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ABP1 Mediates Auxin Inhibition of Clathrin-Dependent Endocytosis in *Arabidopsis*

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Summary

Spatial distribution of the plant hormone auxin regulates multiple aspects of plant development. These self-regulating auxin gradients are established by the action of PIN auxin transporters, whose activity is regulated by their constitutive cycling between the plasma membrane and endosomes. Here, we show that auxin signaling by the auxin receptor AUXIN-BINDING PROTEIN 1 (ABP1) inhibits the clathrin-mediated internalization of PIN proteins. ABP1 acts as a positive factor in clathrin recruitment to the plasma membrane, thereby promoting endocytosis. Auxin binding to ABP1 interferes with this action and leads tothe inhibition of clathrin-mediated endocytosis. Our study demonstrates that ABP1 mediates a nontranscriptional auxin signaling that regulates the evolutionarily conserved process of clathrin-mediated endocytosis and suggests that this signaling may be essential for the developmentally important feedback of auxin on its own transport.

Introduction

The plant signaling molecule auxin is an important regulator of plant developmental processes, including embryogenesis, organogenesis, tissue patterning, and growth responses to external stimuli (Santner and Estelle, 2009; Vanneste and Friml, 2009). Current models

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on auxin signaling and action focus on the paradigm that auxin regulates the expression of subsets of genes, thus eliciting different cellular and, consequently, developmental responses. Nuclear auxin signaling involves the F box protein transport inhibitor response 1 (TIR1), which acts as an auxin coreceptor (Kepinski and Leyser, 2005; Dharmasiri et al., 2005a, 2005b; Tan et al., 2007), and downstream Aux/ IAA and ARF transcriptional regulators (Dharmasiri and Estelle, 2004). This pathway controls a remarkable number of auxin-mediated processes, but some rapid cellular responses to auxin are not associated with TIR1-based signaling (Badescu and Napier, 2006; Schenck et al., 2010).

Decades ago, the plant-specific protein AUXIN-BINDING PROTEIN 1 (ABP1) was proposed to be an auxin receptor (Hertel et al., 1972; Löbler and Klämbt, 1985). ABP1 in both monocot and dicot plant species shows physiological affinities toward natural and synthetic auxin ligands (Jones, 1994). ABP1, despite carrying a KDEL-endoplasmic reticulum (ER) retention motif, is secreted to some extent to the extracellular space where it is active (Jones and Herman, 1993; Tian et al., 1995; Henderson et al., 1997). ABP1 is essential for embryogenesis (Chen et al., 2001) and postembryonic shoot and root development (Braun et al., 2008; Tromas et al., 2009) and mediates auxin effect on cell elongation, but the underlying mechanism remains unclear (Jones et al., 1998; Leblanc et al., 1997).

An important regulatory level in auxin action is its differential distribution within tissues (Vanneste and Friml, 2009). Such auxin gradients result from local auxin biosynthesis and directional, intercellular auxin transport (Petrásek and Friml, 2009) that is triggered by a network of carrier proteins (Swarup et al., 2008; Geisler et al., 2005; Petrásek et al., 2006; Vieten et al., 2007; Yang and Murphy, 2009). The directionality of auxin flow depends on the polar plasma membrane distribution of PIN-FORMED (PIN) auxin efflux carriers (Wiśniewska et al., 2006). In addition to PIN phosphorylation that directs PIN polar targeting (Friml et al., 2004; Michniewicz et al., 2007), PIN activity can be regulated by constitutive endocytic recycling from and to the plasma membrane (Geldner et al., 2001; Friml et al., 2002; Dhonukshe et al., 2007). Auxin itself inhibits the internalization of PIN proteins, increasing their levels and activity at the plasma membrane (Paciorek et al., 2005). The molecular mechanism of this auxin effect remains unknown, but it has been proposed to account for a feedback regulation of cellular auxin homeostasis and for multiple auxin-mediated polarization processes (Leyser, 2006). Here, we show that auxin regulation of PIN internalization involves the ABP1-mediated signaling pathway that targets clathrin-mediated endocytosis at the plasma membrane.

Results

Auxin Inhibits PIN Internalization by a Rapid, Nontranscriptional Mechanism

PIN proteins dynamically cycle between the endosomes and the plasma membrane (Geldner et al., 2001; Dhonukshe et al., 2007). Plasma membrane-localized PIN1 rapidly internalizes in response to the vesicle trafficking inhibitor brefeldin A (BFA) (Geldner et al., 2001), and this intracellular PIN accumulation is inhibited by auxins (Paciorek et al., 2005). In addition, auxin mediates with slower kinetics the degradation of PIN proteins (Sieberer et al., 2000; Abas et al., 2006). The auxin effects on PIN internalization and PIN degradation involve distinct mechanisms (Sieberer et al., 2000; Paciorek et al., 2005; Abas et al., 2006). These processes can be largely distinguished by BFA treatments at 25 and 50 μM that inhibit preferentially recycling or also vacuolar targeting for degradation, respectively (Sieberer et al., 2000; Abas et al., 2006; Kleine-Vehn et al., 2008).

We addressed the characteristics of the auxin signaling mechanism for inhibiting PIN internalization. It is experimentally established that the auxin regulation based on nuclear

signaling requires at least ~10–15 min for execution (Badescu and Napier, 2006), whereas auxin inhibited the PIN2-GFP internalization more rapidly (<5 min) (Figures 1A and 1B). This suggests that this process does not involve auxin-dependent regulation of gene expression. Consistently, chemical inhibition of transcription (cordycepine or actinomycin D treatment) (Figure S1 available online) or de novo protein synthesis (cycloheximide treatment) (Figure 1C) does not prevent the auxin-mediated inhibition of PIN internalization.

Auxin Inhibits PIN Internalization by a TIR1-Independent Pathway

To elucidate the molecular mechanism by which auxin inhibits PIN internalization, we first tested the involvement of the TIR1-mediated signaling by genetical or chemical interference with different steps of this pathway. We analyzed (1) the quadruple *tir1/afb* mutant deficient in most of the TIR1/AFB auxin receptors function, (2) dominant lines conditionally expressing the stabilized transcriptional inhibitor IAA17 (*HS::axr3-1*), (3) stabilized mutations in other Aux/IAA-encoding genes (*axr2-1*, *axr3-1*, *shy2-2*, and *slr-1*), and (4) silenced lines for multiple ARFs *(2X35S::miRNA160*), as well as (5) seedlings treated with the proteasome inhibitor MG132 that interferes with auxin-mediated degradation of Aux/IAA repressors (Figures 1D – 1H and Figure S1). These manipulations have all been shown to strongly inhibit TIR1-mediated transcriptional auxin responses (Timpte et al., 1994; Fukaki et al., 2002; Tian et al., 2002; Knox et al., 2003; Dharmasiri et al., 2005b). Moreover, interference with the TIR1 pathway can be visualized (Figures 1I – 1M and Figure S1) by monitoring the activity of the synthetic auxin-responsive promoter DR5, which is an indicator for TIR1-dependent gene expression (Ulmasov et al., 1997). As expected, treatments with different auxins increased the *DR5::GUS* expression in the wild-type root, but following interference with the TIR1 pathway, auxin was ineffective in inducing DR5 activity (Figures 1I – 1M). In contrast, all of these manipulations did not interfere with the auxin inhibition of PIN internalization as monitored by BFA-induced intracellular PIN1 accumulation (Figures 1D – 1H and Figure S1). In addition, the kinetics of the auxin effect on endocytosis in the quadruple *tir1/afb* mutant was indistinguishable from that of the wild-type (Figures 2A – 2L and Figure S2). Together, these findings show that the auxin effect on PIN internalization does not require TIR1-mediated auxin signaling.

This conclusion is seemingly contradictory to a previous report that proposed TIR1 involvement in auxin effect on BFA-induced PIN internalization (Pan et al., 2009). However, given the experimental conditions used (BFA at 50 μM), the Pan et al. report primarily addressed the auxin effect on PIN vacuolar trafficking that, in terms of kinetics and molecular mechanisms involved, is distinct from the regulation of PIN internalization (Figure S2 and Figure S3).

Auxin Effects on Transcription and PIN Internalization Involve Distinct Perception Mechanisms

To independently test whether auxin regulation of gene expression and inhibition of PIN internalization require independent signaling pathways, we tested a number of structural analogs of the natural auxin indole-3-acetic acid (IAA) for both effects. As expected, most analogs tested affected both gene expression and PIN internalization, albeit often at different effective concentrations. Importantly, we also identified auxin-like compounds that were specific for one or the other process only. For example, α-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA) (Figure S3) did not induce the expression of *DR5rev::GFP* reporter (Figure 3C) nor transcription of auxin-inducible genes related to the TIR1-dependent signaling pathway (Figure S3). However, similar to classical auxins, PEO- IAA inhibited the BFA-induced PIN internalization (Figure 3H). In contrast, another auxin analog, 5-fluoroindole-3-acetic acid (5-F-IAA), activated *DR5rev::GFP* already at 5 μM (Figure 3D

and Figure S3) but failed to inhibit PIN internalization, even at 25 μM (Figure 3I). These nonover- lapping effects of compounds structurally related to auxin suggest that auxin perception upstream of either regulation of gene expression or PIN internalization involves distinct auxin-binding sites, confirming independently that auxin utilizes different signaling pathways for mediating these effects.

abp1 Knockdown Lines Have Decreased PIN Internalization

As the effect of auxin on PIN internalization is not mediated by TIR1-dependent signaling, we addressed the possible role of the putative auxin receptor, ABP1 (Jones, 1994; Napier et al., 2002). To test the involvement of ABP1 in PIN1 internalization, we monitored PIN subcellular dynamics in conditional immunomodulation and antisense *abp1* knockdown lines (Braun et al., 2008; Tromas et al., 2009). Following downregulation of ABP1, the intracellular accumulation of PIN proteins in response to BFA treatment was diminished (Figures 4A – 4D and data not shown). Similarly, pulse-labeling and time-lapse monitoring intracellular fluorescence revealed that uptake of the endocytic tracer FM4-64 was clearly reduced in roots of both immunomodulated and antisense *abp1* knockdown lines as compared to the wild-type (Figure S4 and data not shown). In addition, a genetic interaction between *abp1* knockdown lines and *pin* mutants (*pin1-1* or *eir1-1*) was demonstrated by the enhancement of the single mutant phenotypes (Figure S4). Thus, the ABP1 function is required for PIN internalization and overall endocytosis, indicating that ABP1 plays a positive role in regulating endocytosis in plants.

ABP1 Gain-of-Function Alleles Have Increased PIN Internalization

Next, we tested the effect of *ABP1* gain of function on PIN internalization. ABP1 is predominantly located in the lumen of the ER due to a C-terminal ERretention signal (KDEL), but some ABP1 is secreted and has been shown to be closely associated with the plasma membrane (Jones and Herman, 1993; Henderson et al., 1997; Shimomura et al., 1999).

To investigate the potential role of ABP1 outside of the ER lumen, tobacco (*Nicotiana tabacum;* Bright Yellow 2 (BY-2)) suspension-cultured cells were transfected with PIN1 (*35S::PIN1-RFP*) and the *Arabidopsis* ABP1 variant lacking the KDEL ER retention signal (*35S::ABP1$^{\Delta KDEL}$-GFP*). When the full-length ABP1 protein was expressed (*35S::ABP1-GFP*), the PIN1-RFP localized largely to the plasma membrane, similarly to the control experiments (Figures 4E, 4F, and 4H). In contrast, coexpression of *PIN1-RFP* with the secreted *ABP1$^{\Delta KDEL}$-GFP* version resulted in a strong internalization of PIN1-RFP (Figures 4G and 4H), indicating that ABP1 exported from ER regulates endocytosis.

When introduced into *Arabidopsis* seedlings, *ABP1$^{\Delta KDEL}$-GFP* expression led to auxin-related phenotypes, such as three cotyledons, shorter roots, and reduced apical dominance, but frequently resulted into seedling lethality or sterile development already in the T1 generation (Figure 4I and data not shown). To further characterize the role of ABP1 gain of function in PIN1 internalization, we monitored the subcellular dynamics of PIN1 proteins in the seedlings moderately expressing *ABP1$^{\Delta KDEL}$-GFP.* In accordance with the transient BY-2 assays, the *ABP1$^{\Delta KDEL}$-GFP* expression increased PIN1 internalization in *Arabidopsis* root cells treated with 25 μM BFA for 30 min (Figures 4J – 4L). In summary, ABP1 gain of function induces PIN internalization, whereas reduced expression of *ABP1* leads to reduced PIN internalization. These results strongly suggest that ABP1 acts as a positive effector of endocytosis in plants.

Auxin Negatively Regulates ABP1 Action on PIN Internalization

To study the potential role of ABP1 in mediating auxin inhibition of PIN internalization, we tested the auxin effect in BY-2 cells coexpressing *PIN1-RFP* and $ABP1^{\Delta KDEL}$*-GFP* (Figure 5). Of note, NAA treatment counteracted the positive effect of secreted ABP1 on PIN internalization, leading to a preferential retention of PIN proteins at the plasma membrane (Figure 5E). In contrast, the structurally similar auxin analog 5-F-IAA, which promotes auxin-dependent gene transcription but does not inhibit PIN1 endocytosis (Figure 3 and Figure S3), showed also no detectable effect on ABP1-mediated PIN internalization (Figure 5F). This observation is consistent with the reported weak affinity for 5-F-IAA of the plasma membrane-associated auxin-binding site, which is likely related to ABP1 (Zažímalova and Kutáček, 1985). These results, as well as similarities between knockdown lines and auxin treatment, suggested a model in which auxin inhibits ABP1-mediated stimulation of PIN internalization.

To test this scenario, we used the *abp1-5* mutant allele (Xu et al., 2010) with a point mutation in the conserved auxin-binding pocket (Napier et al., 2002). Conversion of the conserved histidine to tyrosine (H94Y) weakens the Pi interaction between the side-chain ring and the indole ring and is, therefore, predicted to reduce the auxin-binding affinity without major steric hindrance or changes in domain structure (Woo et al., 2002). In contrast to ABP1 knockdown lines that showed an “auxin-like” inhibitory effect on PIN internalization, the *abp1-5* allele was partially resistant to auxin with respect to its effect on PIN internalization. Auxins, such as NAA or IAA, in *abp1-5* root cells were much less effective in inhibiting BFA-induced internalization of PIN proteins than the wild-type roots (Figures 5H – 5L).

Next, we deleted the KDEL ER retention signal in the *abp1-5* mutant sequence. Similarly to $ABP1^{\Delta KDEL}$*-GFP*, the overexpression of $ABP1\text{-}5^{\Delta KDEL}$ induced the PIN1-RFP internalization in tobacco BY-2-cultured cells. But, in contrast to $ABP1^{\Delta KDEL}$*-GFP,* the $ABP1\text{-}5^{\Delta KDEL}$-promoted PIN1 internalization was not counteracted by exogenous auxin application, indicating an auxin resistance due to a decreased affinity of auxin binding to the auxin-binding pocket in the $ABP1\text{-}5^{\Delta KDEL}$ modified version. This result shows that mutations in the auxin-binding pocket of ABP1 led to a decrease in auxin sensitivity of auxin-mediated inhibition of PIN internalization, supporting our hypothesis that auxin binding to ABP1 inhibits the positive action of ABP1 on endocytosis.

Auxin Specifically Targets Clathrin-Based Mechanism of Endocytosis

Previous work using single cells suggested that PIN proteins are cargos of endocytic mechanism involving the vesicle coat protein clathrin (Ortiz-Zapater et al., 2006; Dhonukshe et al., 2007). Thus, we examined the role of clathrin in PIN internalization in planta by conditionally overexpressing the C-terminal part of clathrin heavy chain (termed HUB1) that exerts a dominant negative effect on clathrin function by binding and consequently depleting clathrin light chains (Liu et al., 1995). This interference with the clathrin function inhibited the BFA-induced PIN internalization, confirming that PIN proteins are internalized in *Arabidopsis* root cells by the clathrin-based mechanism of endocytosis (Figure S5).

To specifically test whether auxin inhibits clathrin-mediated endocytosis, we monitored the internalization of a well-established and specific cargo of clathrin-dependent endocytosis, the human transferrin receptor (hTfR) and its ligand transferrin. In *Arabidopsis* protoplasts, which heterologously expressed hTfR, exogenously applied transferrin was efficiently internalized (Figures 6A), as shown previously (Ortiz-Zapater et al., 2006). As expected, this internalization was completely blocked by tyrphostin A23, a known inhibitor of clathrin-

mediated processes (Banbury et al., 2003; Konopka et al., 2008) (Figure 6B and Figure S5). Physiological levels of natural (IAA; data not shown) and synthetic (NAA; Figure 6C) auxins rapidly and efficiently inhibited transferrin internalization in hTfR-expressing *Arabidopsis* protoplasts, demonstrating that auxin-mediated inhibition of endocytosis targets a general clathrin mechanism and is not cargo specific. In contrast, NAA was ineffective in inhibiting the hTfR internalization in HeLa cells (data not shown), suggesting that the effect of auxin on the clathrin endocytotic pathway requires plant-specific factors. These auxin effects on internalization of both endogenous and heterologous cargos of the clathrin pathway suggest that auxin targets the clathrin-mediated mechanism of endocytosis.

Auxin Interferes with Clathrin Recruitment to the Plasma Membrane

To address a possible mode of auxin action on clathrin-mediated endocytosis, we tested for an auxin effect on clathrin localization. As previously described (Konopka et al., 2008), clathrin light chain fused to GFP (CLC-GFP) is associated with intracellular endomembranes (presumably TGN) and with dynamic foci at the plasma membrane (Figure 6D). The amount of clathrin detected at the plasma membrane was variable and strongly depended on growth conditions. Nonetheless, both anti-CHC immunolocalizations (Figure S6) and time-lapse visualizations of CLC-GFP revealed that auxin treatments led to a decrease in the fluorescence associated with the plasma membrane but had no detectable effect on clathrin association with intracellular endomembranes (Figures 6D – 6G and 6K and Figure S6). The effect of auxin on clathrin recruitment to the plasma membrane was rapid and transient and displayed kinetics similar to those of the auxin-mediated inhibition of PIN internalization (Figure S6). In contrast, auxin did not visibly affect other regulators of the early and late endosomal trafficking (Figure S5), including RabF2b (Rab5/Ara7) that is required for PIN internalization, presumably at later steps of endocytosis (Ueda et al., 2001; Dhonukshe et al., 2008). In addition, PEO-IAA, the effective inhibitor of PIN protein internalization, also showed an effect on clathrin incidence at the plasma membrane (Figures 6I and 6K), whereas 5-F-IAA, which is ineffective in the inhibition of PIN protein internalization, showed no detectable effect on CLC incidence at the plasma membrane (Figures 6J and 6K). These experiments demonstrated that auxin specifically interferes with the clathrin recruitment to the plasma membrane, providing a plausible mechanism for auxin effect on the endocytosis of PIN1 and other cargos.

Auxin Negatively Regulates ABP1 Action on Clathrin-Dependent PIN Internalization

Next, weaddressed the potential role of ABP1 in mediating auxin effect on the clathrin-dependent endocytosis. First, we tested the effect of the interference with the clathrin function on ABP1-mediated PIN1 internalization. In BY-2 cells, the ABP1-mediated internalization of PIN1 proteins was abrogated by the inhibition of clathrin-mediated endocytosis either by expression of the dominant-negative clathrin HUB1 (*35S::HUB1-GFP*) or by treatment with tyrphostin A23 (Figures 7A – 7D), indicating that the functional clathrin machinery is required for ABP1 effect on PIN internalization.

In addition, the effect of ABP1 downregulation on the clathrin abundance at the plasma membrane was examined. The plasma membrane association of clathrin was strongly reduced in both immunomodulated (Figures 7E – 7H and Figure S6) and antisense *abp1* knockdown lines (data not shown) when compared to wild-type or noninduced controls. The auxin effect on clathrin abundance at the plasma membrane was significantly lower in *abp1-5* mutant seedlings than in wild-type seedlings (Figures 7I – 7M). Remarkably, these results correlate well with the auxin resistance observed in the *abp1-5* line for the effect on PIN internalization.

These multiple lines of observation clearly linked ABP1 action and clathrin mechanism of PIN internalization: (1) the positive effect of ABP1 on PIN protein internalization requires the clathrin-dependent endocytosis; (2) ABP1 action is required for clathrin localization at the plasma membrane; and (3) a mutation in the auxin-binding pocket of ABP1 conveys decreased auxin sensitivity of auxin effect on clathrin abundance at the plasma membrane. All of these results suggest that auxin binding to ABP1 inhibits the positive action of ABP1 on clathrin-mediated endocytosis.

Discussion

Nonnuclear Auxin Signaling Targets Clathrin-Dependent Mechanism of Endocytosis in Plants

In plants, the existence of endocytosis has been a matter of debates for decades, but in recent years, its physiological importance has become increasingly obvious, and a number of endocytic cargos have been identified (Robinson et al., 2008). The pronounced inhibition of the bulk of the endocytic processes after interference with the clathrin pathway (Dhonukshe et al., 2007) and its accessory protein (such as dynamin-related proteins) (Collings et al., 2008; Konopka et al., 2008) suggests that most endocytic processes in plants depend on an evolutionarily conserved mechanism involving clathrin.

We demonstrated through multiple approaches that clathrin-mediated endocytosis is rapidly inhibited by auxin and that auxin promotes the rapid disappearance of plasma membrane-associated clathrin. Of note, this auxin signaling does not involve the molecular components of the nuclear TIR1/AFB pathway (Kepinski and Leyser, 2002; Dharmasiri and Estelle, 2004) and does not require gene transcription or protein synthesis. This auxin effect on endocytosis is not specific to PIN proteins but regulates a number of endogenous and heterologous cargos. These observations strongly suggest that nontranscriptional auxin signaling interferes specifically with the general process of clathrin-mediated endocytosis in plant cells.

ABP1 Acts as an Auxin-Sensitive, Positive Regulator of Clathrin-Mediated Endocytosis

To identify the molecular mechanism underlying auxin effect on endocytosis, we tested the involvement of the putative auxin receptor ABP1 that is essential, but the mechanism of its action remained unclear (Badescu and Napier, 2006). Our loss- and gain-of-function analyses show that ABP1 acts as a positive regulator of clathrin-mediated endocytosis. ABP1 seems to be a plant-specific regulatory element of the evolutionary conserved clathrin-mediated endocytic mechanism. Because ABP1 binds auxin with high affinity (Jones, 1994; Napier et al., 2002), it is suggestive that auxin mediates its effect on clathrin-mediated endocytosis via ABP1. In this scenario, given the positive effect of ABP1 but the negative effect of auxin on endocytosis, auxin binding to ABP1 inhibits rather than activates the ABP1 action in endocytosis. This model (see Graphical Abstract) is supported by several independent lines of evidence: (1) the stereo-selectivity of auxins correlates with ABP1 binding (Zažímalová and Kutáček, 1985) and inhibition of endocytosis; (2) both increasing auxin or decreasing the active pool of ABP1 diminishes the clathrin incidence at the plasma membrane and inhibits the clathrin-dependent endocytosis; (3) increasing levels of secreted ABP1 lead to enhanced endocytosis that can be reversed by auxin treatment; and (4) an ABP1 with a mutated auxin-binding site is less effective in mediating auxin effect on clathrin incidence at the plasma membrane and on inhibition of endocytosis.

These observations and, in particular, the remarkable difference between the knockdown *abp1* and *abp1-5* mutants provide strong support for the model that auxin binding to ABP1

interferes with its positive action on clathrin-mediated endocytosis. However, it remains open by which mechanism this regulation occurs.

Physiological Role of the ABP1 Pathway for Regulation of Clathrin-Dependent Endocytosis

Our studies here have primarily focused on PIN auxin transporters as targets for auxin- and ABP1-mediated regulation of endocytosis. By this mechanism, auxin increases the incidence of PIN proteins at the cell surface, stimulating auxin efflux (Paciorek et al., 2005) and providing developmentally important feedback of auxin on the rate of its intercellular flow. However, a number of additional membrane proteins and other cargos of clathrin-mediated endocytosis might be regulated in a similar manner. A more general auxin effect on clathrin-dependent endocytosis might be related to its phylogenetically ancient role in the control of cell expansion (Lau et al., 2009), whereby ABP1 also plays a crucial role (Jones et al., 1998). During this process, when the cell surface rapidly increases, generally the endocytosis rate is attenuated to retain the essential signaling and structural components at the cell surface. Of note, ABP1 has also been connected to the ROP-GTPase pathway involved in the interdigitating growth of epidermal pavement cells (Xu et al., 2010), but the mechanistic link of this ABP1 function with its role in the clathrin-mediated endocytosis is still missing. Future work that builds on the proposed framework of the ABP1 action in clathrin-mediated endocytosis is necessary in order to understand how and with which components of the clathrin machinery ABP1 communicates. The intriguing possibility that the ABP1-mediated regulation of endocytosis is a part of a long-looked mechanism for auxin-mediated cell expansion and tissue polarization also remains open.

Experimental Procedures

Material and Growth Conditions

Arabidopsis thaliana (L.) Heyhn. seedlings, Columbia ecotype (Col-0), were grown on vertical half-strength Murashige and Skoog (0.5 MS) agar plates at 22°C for 4 days. BFA (Molecular Probes and Sigma), tyrphostin A23 (Sigma), tyrphostin A51 (Sigma), cycloheximide (Sigma), cordecypin (Sigma), or actinomycin (Sigma) were used from 50 mM dimethylsulfoxide stock solutions and added to the liquid 0.5 MS growth medium for the indicated times, if not mentioned otherwise: 90 min with 25 μM BFA; 30 min with 5, 10, or 30 μM of NAA; 30 min with 30 μM tyrphostin A23 or tyrphostin A51; and 30 min with 50 μM cordycepin or actinomycin followed eventually by 90 min NAA or NAA/BFA cotreatment. In control treatments, equal amounts of solvent were used.

Transferrin Uptake Assays in *Arabidopsis*

Transferrin uptake in *Arabidopsis* protoplasts expressing hTfR was assayed as described (Ortiz-Zapater et al., 2006) with transferrin-Alexa Fluor 546 (500 μg/ml, 28°C, 45 min). Tyrphostin A23 (350 μM) or auxin (10 μM NAA or 10 μM IAA) were added 15 min before transferrin and remained present during the internalization period.

Immunodetection and Microscopy

Immunofluorescence in *Arabidopsis* roots was analyzed as described (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) (Kim et al., 2001) were used, and the fluorochrome-conjugated secondary antibodies Alexa488 and the anti-rabbit-Cy3 (1:600) (Dianova) were used. Live-cell microscopy was done as described (Kleine-Vehn et al., 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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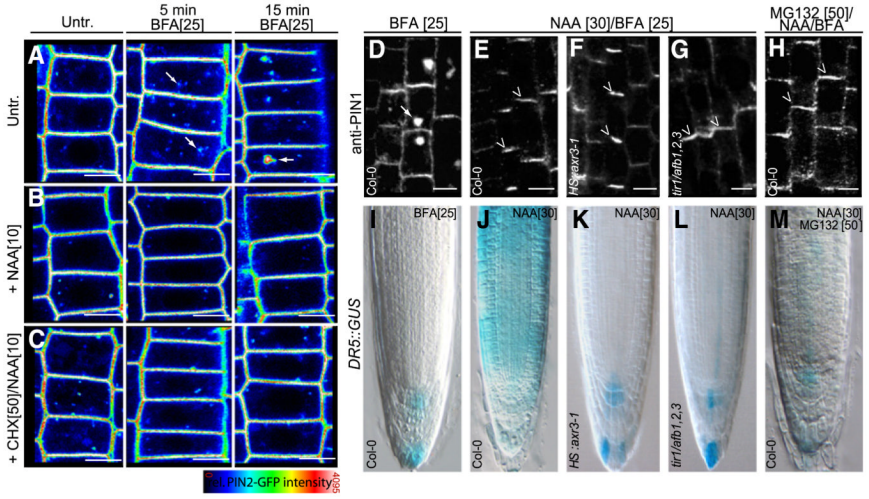

Figure 1. Auxin-Mediated Inhibition of Endocytosis by Nontranscriptional, TIR1-Independent Mechanism

(A–C) Time lapse showing BFA-induced increase of PIN2-GFP endosomal signal and its intracellular accumulation within minutes (A). NAA treatment effectively and rapidly inhibits BFA-induced PIN2-GFP internalization (B) also when protein synthesis is inhibited by cycloheximide (CHX) (C).

(D–H) BFA treatment for 90 min induces intracellular accumulation of PIN1 (D). Auxins, such as NAA (30 min pretreatment), inhibit BFA-induced PIN1 internalization in the wild-type (E); in the TIR1-mediated auxin signaling-deficient mutants, such as overexpressors of stabilized IAA17 (*HS::axr3-1;* induced for 2 hr at 37°C) (F); in the *tir/afb* quadruple mutant (G); and after MG132-mediated inhibition of proteasome function (H). See also Figure S1.

(I–M) Auxin treatments for 3 hr, such as NAA (J), but not BFA alone (I), induce transcriptional auxin response monitored by *DR5::GUS* in the wild-type (J), but not in the *HS::axr3-1* (K) and *tir/afb* quadruple (L) mutants or after MG132 treatment (M). See also Figure S1.

Arrows mark PIN proteins internalized into BFA compartments. Arrowheads highlight PIN retention at the plasma membrane. Scale bar, 10 μm.

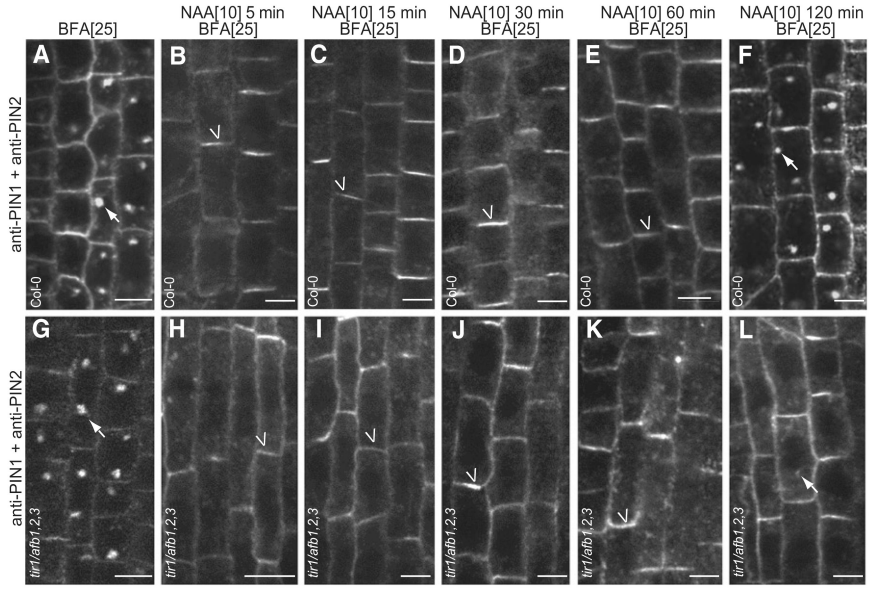

Figure 2. Auxin Effect on BFA-Induced PIN Internalization

Kinetics of auxin effect on 25 μM BFA-induced PIN internalization with different time points of auxin pretreatment (0, 5, 15, 30, 60, and 120 min) in the wild-type (A–F) and in the quadruple *tir/afb* mutant (G–L). Note the comparable sensitivity of the quadruple *tir/afb* mutant and wild-type to auxin effect on PIN internalization. Auxin effect on PIN protein internalization was immediate (within minutes) but transient: prolonged auxin treatments from 1 to 2 hr resulted in reduced inhibition of PIN internalization (arrows). Scale bar, 10 μm. See also Figure S2.

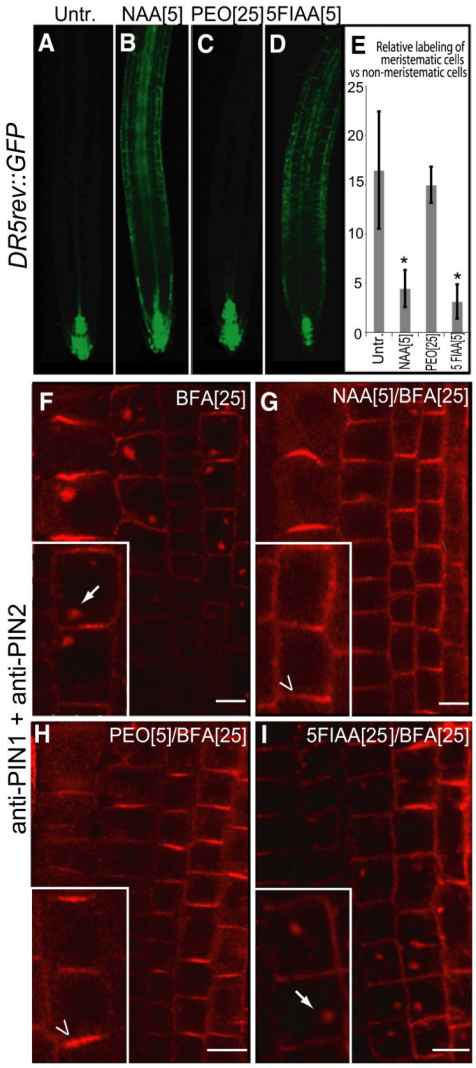

Figure 3. Distinct Auxin Perception Mechanisms for the Regulation of Transcription and Endocytosis

(A–D) Activity of auxin-responsive promoter *DR5rev::GFP* (A) induced by treatment with auxin analogs, such as NAA (B) and 5-F-IAA (D) at 5 μM for 3 hr, but not by PEO-IAA even at concentrations up to 25 μM (C).

(E) Relative *DR5rev::GFP* signal of meristematic cells versus nonmeristematic cells. n = 3 independent experiments with at least 21 roots analyzed for each assay. See also Figure S3.

(F–I) BFA-induced internalization of PIN1 and PIN2 (F) inhibited by NAA (G) and PEO-IAA (H) at 5 μM, but not by 5-F-IAA (all 30 min pretreated), even at concentrations up to 25 μM (I).

Arrows mark PIN proteins internalized into BFA compartments. Arrowheads mark the PIN retention at the plasma membrane. Scale bar, 10 μm. Error bars represent standard deviation. *$p < 0.05$.

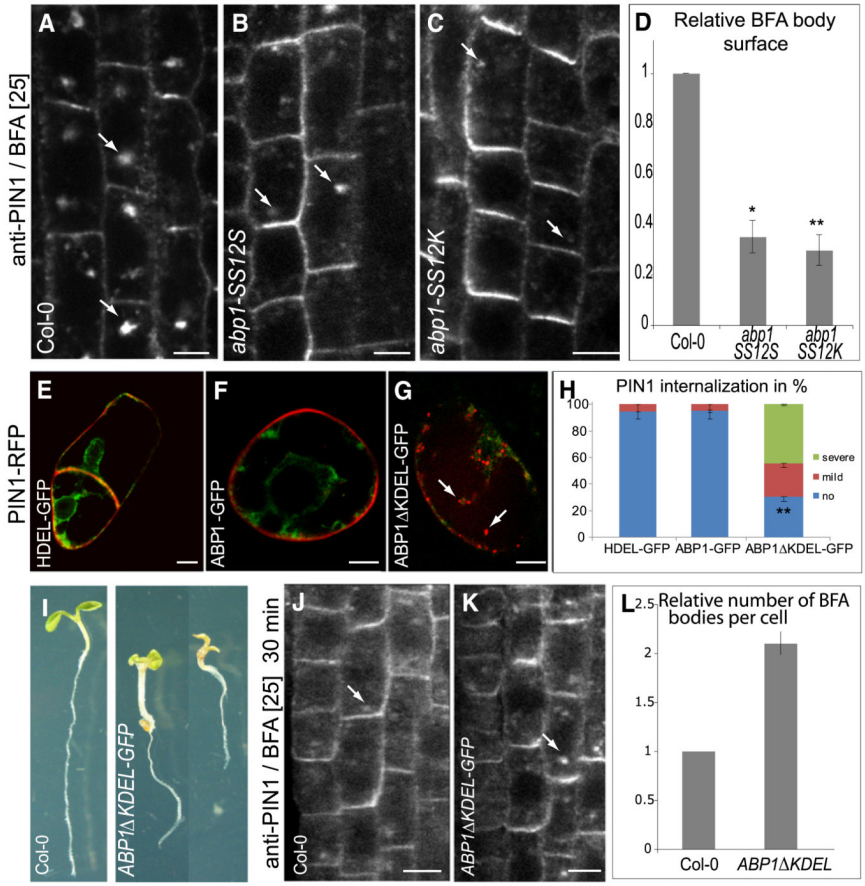

Figure 4. Positive ABP1 Role in PIN Internalization

(A–D) Reduced BFA-induced PIN1 internalization in inducible *abp1* knockdown lines *SS12S* (B) and *SS12K* (C) as compared to the induced wild-type (A). Number of BFA compartments was reduced after ABP1 downregulation in immunomodulation (*SS12S* and *SS12K*) (D). Values in (D) represent the relative mean surface area (pixels^2) in comparison with the wild-type for each individual experiment. n > 3 independent experiments with a least 60 cells measured for each assay. See also Figure S4.

(E–H) Cotransfection of tobacco BY-2 cells with *PIN1-RFP* (0.05 mg) (in red) and ER marker *HDEL-GFP* (0.05 μg) (E), full-length *ABP1-GFP* (0.5 μg) (F), and ABP1 with deleted ER retention signal (*ABP1$^{\Delta KDEL}$-GFP*) (0.05 μg) (G) (all in green). In contrast to the full-length *ABP1-GFP*, the secreted *ABP1$^{\Delta KDEL}$-GFP* induced pronounced PIN internalization. Percentage of cells displaying severe (green), mild (red), or no detectable (blue) PIN1-RFP internalization (H). n > 3 independent experiments and at least 60 cells counted for each assay. (I–L) Phenotypes of 4-day-old *35S::ABP1$^{\Delta KDEL}$-GFP* stable transformed Col-0 seedlings. Primary root growth defects and aberrant cotyledon number observed in the primary transformants. See also Figure S4. (I) BFA-induced internalization of PIN1 within 30 min is promoted in *35S::ABP1$^{\Delta KDEL}$-GFP* seedlings (K) versus the Col-0 control (J). Relative number of BFA bodies per cell (L). n = 3 independent experiments on two different transformants and at least 150 cells counted for each assay.

Arrows mark PIN protein internalization. Scale bar, 10 μm. Error bars represent standard deviation. *p < 0.05; **p < 0.001.

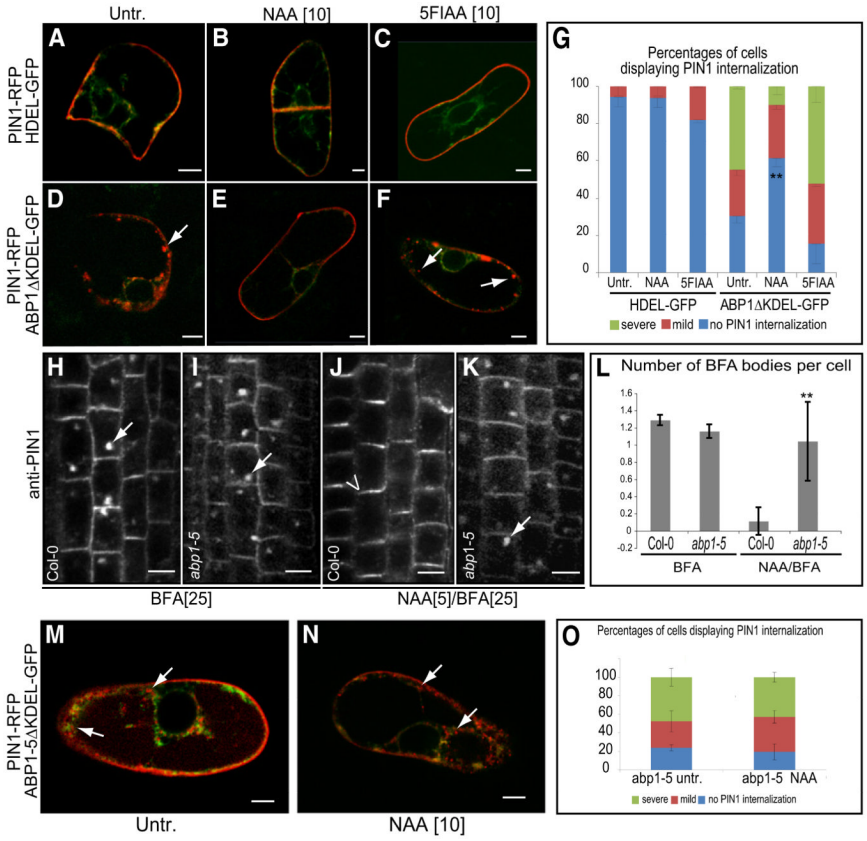

Figure 5. ABP1 Involvement in Auxin-Mediated Inhibition of PIN Protein Internalization

(A–G) Cotransfection of tobacco BY-2 cells with *PIN1-RFP* (0.05 μg) (in red) (A-F) and ER marker *HDEL-GFP* (0.05 μg) (A and B) or $ABP1^{\Delta KDEL}$-*GFP* (0.05 μg) (D–F) (all in green). After transfection, BY-2 cells were treated with NAA (B and E) or 5-F-IAA (C and F). NAA, but not 5-F-IAA, sup-pressed the $ABP1^{\Delta KDEL}$-dependent effect on PIN1 internalization. Percentage of cells displaying severe (green), mild (red), or not detectable (blue) PIN1-RFP internalization (G). n > 3 independent experiments with at least 60 cells counted for each assay.

(H–L) BFA-induced PIN internalization in wild-type (H) and *abp1-5* lines with mutation in auxin-binding site of ABP1 (I). Whereas NAA (5 μM, 30 min pretreatment) reduced the BFA-induced PIN protein internalization in the wild-type (J), the *abp1-5* mutant seedlings were partially resistant to this auxin effect (K). Average number of BFA bodies per root cell in BFA- or NAA/BFA-treated wild-type and *abp1-5* mutant seedlings (L). n = 3 independent experiments with at least 150 cells counted for each assay.

(M–O) Cotransfection of tobacco BY-2 cells with *PIN1-RFP* (0.05 μg) (in red) (A–F) and mutated $ABP1\text{-}5^{\Delta KDEL}$ (0.05 μg) (in green) (M and N). After transfection, BY-2 cells were treated with NAA (N). NAA did not suppress the positive effect of $ABP1\text{-}5^{\Delta KDEL}$ with mutated auxin-binding site on PIN1 internalization.Percentageofcells displaying severe (green), mild (red), ornot detectable (blue) PIN1-RFP internalization (O). n > 3 independent experiments, and at least 60 cells counted for each assay.

Arrows mark PIN proteins internalized into BFA compartments. Arrowheads mark the PIN retention at the plasma membrane. Scale bar, 10 μm. Error bars represent standard deviation. *p < 0.05; **p < 0.001.

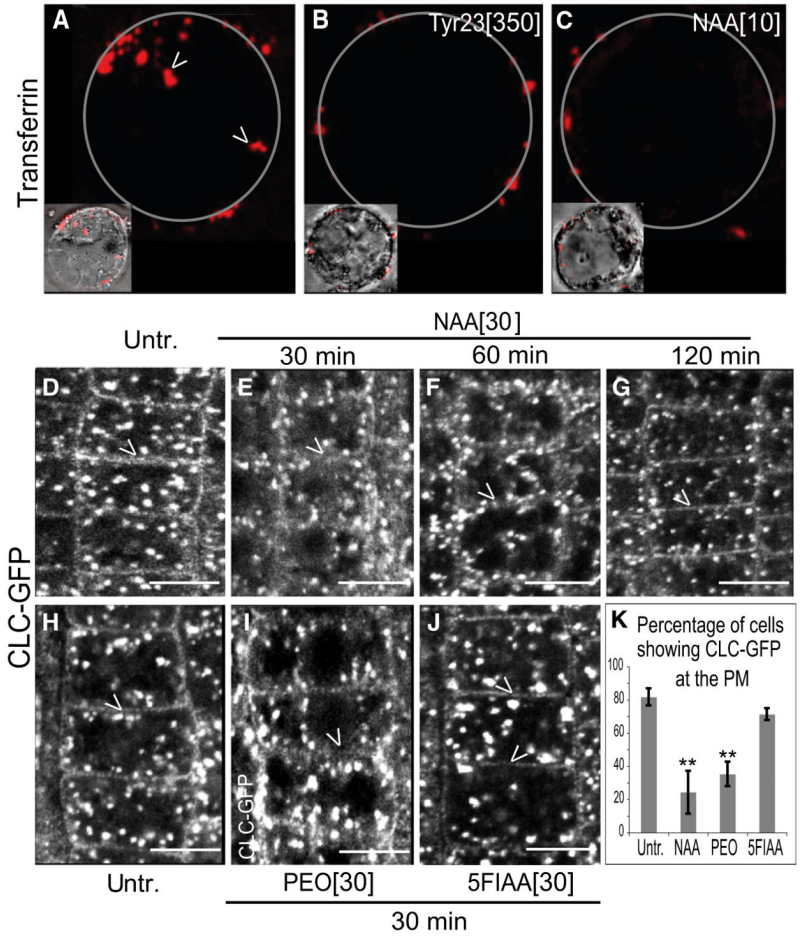

Figure 6. Auxin Effect on Clathrin-Dependent Endocytosis and Clathrin Recruitment to the Plasma Membrane

(A–C) Heterologous expression of human transferrin receptor in protoplast enabled Alexa633-labeled transferrin internalization (A). Transferrin uptake was blocked by both tyrphostin A23 (B) and auxin (NAA) (C). See also Figure S5. Arrowheads mark internalized proteins.

(D–K) Clathrin light-chain GFP (CLC-GFP) localization at the *trans*-Golgi network (TGN) and the plasma membrane (D). After auxin treatment for 30 (E) to 60 min (F), the CLC-GFP transiently disappeared from the plasma membrane but stayed at the TGN. After longer auxin treatments (2 hr), CLC-GFP reappeared at the plasma membrane (G). Arrowheads mark CLC-GFP intensity at the plasma membrane. See also Figure S5.

(H–J) PEO-IAA (30 μM for 30 min) inhibited the CLC-GFP localization at the plasma membrane (I), whereas treatment with 5-F-IAA (30 μM for 30 min) had no visible effect (J, arrowheads).

(K) Percentage of cells showing CLC-GFP labeling at the plasma membrane in untreated seedlings and treated with NAA, PEO-IAA, and 5-F-IAA for 30 min. The percentage of cells showing a plasma membrane localization of CLC-GFP was calculated for at least 21 roots for each condition. Arrowheads mark CLC-GFP intensity at the plasma membrane. Scale bar, 10 μm. Error bars represent standard deviation. **$p < 0.001$.

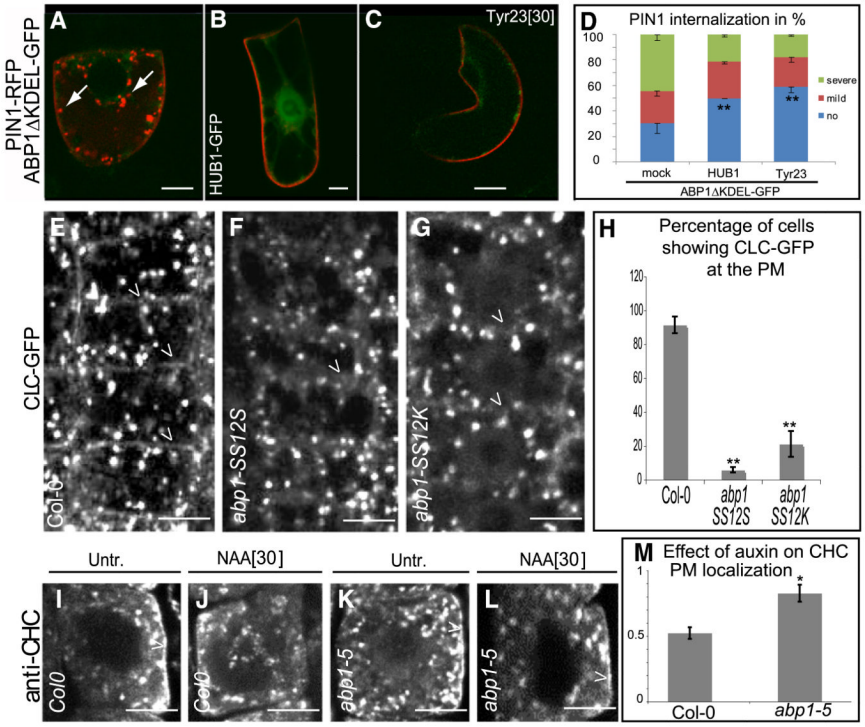

Figure 7. ABP1 Mediates Auxin Effect on Clathrin-Dependent PIN Internalization

(A–D) Cotransfection of tobacco BY-2 cells. ABP1^{DKDEL}-GFP-dependent (green) promotion of PIN1-RFP (red) internalization (A) is reduced after inhibition of clathrin-dependent endocytosis by HUB-GFP (green) (B) or tyrphostin A23 (C). Percentage of cells displaying severe (green), mild (red), or no detectable (blue) PIN1-RFP internalization (D). n > 3 independent experiments and at least 60 cells counted for each assay. Arrows mark PIN proteins internalization. Arrows indicate PIN internalization.

(E–H) Localization of clathrin as visualized by CLC-GFP at the TGN and the plasma membrane (E, arrowheads). In the *abp1* knockdown immunomodulation lines, CLC-GFP labeling remained at the TGN but decreased at the plasma membrane (F and G). Percentage of the cells showing CLC-GFP localization at the plasma membrane (H). n = 3 independent experiments with at least 18 roots analyzed for each assay. See also Figure S6. Arrowheads mark CLC-GFP at the plasma membrane.

(I–M) Localization of clathrin as visualized by immunodetection with an anti-CHC antibody at the TGN and the plasma membrane in Col-0 and the *abp-1-5* lines mutated in the auxin-binding site. In the *abp1-5* mutant, depletion of clathrin from the plasma membrane was less sensitive to NAA. (M) Percentage of the cells showing CHC localization at the plasma membrane. n = 3 independent experiments with at least 15 roots analyzed for each assay. Arrow-heads mark CHC immunolabeled intensity at the plasma membrane.

Scale bar, 10 μm. Error bars represent standard deviation. *p < 0.05; **p < 0.001.