition of the PIN internalization in tobacco BY-2 suspension cells (Robert *et al.*, 2010), the ABP1-M2X mutant variant targeting the metal core of the auxin-binding site exhibited the most serious defects in the transmitting of the auxin signal. The ABP1-M1X mutant targeting only a single amino acid similar to the known *abp1-5* point mutation (Xu et al., 2010, 2014) showed weaker effects, and mutations in hydrophobic amino acids of the binding site had even milder effects.

Next we wanted to test which aspects of the strong lethal phenotypes of the *abp1* knockout lines (Chen *et al.*, 2001) can be complemented by our *abp1* variants with mutations in the auxin-binding pocket. Unfortunately, we were unable to complement the embryo lethal *abp1-1* allele with any of the wild-type or mutated ABP1 constructs. This is in line with a recent identification of new *abp1* knockout mutants without strong phenotypes suggesting that the embryo lethal phenotype of the *abp1-1* mutant might not be linked with mutation in the *ABP1* locus (Gao *et al.*, 2015).

Given the inability of our ABP1 constructs to complement the reported strong *abp1-1* allele, we focused on analysis of the importance of auxin binding for the gain-of-function phenotypes. The overexpression of the strong ABP1-M2X mutant variant in the wild-type *Arabidopsis* failed to generate cellular gain-of-function phenotypes caused by the overexpression of the wild-type ABP1 protein including promotion of endocytosis along with the increased association of clathrin heavy chain with the PM and TGN/EE. Moreover, ABP1-M2X mutant overexpression rendered these processes largely auxin-insensitive. Similar observations for clathrin-mediated endocytosis have been made for the auxin-mediated lateralization of PIN proteins in roots, which has been shown to depend on both extracellular ABP1 and nuclear TIR1 signalling (Sauer *et al.*, 2006; Rakusová *et al.*, 2015). Following ABP1 overexpression, lateralization of PIN proteins occurred even without auxin treatment and could not be further enhanced by the addition of auxin. In contrast, following ABP1-M2X overexpression, PIN lateralization was visible only after auxin treatment, which shows the importance of auxin sensing for ABP1 in this process. This persistent auxin sensitivity in the ABP1-M2X overexpression line can be explained by the presence of wild-type ABP1 protein from the Col-0 background.

The analysis of conditional *abp1* loss-of-function lines and the weak *abp1-5* allele suggested an important role of ABP1 in several developmental processes including interdigitation of leaf epidermal cells, cotyledon vasculature formation, root growth and root meristem activity and lateral root formation (Braun *et al.*, 2008; Tromas *et al.*, 2009; Xu *et al.*, 2010; Rakusová *et al.*, 2015). In all these processes, overexpression of the wild-type version generates much more pronounced gain-of-function phenotypes than expression of the *ABP1-M2X* version with the strong mutation in auxin binding site.

Altogether, our analyses demonstrate the crucial importance of the ABP1 auxin-binding pocket for the cellular roles of ABP1 in auxin-mediated processes including activation of downstream ROP GTPases, inhibition of endocytosis and repolarization of PIN auxin transporter localization. In addition, the auxin-binding pocket is important for all the *in planta abp1* gain-of-function phenotypes tested. Thus despite the current controversy about the *abp1* knockout mutant phenotypes, our observations clearly support a role for ABP1 in multiple auxin-mediated processes both at the cellular level and *in planta*.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Involvement of seven hydrophobic amino acids on inhibition of endocytosis.

Supplementary Fig. S2. Complementation of *abp1-1* mutant.

Supplementary Fig. S3. Inhibition of PIN endocytosis in *Arabidopsis* by auxin analogues.

Supplementary Table S1. Primers and synthesized fragments used for construct preparation.

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