Potential role of ABP1 (AUXIN BINDING PROTEIN1) as a receptor for regulation of auxin-related functions and modulation of light signaling

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ABSTRACT

Based on early studies AUXIN BINDING PROTEIN 1 (ABP1) was characterized as receptor for a number of rapid auxin responses particularly in cells and the plasma membrane cell. Recent studies demonstrated downstream elements which were suggested to be linked directly to an ABP1-mediated signaling pathway (Robert et al., 2010; Xu et al., 2010). The embryo lethality of ABP1-null mutants indicates that *ABP1* is an important gene in plant although in the same time it thwarts post-embryonic investigations. This present study aims to investigate auxin responses of several available types of *abp1* mutants. Besides investigating the presently available *abp1* mutants, heterozygous *abp1/+* and *abp1-5,* new *ABP1* mutants containing a point mutation in the auxin binding site were designeed and characterized in this study. These mutants were named *abp1-8, abp1-9*, and *abp1-10* and *ABP1-OX*, an over-expressing wild type cDNA plant. Auxin-related functions and responses to auxin in these mutants at molecular and morphological levels were investigated. Furthermore, response to monochromatic light and shade avoidance condition were also investigated. Our data show that typical auxin related-responses in all *abp1* mutants are defect. Reduction in tropistic responses such as gravitropism of roots and hypocotyls or phototropism, decrease in apical dominance and early flowering were found in all *abp1* mutants indicate decreasing in auxin sensitivity of the mutants. Interestingly, transcriptional regulation of auxin-induced genes that previously has been known as domain of TIR1 function was decreased in all *abp1* mutants, suggesting ABP1 somehow is functionally linked to this process. Based on current studies of ABP1 and the data in this study, for example the similar pattern of transcriptional expression of auxin-related genes in *abp1-5* and *pin2/eir1-1* mutants and slower of auxin transport in heterozygous *abp1/+*, it is suggested that there are molecular links between ABP1 and auxin polar transport regulation. This ABP1-dependent polar auxin transport may modulate cytocolic concentration of auxin and affects control of gene regulation. Light responses in all *abp1* mutants were defect which is indicated by hypersensitive hypocotyl growth under red and far-red light and far-red rich light (shade avoidance) condition. Additionally, similarities in transcriptional pattern of light-induced genes in the *abp1-5* mutant and all in-vitro generated *abp1* mutants to the pattern provided by *phyB* mutant provide initial evidence that PHYB-related function in the *abp1* mutants may be impaired. All together it is suggested that *abp1* mutants not only have auxin-impaired properties but also light-impaired properties that are particularly connected to PHYB-related functions. At the end, this study points out that ABP1 may function as a link of the auxin-signal pathway and the red light-signal pathway.

Keywords: AUXIN BINDING PROTEIN1 (ABP1), auxin receptor, light.

Zusammenfassung

Basierend auf frühen Studien wurde das AUXIN BINDING PROTEIN 1 (ABP1) als Rezeptor für eine Reihe von schnellen Auxin-Antworten, vor allem in Einzelzellen und der Plasmamembran, charakterisiert. Neuere Studien zeigen dem Rezeptor nachgeschaltete Elemente, die eine Funktion im ABP1-vermittelten Signalweg haben (Robert et al., 2010; Xu et al., 2010). Die Abwesenheit von ABP1 in der homozygoten T-DNA-Insertionsmutante ist im Embryo letal, was auf eine wichtige Funktion des *ABP1*-Gens in der Pflanze schließen lässt. Gleichzeitig erschwerte dies den Zugang zu *abp1* Mutanten. Das Ziel der vorliegenden Arbeit war die Untersuchung der Reaktionen von verschiedenen Typen verfügbarer *abp1*-Mutanten auf Auxin. Neben der Untersuchung der vorhandenen Mutanten, der heterozygoten *abp1/+* und *abp1-5*, wurden Mutanten mit einer Punktmutation in der Auxin-Bindungsstelle hergestellt und charakterisiert. Diese Allele wurden *abp1-8*, *abp1-9, abp1-10* und *ABP1-OX* (eine Wildtyp-cDNA überexpremierende Mutante) genannt. Die Auxin-bezogenen Funktionen und Reaktionen auf Auxin wurden in diesen Mutanten auf molekularer und morphologischer Ebene untersucht. Darüber hinaus wurden die Reaktionen auf monochromatisches Licht und auf simulierten Schatten untersucht. Unsere Daten zeigen, dass Auxin-gesteuerte Reaktionen in allen *abp1*-Mutanten weitgehend defekt sind. Reaktionen wie Gravitropismus von Wurzeln und Hypokotylen, Phototropismus, Apikaldominanz, und relativ frühe Blüte sind in allen *abp1*-Mutanten gestört, was sich durch verringerte Hormon-Sensitivität erklären lässt. Interessanterweise war die transkriptionelle Regulation von Auxin-induzierten Genen, die zuvor als Funktion von TIR1 beschrieben war, deutlich in allen *abp1*-Mutanten verringert. Dies deutet darauf hin, dass *abp1* zur Genregulation beiträgt. Basierend auf aktuellen Studien von *abp1*-Mutanten und deren Daten, die zum Beispiel sowohl ein ähnliches Muster der Regulation der Expression von Auxin-induzierten Genen in *abp1/+, abp1-5,* der *pin2/eir1-1*-Mutante als auch einen langsameren Auxintransport in der heterozygoten *abp1/+* zeigten, wird postuliert, dass die Regulation des Auxintransports durch ABP1 zu Konzentrationsänderungen des Hormons im Cytosol führt, so dass ABP1 und PIN2 an der Genregulation beteiligt sein können. Zudem waren die Reaktionen auf Licht in allen *abp1*-Mutanten gestört. Eine überempfindliche Reaktion des Hypokotylwachstums auf Rotlicht, Dunkelrotlicht und in simulierten Schatten wurde gezeigt. Darüber hinaus sind Ähnlichkeiten in der Regulation der Transkription Licht-induzierter Gene in *abp1-5* und allen in vitro erzeugten Mutanten zu denen einer *phyB*-Mutante ein erstes Anzeichen dafür, dass

PHYB-bezogene Funktionen in den *abp1*-Mutanten beeinträchtigt sind. Zusammenfassend wird vermutet, dass *abp1*-Mutanten nicht nur Beeinträchtigungen in Auxin-Funktionen aufweisen, sondern auch in lichtphysiologischen Eigenschaften, besonders im Zusammenhang mit PHYB. ABP1 könnte demnach eine Funktion in der Verbindung des Auxin-Signalwegs und des Rotlicht-Signalwegs haben.

Schlageworte: AUXIN BINDING PROTEIN1 (ABP1), Rezeptor des Auxin, licht.

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Abbreviations

ABP1	AUXIN BINDING PROTEIN1
TIR1	TRANSPORT INHIBITOR RESISTANT1
AFBs	Auxin Signaling F-BOX proteins
PHYA	phytochrome A
PHYB	phytochrome B
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qRT-PCR	Qualitative Real Time Polymerase Chain Reaction
IAA	indoleacetic acid
1-NAA	1-naphthaleneacetic acid
R:FR	red:far red
Ws	Wassilewskija
Col-0	Columbia-0

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Auxin is a vital hormone that regulates many aspects of plant development. It coordinates many growth and developmental processes at different levels, such as embryogenesis, morphogenesis, organogenesis, reproduction, cell elongation and cell division, and cell differentiation. In addition, auxin mediates responses to environmental cues such as responses to gravity and light by developing certain tropisms (Davies, 1995; Leyser, 2006; Benjamins and Scheres, 2008; Mockaitis and Estelle, 2008; Chapman and Estelle, 2009). However, in comparison to the tremendous information that are accumulated about auxin function, the molecular mechanism(s) of auxin signaling pathways are still poorly understood (reviewed in Leyser. 2001; Scherer, 2002; Dharmasiri and Estelle, 2004; Mockaitis and Estelle, 2008; Tromas et al., 2009; Scherer, 2011).

Auxin receptor

The binding of specific ligand to given receptors is usually considered as the begining of signal transduction network. The receptor can either be localized in a membrane or in a cytosolic compartment (Scherer, 2011). As a major plant hormone, auxin receptor(s) has been investigated since five decades, and several auxin-binding proteins have been identified (Hertel et al., 1972; Melhado et al., 1982; Shimomura et al., 1986; Klämbt, 1990). The first characterized auxin binding protein that was considered to be a potential auxin receptor is AUXIN BINDING PROTEN1 (ABP1). ABP1 initially was isolated from maize coleoptiles membrane (Löbler and Klämbt, 1985; Venis and Napier, 1991; Napier, 1995) and has been demonstrated to have high specificity of binding and affinity to auxin (Löbler and Klämbt, 1985; Inohara et al., 1989; Hesse et al., 1989; Jones and Venis, 1989). Most of ABP1 potential functions as an auxin receptor remained largely unclear (review in Leyser, 2001; Dharmasiri and Estelle, 2004; Quint and Gray, 2006; Sauer and Kleine-Vehn, 2011). Recent experimental data proposed that ABP1 is a key player of auxin signaling and considered as a auxin receptor in plasma membrane (Robert et al., 2010; Tromas et al., 2010; Xu et al., 2010; Effendi et al., 2011; Sauer and Kleine-Vehn, 2011; Scherer, 2011). The second auxin receptor is TRANSPOR-INHIBITOR-RESISTANT1 (TIR1) and the closely related AUXIN SIGNALING F-BOX proteins (AFBs), of which in *Arabidopsis* five AFB proteins were identified (Parry et al., 2009). Currently, TIR1/AFBs proteins have been accepted as auxin receptor by scientific community (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b;

Kepinsky and Leyser, 2005). TIR1/AFBs are well known to regulate auxin-induced gene expression by forming a complex with auxin and members of a family of IAA protein repressors to enhance the E3 ligase activity of TIR1 and induce activation of auxin-responsive genes by destruction of the repressors in the proteosome (Mockaitis and Estelle, 2008; Calderon-Villalobos et al., 2010). The success in elucidating of the nuclear auxin pathway via TIR1/AFB action was always interpreted so that TIR1/AFB is the main auxin receptor. At the same time, the other cellular auxin effects which are not obviously connected to gene regulation have received less attention (Badescu and Napier, 2006; Paciorek and Friml, 2006). Activation of phospholipase A, changes in membrane K^+ currents, membrane hyperpolarisation, activation of proton pumping, MAP kinase, and inhibition of endocytosis (Scherer, 1989; Scherer and Andre, 1989; Rück et al., 1993; Hager, 2003; Mizoguchi et al., 1994; Paul et al., 1998; Paciorek et al., 2005) are rapid auxin-dependent processes that mainly are happened in the plasma membrane and obviously do not involve regulation of gene expression (reviewed Scherer, 2011). Furthermore, a quadruple *tir1/afb1/afb2/afb3* mutant survived and was able to develop completely (Dharmasiri et al., 2005b), suggesting that auxin signaling is not completely abolished in this plant and provide evidence that besides TIR1/AFB-dependent signaling, another auxin pathway mediates auxin effects is present (Quint and Gray, 2006).

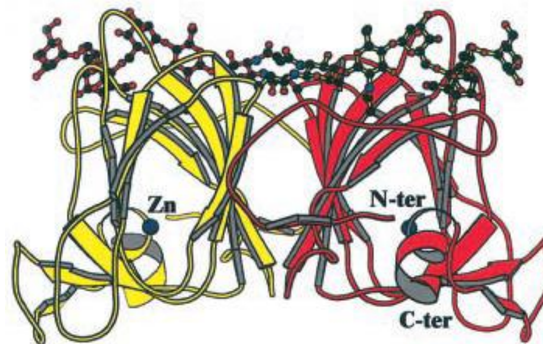

Figure .1. Schematic representation of the overall fold of the ABP1 dimer. Picture was taken from Woo *et al.* (2002).

Structure and character of ABP1

ABP1 was initially identified from crude membrane preparation of maize (Hertel et al., 1972) and then its binding activity was characterized (Batt and Venis, 1976; Ray, 1977; Venis,

1977) before in 1985 first purification of ABP1 was done by Löbler and Klämbt (see review by Napier et al., 2002). Later on, molecular cloning of maize ABP1 was achieved (Hesse et al., 1989; Inohara et al., 1989; Tillmann et al., 1989). In vitro binding assays of purified ABP1 showed that ABP1 had strong binding to 1-NAA (1-naphthaleneacetic acid) with a *Kd* from 2.10-7 to 5.10-7 M and it is pH-dependent with an optimum pH at 5.5 and binding activity drops until near no binding at pH 7.0 (Dohrmann et al., 1978; Löbler and Klämbt, 1989; Tian et al., 1995).

ABP1 is a dimer of 22 kDa protein consisting of 164 amino acid residues (*Zea mays* ABP1) which lacks a transmembrane domain that is generally present in a plasma membrane-bound receptor (see reviews by Jones, 1994; Macdonald, 1997), suggesting that ABP1 might require another protein as 'docking' to bind to the plasma membrane (Klämbt, 1990; Diekmann et al., 1995). Sequence analysis of ABP1 showed that its C-terminus contains a KDEL, an endoplasmic reticulum retention signal, suggesting that ABP1 is localized in the ER lumen where the neutral pH (7.0) provides an environment for very low auxin-binding affinity or near none (Tian et al., 1995). However, some experimental data demonstrated that only a small amount of ABP1 is secreted to the plasma membrane and/or the extracellular matrix (Jones and Herman, 1993; Henderson et al., 1997; Bauly et al., 2000). Furthermore, auxin application to maize coleoptiles protoplast induces a clustering of ABP1 at the outer face of the plasma membrane (Diekmann et al., 1995). Thus, ABP1 is localized in the ER and the plasma membrane.

Auxin-binding site of ABP1

The amino acid residues in three domains of the ABP1 protein are highly conserved among all higher plants. These domains are labeled as box A (or D16), box B and box C (or peptide 11) (Napier et al., 2002). The highly conserved residues D16 or Box A, Box B and C or peptide 11 (Brown and Jones, 1994) were suggested to provide a major contribution to the auxin binding site (Napier et al., 2002). The Box A (Brown and Jones, 1994) is located between Thr54 and Gly70 and has been proposed to contain the auxin-binding site (Venis et al., 1992). This was concluded from experiments using antibodies against a synthetic peptide of box A. In the absence of auxin the antibody stimulated hyperpolarization of tobacco protoplast plasma membranes in a manner similar to auxin stimulus, so that these antibodies mimic the auxin effect and the box A is likely the auxin-binding site (Venis et al., 1992).

Photoaffinity labeling assay using an active auxin analog, 5-(7-3H) azidoindole-3-acetic acid (5-(3H)N3IAA and analysis of tryptic fragments of ABP1 in maize showed that a 17-mer in the carboxyl terminus of ABP1, designed as peptide 11, incorporated specifically (5-(3H)N3IAA and is suggested contain at least one determinant of the auxin-binding site (Brown and Jones, 1994). Furthermore, the relevant residues were suggested to be Asp134 and Trp136 to form the hydrophobic platform determined as the auxin binding. David *et al.* (2001) using tobacco mesophyll protoplast demonstrated that highly conserved residues at the C-terminus of ABP1 were important in protein folding and activation at plasma membrane. The Cys177 as well as Asp175 and Glu176 were to be identified substantially involved in these functions. Even though it was shown that the KDEL sequence was not taking part in auxin binding, interaction with plasma membrane or even activation of transduction cascade, the KDEL appears to determine the stability of ABP1 (David et al., 2001; Robert et al., 2010). Moreover, using a monoclonal antibody mAb12 raised against tobacco ABP1, a discontinuous epitope embracing residues in both Box A and C can be recognized (Leblanc et al., 1999).

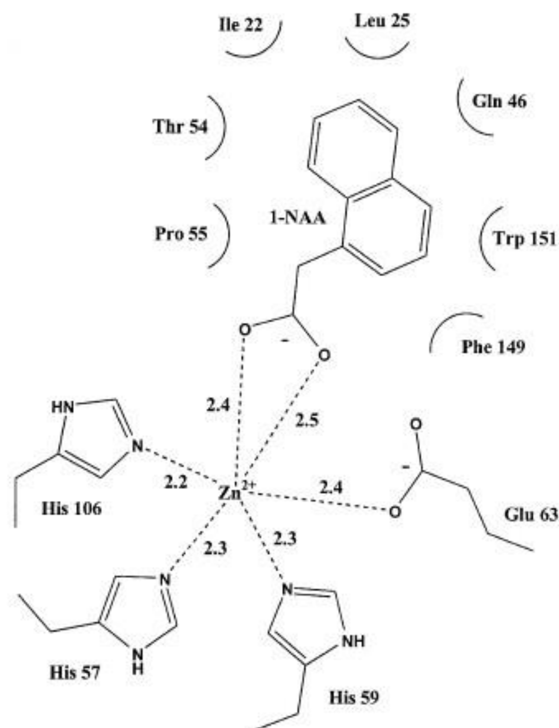

Figure 2. Auxin-binding site of ABP1. Simplifed representation of the contacts between 1-NAA and ABP1 and between the zinc ion and ABP1 in the complex show some residues

providing auxin binding pocket and some residues face to zinc ion. Distances shown are in angstroms. Picture was taken from Woo *et al*. (2002).

Using a molecular model of ABP1, Warwicker (2001) proposed a model of auxin-binding site of *Zea mays* ABP1. This model pointed out that Trp44 residue might form part of the auxin-binding site rather than Trp136 and Trp151. One of these C-terminal tryptophans was proposed to occupy the binding pocket in the absence of auxin, thereby providing a model for a potential conformational change.

Later on, using a crystal structure of ABP1 protein Woo *et al.* (2002) successfully revealed the architecture, disulfide arrangement and glycosylation of ABP1 as well as the structure of the ABP1 auxin-binding site, which in general, is consistent with previous models and experimental data of ABP1 auxin-binding (Flecker et al., 2001; David et al., 2001). The presence of a zinc ion is coordinated by three histidines (His57, His59 and His106) and a glutamate (Glu63) (Fig. 2). Note that the His57, His59 and Glu69 are localized in box A, that previous has been suggested to be involved in auxin-binding (Brown and Jones, 1994; Napier et al., 2002). Interaction of ABP1 crystal with 1-NAA revealed that there are some residues that interact with ABP1 such as Trp151, Thr54, Pro55, Ile22 and Leu25 (Fig.2). No change in the conformation of ABP1 was observed in the crystal when auxin binds (Woo et al., 2002). Recent studies point out that this proposed auxin binding site might be correct, thus alterations in certain amino acids in the auxin binding site or the metal binding site might imply to impair responses in the auxin-related functions. Using a weak *abp1-5* mutant containing a point mutation in His59>Tyr59, two different groups have demonstrated that *abp1-5* mutant plants showed defects in the PIN1 polarization as well as in the epidermal cell pattern of leaves and resistance to the auxin effect on clathrin-dependent endocytosis (Xu et al., 2010; Robert et al., 2010).

ABP1 as auxin receptor for rapid auxin-induced processes and potential functions in the regulation of auxin polar transport

As already mentioned, several lines of evidence indicate that some rapid auxin-induced processes do not likely involve regulation of gene expression which in turn also indicates that these processes might not be TIR1/AFB-dependent signaling. This suggests other pathway(s) mediating these non-transcriptional auxin effects (Paciorek, 2006; Tromas et al., 2009;

Scherer, 2011). Since those processes mostly happened in the plasma membrane, then ABP1 is the best candidate receptor for these processes. Most early experimental data of ABP1 functions in mediating of auxin-induced processes such as activation of the proton pump ATPase, activation of an inward rectifying K^+ channel in *Vicia* guard cells, increases of proton current in maize protoplast, and protoplast swelling induced by auxin (see review Scherer, 2011) are mainly based on electrophysiological and biochemical experiments. Another recent study using various plants expressing modified ABP1 (Chen et al., 2001a, 2001b; Chen et al., 2006; Braun et al., 2008; Tromas et al., 2009) provide more evidence that ABP1 is involved in the regulation of auxin-related functions. However, the large body of evidence of ABP1 as presumed auxin receptor for the rapid auxin-induced processes does not directly place this protein as auxin receptor equivalent in functional importance to as TIR1/AFB. There are remaining questions about ABP1 as an auxin receptor that emerges mainly from the unknown downstream signaling responses that should be clearly linked to these very rapid responses (Scherer, 2011). Two recent studies have shown initial evidences of downstream responses that might be linked to ABP1 as auxin receptor in the plasma membrane (Robert et al., 2010; Xu et al., 2010).

Auxin is unique phytohormone that has been demonstrated to be specifically and actively transported. Its asymmetric transport in specific cells is required for various developmental roles of auxin (Sabatini et al., 1999; Friml et al., 2002; Benková et al., 2003). Although the rate of auxin synthesis and its conjugates are important in determining the auxin status in plants, the pattern of responses to auxin in plant is also much determined by relative accumulation of auxin depending on auxin influx and efflux (review in Teale et al., 2006). PIN proteins are one of the important molecular components for these processes and their polar as well as subcellular distribution determines the direction of auxin flow out of the cells (Wisniewska et al., 2006). PINs are auxin efflux carriers which dynamically cycle between plasma membrane and the endosomal compartment (Kleine-Vehn and Friml, 2008). Auxin inhibits internalization of PIN protein which in turn enhances the increase of auxin efflux due to accumulation of more of this protein in the plasma membrane (Paciorek et al., 2005). Interestingly, this inhibition of PIN internalization occurs within minutes after auxin application (Paciorek et al., 2005; Robert et al., 2010), suggesting that it does not involve gene regulation and protein synthesis as the basic mechanism of TIR1/AFB function. Later on, Robert *et al.* (2010) demonstrated that *abp1-5* plants shows lack of regulation of PIN internalization cycling and proposed that ABP1 mediate this process by binding to auxin and

inhibiting PIN internalization. By perceiving apoplastic auxin, ABP1 could contribute to controlling of auxin concentration in the cytosolic compartment via regulating the PIN subcelluar localization. The resulting dynamic changes of intracellular auxin then could mediate regulation expression of auxin-induced genes via TIR1/AFB action. Thus, this idea proposes a link between ABP1 as plasma membrane receptor and TIR1/AFB as cytocolic receptor (Scherer, 2011). However, this hypothesis needs experimentally to be investigated further.

Genetically modified *ABP1* plants as material for *ABP1* future research

From the evidence on ABP1 functions that have been collected so far, it may be concluded that ABP1 is a membrane-bound auxin receptor for perceiving auxin at the cell surface. A link to functions of the nuclear auxin receptor, TIR1/AFB, perhaps also needs to be considered. Also, biochemical signaling at some point must cross the plasma membrane. So, the question how this happens still need to be elucidated since no transmembrane domain in ABP1 protein was identified, thus, a docking protein could be present for binding ABP1 to the plasma membrane (Klämbt, 1990), although this idea experimentally needs to be investigated. Recent studies had already shown, that using *abp1* mutant plants as well as engineered genetically modified expression of *ABP1* in plants, several ABP1 functions were revealed (Chen et al., 2001a, 2001b; Chen et al., 2006; Braun et al., 2008; Tromas et al., 2009; Robert et al., 2010; Xu et al., 2010). Embryo lethality in null *ABP1* mutant point out that ABP1 is an important gene (Chen et al., 2001b) although at the same time this thwarts further ABP1 research (Scherer, 2011). Nevertheless, these studies showed that *ABP1* mutants and engineered genetically modified *ABP1* mutants may be necessary for further investigation ABP1 functions. Here in this study, using heterozygous *abp1/+* mutant (Chen et al., 2001b), *abp1-5* mutant and new *abp1* mutants, we demonstrate more evidence that ABP1 is not only required in mediating of some auxin-related functions but also in regulation of gene expression of early auxin-induced genes. Interaction between ABP1 and auxin transport is also shown. Moreover, our data also indicate that there is molecular interaction between auxin signaling network and light signaling network, particularly PHYB-related signaling.

Objectives of the thesis

Potential role of ABP1 as an auxin receptor

The main objective of this study is to investigate potential role of ABP1 as an auxin receptor in *Arabidopsis*. This study will be focused on investigation of auxin-related physiological functions in *abp1* mutants and the involvement of ABP1 in regulation of early auxin-induced genes, analysis of functional role of ABP1 in the regulation of auxin transport, designing and characterization of new *abp1* mutants, and investigation of potential functions of ABP1 in light signaling, particularly in shade avoidance responses.

Outline of the thesis

The aim of this thesis is to investigate the role of AUXIN BINDING PROTEIN1 (ABP1) as an auxin receptor in *Arabidopsis thaliana* by quantifying responses of *abp1* mutants in their auxin-related functions such as tropic responses, auxin sensitivity, auxin transport as well as the physiological processes related to functions such as expression of early auxin-induced genes and PIN genes, and flowering time. Polar auxin transport recently has been shown to be regulated by light. Thus, we investigated also the connection of auxin and light by investigating responses of *abp1* mutants to different light spectra and shade simulated condition.

Chapter 2 (Effendi et al., 2011) will present data on the heterozygous mutant of T-DNA insertion mutant of *ABP1* (Chen et al., 2001b) and its responses to auxin stimulation. Auxin-related physiological functions such as gravitropism in roots and hypocotyls, phototropism in hypocotyls, auxin sensitivity, apical dominance, and flowering time on *abp1/+* will be presented. Auxin transport and auxin up-take assays will be also shown. Furthermore, expression of auxin-regulated genes in heterozygous *abp1/+* will be compared to wild-type plants.

Chapter 3 (Effendi and Scherer, 2011) will explore the role of ABP1 on the auxin transport by measuring expression of PINs genes in heterozygous *abp1/+* seedlings and in *eir1-1*, a loss-of-function *pin2* mutant. It is suggested that ABP1 affects the auxin-regulated functions by regulating polar auxin transport or auxin efflux. This process is known to involve cycling regulation of PIN protein from plasma membrane to endosomal compartments (Robert et al., 2010). Thus, it is suggested that the defect on the *ABP1* might be also contributing in the impaired transcriptional expression of PIN genes.

Chapter 4 will focus on elucidation of the effect of a point mutation in *abp1-5* and of active alleles in heterozygous *abp1/+* plants in responses to auxin and light stimulation. The effects of impairing *ABP1* on hypocotyls length, in particular in response to simulated shade light condition will be observed. The expression of shade marker genes will be investigated in the responses to red-enriched white light (high R:FR ratio) and far red-enriched white light (low R:FR ratio light). A comparison to *tir1-1* and *tir1-9* mutants will be also presented.

Chapter 5 will show characterization of new in-vitro generated *abp1* mutants in *Arabidopsis*. Auxin-responsive phenotypes and auxin-induced gene expression in three *abp1* mutant lines, *abp1-8, abp1-9, abp1-10,* and an ABP1 overexpression line (*ABP1-OX*) will be investigated. The physiological light responses of these *abp1* mutants will be also explored by exposing these mutants to far red-enriched light (low R:FR ratio) and red-enriched light (high R:FR ratio light). The expression of shade marker genes will be quantified.

Chapter 6 will present the general discussion and the summary of the previous chapters.

CHAPTER 2

The heterozygous *abp1/ABP1* insertional mutant has defects in functions requiring polar auxin transport and in regulation of early auxin-regulated genes

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The heterozygous *abp1/ABP1* insertional mutant has defects in functions requiring polar auxin transport and in regulation of early auxin-regulated genes

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SUMMARY

AUXIN-BINDING PROTEIN 1 (ABP1) is not easily accessible for molecular studies because the homozygous T-DNA insertion mutant is embryo-lethal. We found that the heterozygous *abp1/ABP1* insertion mutant has defects in auxin physiology-related responses: higher root slanting angles, longer hypocotyls, agravitropic roots and hypocotyls, aphototropic hypocotyls, and decreased apical dominance. Heterozygous plants flowered earlier than wild-type plants under short-day conditions. The length of the main root, the lateral root density and the hypocotyl length were little altered in the mutant in response to auxin. Compared to wild-type plants, transcription of early auxin-regulated genes (*IAA2, IAA11, IAA13, IAA14, IAA19, IAA20, SAUR9, SAUR15*, *SAUR23, GH3.5* and *ABP1*) was less strongly up-regulated in the mutant by 0.1, 1 and 10 μM IAA. Surprisingly, *ABP1* was itself an early auxin-up-regulated gene. IAA uptake into the mutant seedlings during auxin treatments was indistinguishable from wild-type. Basipetal auxin transport in young roots was slower in the mutant, indicating a *PIN2/EIR1* defect, while acropetal transport was indistinguishable from wild-type. In the eir1 background, three of the early auxin-regulated genes tested (*IAA2, IAA13* and *ABP1*) were more strongly induced by 1 μM IAA in comparison to wild-type, but eight of them were less up-regulated in comparison to wild-type. Similar but not identical disturbances in regulation of early auxin-regulated genes indicate tight functional linkage of ABP1 and auxin transport regulation. We hypothesize that ABP1 is involved in the regulation of polar auxin transport, and thus affects local auxin concentration and early auxin gene regulation. In turn, *ABP1* itself is under the transcriptional control of auxin.

Keywords: *abp1/ABP1* mutant, AUXIN-BINDING PROTEIN 1, auxin-induced transcription, gravitropism, phototropism, auxin transport.

INTRODUCTION

AUXIN-BINDING PROTEIN 1 (ABP1) was the first protein described as having specific auxin-binding activity (Napier *et al.,* 2002). Previously, auxin-binding activity, probably resulting from ABP1, had been reported for membranes isolated from maize coleoptiles (Hertel *et al.,* 1972). Initially, research on ABP1 functions focused on rapid regulation of membrane potential and potassium channels (Barbier-Brygoo *et al.,* 1989, 1991; Thiel *et al.,* 1993). Clear evidence of a link to typical auxin functions such as cell elongation, cell division or lateral root formation was lacking at first, as no ABP1 mutants or antisense plants were available. The *Arabidopsis thaliana* genome contains only one ABP1 gene, and its knockdown resulted in embryo lethality of homozygous progeny (Chen *et al.,* 2001b). Although the embryo lethality of the Arabidopsis homozygous *abp1* knockout mutant demonstrated the functional importance of ABP1, it hindered investigations on the post-embryonic functions of *ABP1*. Determination of the 3D structure of ABP1 revealed a specific binding site for auxins (Woo *et al.,* 2002). Moreover, ABP1 is a small glycoprotein that is abundant in the ER, with

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only a small proportion exposed on the outer leaflet of the plasma membrane (Napier *et al.,* 2002). As ABP1 has no transmembrane domain, a docking protein was postulated to exist that linked auxin perception to intracellular signaling (Klämbt, 1990). However, no such membrane anchor for ABP1 has yet been identified.

Progress was made by investigating tobacco cell culture cells over-expressing the *ABP1* gene in the sense or antisense orientation (Jones *et al.,* 1998) and tobacco plants over-expressing *ABP1* (Chen *et al.,* 2001a). These studies suggested that ABP1 does indeed positively regulate cell division and cell elongation. In another approach, a specific antibody against ABP1 was expressed in tobacco cell cultures, secreted into the ER, and thus onto the cell surface. This resulted in down-regulation of ABP1 function (David *et al.,* 2007). Down-regulation of ABP1 function was not found when the antibody was expressed in the cytoplasm. The study showed that ABP1 functions as an extra-cytoplasmic protein and that ABP1 inhibition hinders the cell cycle at the G_1/S and G_2/M phase transitions. This concept of suppression of ABP1 function by antibody binding was expanded by ethanol-controlled expression of the antibody *in planta* (Braun *et al.,* 2008; Tromas *et al.,* 2009). Suppres-sion of ABP1 function by ethanol-stimulated antibody expression inhibited both cell expansion and cell division in these plants. Moreover, expression of the anti-ABP1 antibody for 8 h also led to down-regulation of several *IAA* genes, suggesting that ABP1 also functions in auxin-induced gene regulation, which, at that time, was attributed exclu-sively to TIR1 and its homologs (Mockaitis and Estelle, 2008). We previously showed that auxin activates phospholi-pase A, and that inhibitors of phospholipase A inhibited hypocotyl elongation and up-regulation of early auxin-induced genes (Paul *et al.,* 1998; Scherer *et al.,* 2007), although the inhibitors did not directly affect TIR1 activity. In this way, we provided indirect evidence that an auxin receptor other than TIR1 participates in gene regulation of auxin-induced genes.

TIR1, on the other hand, is well established as both an auxin-binding receptor and a ligand-activated E3 ligase. TIR1 activity ubiquitinates IAA proteins, leading to the hydrolysis of these transcriptional co-repressors. Down-regulation of IAA proteins leads to up-regulation of a set of early auxin-activated genes (Mockaitis and Estelle, 2008). This is thought to explain the multitude of auxin functions that require gene regulation to be executed. Whereas TIR1 acts as a receptor enabling gene regulation and induces lasting physiological changes, such functions are less obvious for the small glycoprotein ABP1 dimer.

In an ongoing study to generate viable mutants of ABP1 in order to provide a 'missing link' between the lethal abp1 knockouts and the wild-type, we performed experiments on heterozygous *abp1/ABP1* plants. The heterozygous plants are viable, whereas homozygosity leads to embryo lethality (Chen *et al.,* 2001b). Surprisingly, heterozygous plants showed physiological and morphological features that clearly deviated from wild-type. In addition, even as early as 30 min after auxin challenge, a number of *IAA* genes and other early auxin-regulated genes were up-regulated to a much lower extent in *abp1/ABP1* seedlings compared to wild-type seedlings.

RESULTS

Morphological differences and physiological responses in *abp1/ABP1* mutants

We grew seeds from heterozygous *abp1/ABP1* plants on kanamycin-containing agar plates under a 16 h/8 h light/ dark cycle to eliminate wild-type plants (Ws background), before transferring resistant seedlings to soil. Resistant plants appeared to be smaller than wild-type plants (data not shown). This observation prompted us to investigate phe-notypic properties of the *abp1/ABP1* plants. PCR genotyping with primers against the insertion allele of ABP1 (Chen *et al.,* 2001b) confirmed that resistant plants were heterozygous for *abp1*. Furthermore, viable seeds from *abp1/ABP1* plants segregated 2:1 into resistant and wild-type seedlings on kanamycin-containing plates. Siliques of *abp1/ABP1* contained approximately 25% non-viable white seeds, as described by Chen *et al.* (2001b). Of 700 white seeds plated on agar, only one seed developed into a white but non-viable seedling and another seed resulted in a viable green seedling, which was dwarf and died during early vegetative development (Figure S1).

When we grew seeds from an *abp1/ABP1* plant on kanamycin-free upright agar plates, we observed two seedling phenotypes: seedlings with roots growing down-wards, with only a small slanting angle, and seedlings with a strong slanting angle and roots that grew in a wavy pattern. Seedlings with a strong slanting angle were transferred to new agar plates after 4 days and grown side by side with wild-type seedlings treated the same way (Figure 1a,b). After 7 days, the selected mutant seedlings had a greater slanting angle (Figure 1a,b) and longer hypocotyls (Fig-ure 1c) compared to wild-type seedlings. The greater slanting angle suggested that heterozygous roots might be agravitropic. Therefore, we tested hypocotyls of 3-day-old dark-grown seedlings and roots of 14-day-light-grown seed-lings for defects in gravitropism by tilting the agar plates through 90° (Figure 1c–f). No pre-selection on kanamycin-free plates was performed prior to the experiment in order not to disturb or wound the seedlings as a result of transfer to a second medium. Seeds from wild-type and *abp1/ABP1* plants were tested as separate populations. The distribution of bending angles of wild-type plants had a single peak centred at approximately 80° for hypocotyl gravitropism and approximately 90° for root gravitropism after 24 h. The population segregating for *abp1* showed a peak at an angle

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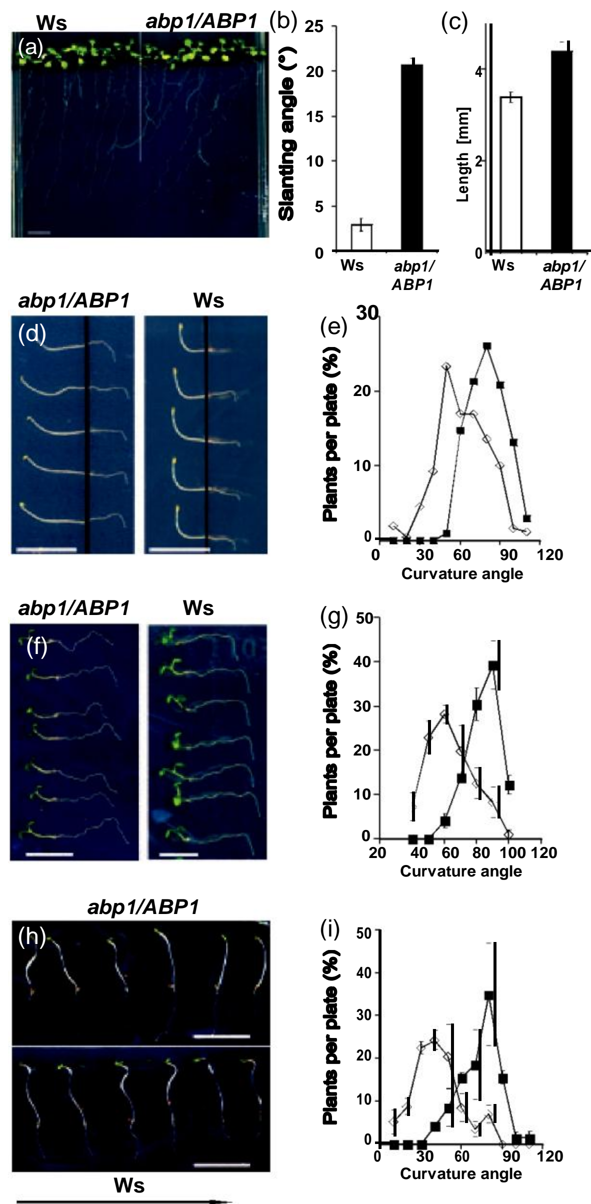


Figure 1. Phenotype and responses to gravity and light in wild-type Ws and heterozygous *abp1/ABP1* seedlings.

(a) Ws seedlings (left) and *abp1/ABP1* seedlings (right). Scale bar = 1 cm.

(b) Slanting angles of seedlings grown for 7 days in the light. White bar, Ws; black bar, *abp1/ABP1*. Values are means ± SE (n = 20, P < 0.01 for mutant versus wild-type).

(c) Hypocotyl length of 7-day-old light-grown seedlings. White bar, Ws; black bar, *abp1/ABP1*. Values are means ± SE (n = 20, P < 0.01 for mutant versus wild-type).

(d) Representative images showing the gravitropic response of 4-day-old light-grown Ws seedlings and an *abp1/ABP1*:Ws segregating population after 24 h. Scale bar = 1 cm.

(e) Gravitropic response of the hypocotyls of dark-grown 4-day-old seedlings. Seedlings were grown on vertical 10 · 10 cm agar plates in the dark, plates were tilted by 90°, and gravitropic angles were determined after 24 h. Intervals were defined comprising all seedlings with angles from 0–10 and 11–20°, etc. The frequency is expressed as the percentage of plants per plate in each group, and the means were calculated from all plates for each data point. Nine plates per assay with >180 total of seedlings per genotype were counted. Values are means ± SE. Open diamonds, seeds from a kanamycin-resistant *abp1/ABP1* plant; filled squares, Ws.

(f) Representative images showing the gravitropic response of 7-day-old light-grown Ws seedlings and an *abp1/ABP1*:Ws segregating population after 24 h. Scale bar = 1 cm.

(g) Gravitropic response of roots of 7-day-old light-grown seedlings after 24 h. Growth and quantification were performed as described in (e). Four plates per genotype with a total of 123 wild-type seedlings and 96 seedlings from seed collected from an *abp1/ABP1* were counted. Values are means ± SE. Open diamonds, seeds from a kanamycin-resistant *abp1/ABP1* plant; filled squares, Ws.

(h) Representative images showing the phototropic response of 4-day-old dark-grown Ws seedlings and an *abp1/ABP1*:Ws segregating population after 10 h. The arrow shows the direction of light. Scale bar = 1 cm.

(i) Phototropic response of the hypocotyls of dark-grown 4-day-old seedlings. Seedling growth and quantification were performed as described in (e). Four plates per genotype with a total of 95 wild-type seedlings and 80 seedlings from seeds collected from an *abp1/ABP1* plant were counted. Values are means ± SE. Phototropism was induced by lateral blue light (10 μE) from an LED light source (CFL, Plant Climatics GmbH, http://www.plantclimatics.de). Open diamonds, seeds from a kanamycin-resistant *abp1/ABP1* plant; filled squares, Ws.

of 50° and a smaller peak at 70–80° for hypocotyls, consistent with a segregation ratio of 2:1 for heterozygous versus wild-type plants (Figure 1d,e). Similar results were obtained for the gravitropic response of roots. While wild-type root bending angles peaked at 90°, the segregating population showed a peak at 60°, with a shoulder at 90° (Figure 1f,g). We then tested hypocotyl phototropism in dark-grown seedlings in a segregating population by application of 10 μmol m^{-2} s^{-1} of lateral blue light for 10 h. We again found a strong and uniform response in wild-type seedlings, with a peak of bending angles at approximately 80°, while a major response angle at 40° and a minor peak at 80° was observed in *abp1/ABP1* progeny seedlings, reflecting the 2:1 segregation of this population (Figure 1h,i). The *abp1* mutation therefore results in defects of gravitropism and phototropism, both developmental processes that are mainly controlled by auxin.

We tested auxin sensitivity in heterozygous and wild-type seedlings placed side by side on upright agar plates containing increasing auxin concentrations. Only small differences between wild-type and mutant in the length of the main root, lateral root formation and lateral root density were found in response to auxin (Figure 2a–c). These small differences were reproducible and may indicate a slight insensitivity of root and hypocotyl growth to auxin in the mutant.

Plants selected on kanamycin agar and later grown in the greenhouse were smaller than wild-type plants. We therefore investigated apical dominance in *abp1/ABP1* plants grown under long- and short-day conditions (Figure 3). The primary inflorescence of wild-type plants was always thicker than the secondary inflorescences (Figure 3a,c). The progeny from an *abp1/ABP1* plant grown under long-day conditions segregated into plants that showed the same distinction between primary and secondary inflorescences

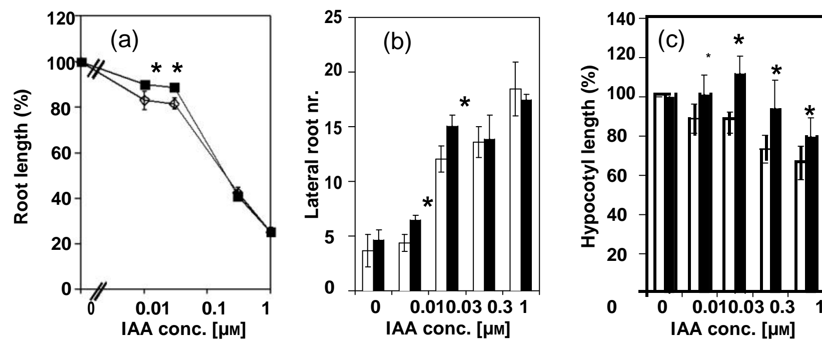


Figure 2. Auxin sensitivity of wild-type Ws and *abp1/ABP1* seedlings.

All seedlings were grown on vertical agar plates without auxin for 4 days, and then transferred to plates containing increasing concentrations of IAA. The *abp1/ABP1* seedlings were selected from the segregating population after 3 days on the basis of their strong slanting angle, and both Ws and mutant seedlings were transferred to a fresh plate for 4 days. Response to auxin of (a) the relative length of the main root, (b) lateral root number, and (c) relative hypocotyl length. Values are means ± SE (n = 20). Filled bars/filled squares, *abp/ABP1* plants; open bars/open diamonds, Ws. Asterisks indicate values that are significantly different from wild-type (P < 0.01).

Figure 3. Apical dominance of wild-type Ws and heterozygous *abp1/ABP1* plants grown under long- (16 h/8 h light/dark) or short-day condi-tions (8 h/16 h light/dark).

Representative wild-type Ws plant (a) and heterozygous *abp1/ABP1* plant (b), both grown under long-day conditions. Red arrow, main inflorescence; yellow arrows, lateral inflorescences. Plant genotypes were determined by PCR. Note the lower number of rosette leaves and absence of a prominent main stem in mutant plants.

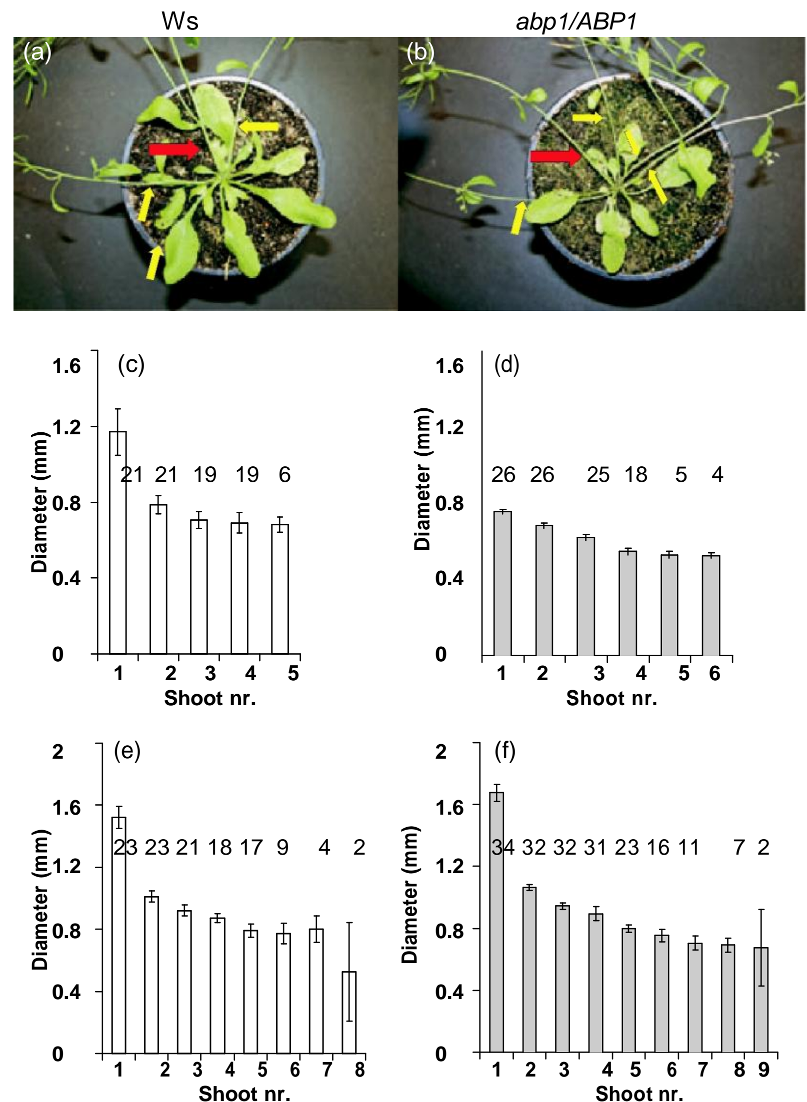
(c) Inflorescence thickness and inflorescence number of wild-type Ws plants grown under long-day conditions. Values are means ± SE (n as indicated).

(d) Inflorescence diameter and total number of inflorescences of heterozygous *abp1/ABP1* plants grown under long-day conditions. Values are means ± SE (n as indicated).

(e) Inflorescence diameter and inflorescence number of wild-type Ws plants grown under short-day conditions (n as indicated).

(f) Inflorescence diameter and total number of inflorescences of heterozygous *abp1/ABP1* plants grown under short-day conditions (n as indicated).

Plant genotypes were determined by PCR. Numbers on top of bars are the total number of branches for the given class. The results shown are from one of two experiments.



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described above, and plants with a strongly reduced diameter of the primary inflorescence but an equal diameter for the secondary inflorescences. PCR genotyping revealed that the plants with a thinner primary inflorescence were heterozygous for abp1 (Figure 3c,d). Under short-day conditions, the diameter of the primary inflorescences was nearly equal for wild-type and heterozygous plants; however, slightly more secondary inflorescences formed in the *abp1/ABP1* plants (Figure 3e,f). Taken together, these results indicate a decrease in apical dominance in heterozygous plants.

Heterozygous plants grown under long-day conditions not only had reduced apical dominance but often had fewer rosette leaves. Therefore, we determined the flowering time and rosette and cauline leaf numbers in plants grown under short- and long-day conditions (Figure 4). The photograph (Figure 4a) taken shortly before the wild-type plants started flowering shows that the population of seeds grown from a kanamycin-resistant *abp1/ABP1* plant segregated into approximately one-third that were not as yet flowering and two-thirds that were flowering. When plants of the segregating population were sorted by PCR genotyping, the early-flowering plants had an *abp1/ABP1* genotype, whereas the late-flowering plants were homozygous for the wild-type allele. Under short-day conditions, heterozygous *abp1/ABP1* plants flowered approximately 5 days earlier than wild-type plants, and rosette leaf numbers were lower in heterozygous plants than in wild-type plants (Figure 4b,c). Under long-day conditions, flowering occurred only slightly earlier in heterozygous plants ($P < 0.05$) and they had fewer rosette leaves (not statistically significant) (Figure 4d,e).

Gene regulation in *abp1/ABP1* mutants

Differences in phenotypes caused by altered auxin-related functions result from differential gene regulation. To investigate transcription of early auxin-regulated genes in response to auxin application, wild-type seedlings were

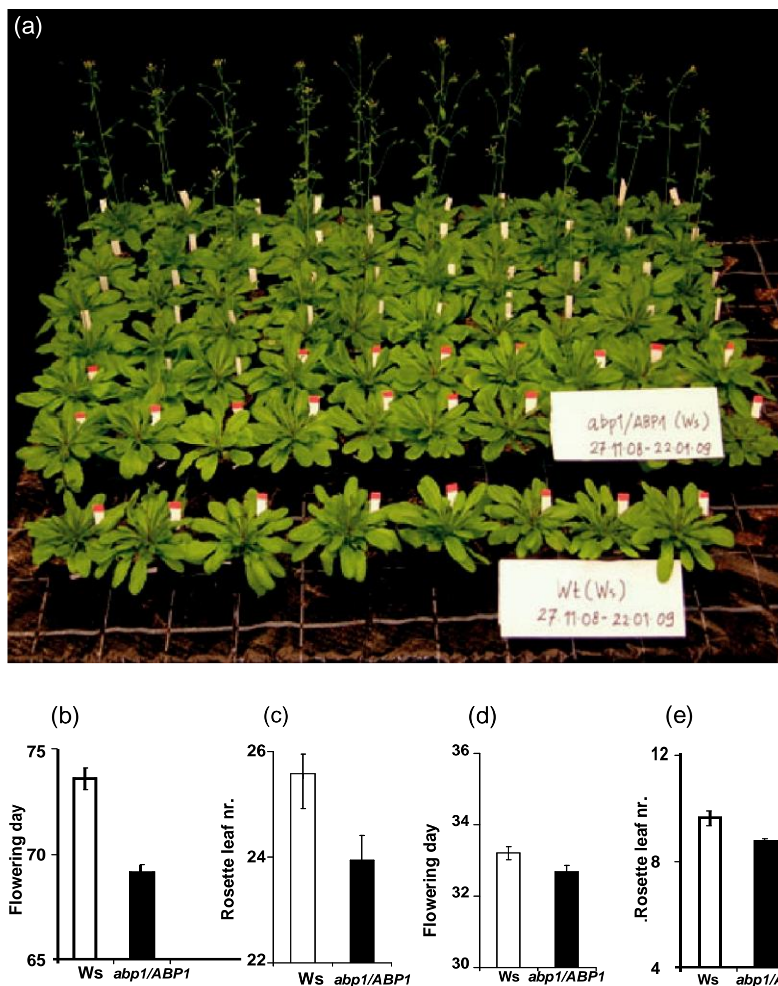

Figure 4. Early-flowering phenotype of wild-type Ws and heterozygous *abp1/ABP1* plants grown under short-day conditions (8 h/16 h light/dark) (a-c) or long-day condition (16h/8 h light/dark) (d, e).

(a) Plants from seeds of a kanamycin-resistant heterozygous *abp1/ABP1* plant and Ws wild-type plants, as indicated, grown under short-day conditions. Plants were ordered as follows: those with open flowers were placed at the back (small white tags) and non-flowering plants were placed at the front (small red tags). All plants were PCR-genotyped prior to statistical analysis. (b, c) Flowering date (b) and number of rosette leaves (c) of plants grown under short-day conditions. Open bars, wild-type Ws (n=31; $P < 0.05$ for flowering date and number of rosette leaves, by Student's t test). Filled bars heterozygous *abp1/ABP1* plants (n = 37; $P < 0.01$ for flowering date and $P < 0.05$ for number of rosette leaves, by Student's t test). The results shown are from one of two experiments.

(d, e) Flowering date (d) and number of rosette leaves (e) of plants grown under long-day conditions. Open bars, wild-type Ws (n = 34; $P < 0.05$ for flowering date and number of rosette leaves, by Student's t test). Filled bars, Heterozygous *abp1/ABP1* (SD: n = 26, $P < 0.05$ for flowering date and number of rosette leaves, by Student's t test). The results shown are from one of three experiments.

Flowering date was recorded as the time of opening of the first flower. Rosette leaves and cauline leaves were counted at the time of bolting. The numbers of cauline leaves did not differ between mutant and wild-type. Values are means ± SE.

grown in half-strength liquid MS medium and *abp1/ABP1* progeny seeds were grown in medium containing kanamycin. After 14 days, resistant seedlings were selected, and grown for 5 more days in kanamycin-free half-strength liquid MS medium, followed by auxin treatment. Wild-type seedlings were processed identically, omitting kanamycin throughout the selection procedure. At 30 min after treatment with 0.1 μM IAA, none of the seven IAA genes tested was up-regulated in *abp1/ABP1* seedlings, but in the wild-type, I*AA19* and *IAA20* were more than tenfold up-regulated and *IAA11, IAA13* and I*AA14* were approximately two- to threefold up-regulated (Figure 5a). When seedlings were treated with 1 μM IAA, 11 of the 12 genes tested showed up-regulation in wild-type seedlings (Figure 5b), while five genes (*IAA2, IAA14, SAUR9, SAUR15* and *SAUR23*) were not up-regulated at all in heterozygous seedlings and six were up-regulated, but to a lesser extent than in wild-type. *IAA12* was down-regulated in wild type (Braun *et al.,* 2008) but not in *abp1/ABP1* seedlings. Only *IAA11* and *IAA19* expression was up-regulated to a similar level in heterozygous and wild-type seedlings in 1 μM IAA. Transcriptional stimulation was again generally higher after treatment with 10 μM IAA, and the expression levels for five of the 12 genes tested (*IAA11, IAA13, IAA14, SAUR15, ABP1*) were similar in heterozygous and wild-type seedlings, while the other genes were transcribed at lower levels in *abp1/ABP1* than in wild-type seedlings (Figure 5c). *IAA12* was slightly down-regulated by auxin in wild-type seedlings and weakly up-regulated in the

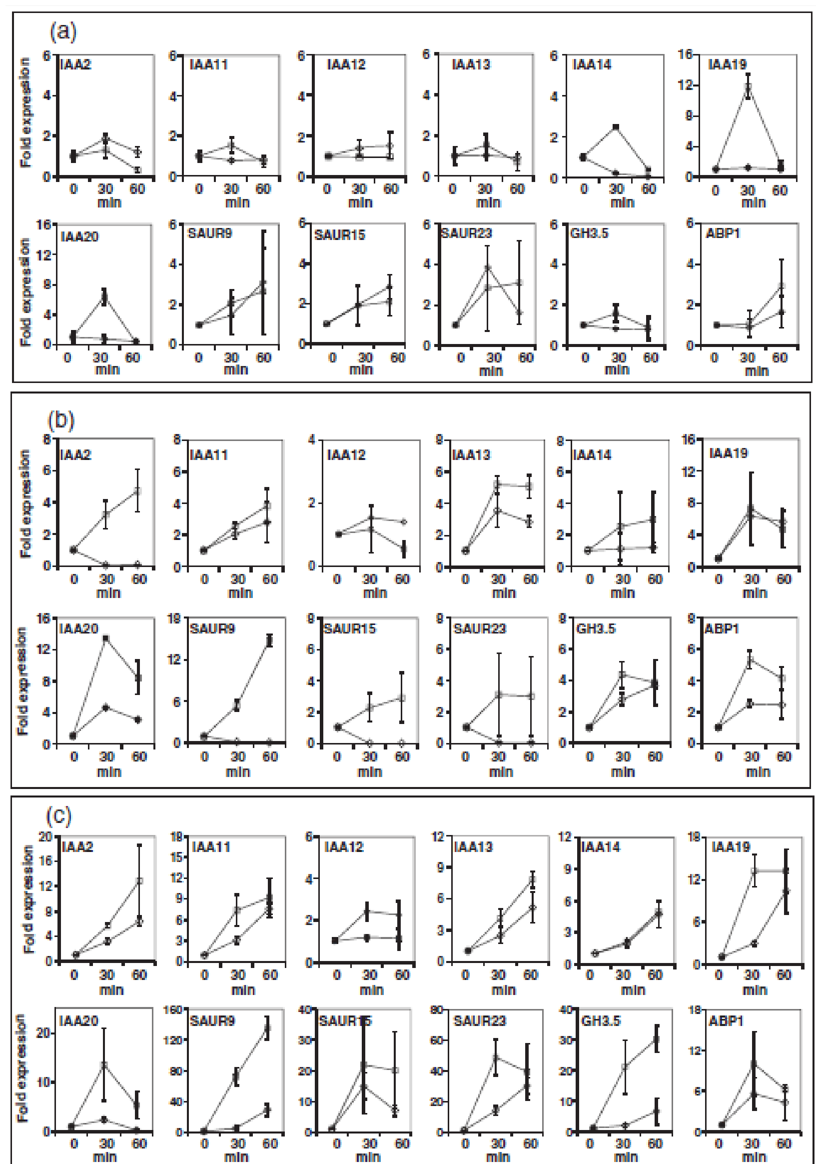

Figure 5. Regulation of early auxin-regulated genes and ABP1 in light-grown wild-type (Ws) and *abp1/ABP1* seedlings.

Wild-type seedlings were grown on half-strength MS agar, and seeds from a kanamycin-resistant *abp1/ABP1* plant were grown in the same medium containing 50 μg ml^{-1} kanamycin. After 14 days, heterozygous seedlings were selected as kanamycin-resistant, washed three times in the medium, and transferred to fresh liquid half-strength MS medium, as were the wild-type plants, but omitting the kanamycin, and grown for another 5 days. The plants were then treated with fresh medium containing either 0.1, 1 or 10 μM IAA for the times indicated. For details of RNA extraction and real-time PCR quantification, see Experimental Procedures. The results are from three (a, c) or two (b) biological treatments with three technical replicates for each measure-ment. Statistical analysis was performed as described by Pfaffl and Horgan G.W. (2002). (a) Treatment with 0.1 μM IAA. (b) Treatment with 1 μM IAA. (c) Treatment with 10 μM IAA. Wild-type, open squares; *abp1/ABP1*, open diamonds.

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mutant seedlings. In conclusion, transcriptional regulation of early auxin-response genes in heterozygous seedlings was less auxin-sensitive than in wild-type seedlings. When we compared expression of all tested genes in wild-type and mutant seedlings without auxin treatment, we found near-identical values for each, with differences of <4%. Surprisingly, ABP1 was itself an early auxin-regulated gene (Figure 5b,c). As for other auxin-inducible genes, ABP1 was up-regulated to a lesser extent by auxin in heterozygous mutant seedlings compared with the wild-type.

Auxin transport in *abp1/ABP1* mutants

The apparent lower auxin sensitivity in heterozygous *abp1/ ABP1* seedlings, as revealed by the transcriptional measurements, could either be explained by ABP1 interference with auxin perception and signalling, or, alternatively, by a lower auxin content in critical tissues or slower uptake by the mutant seedlings. Therefore, we measured the amount of auxin taken up by seedlings treated with various auxin concentrations (0.1, 1 or 10 μM IAA) in the same way as for the quantitative transcript profiling (Figure 5). The initial auxin contents were indistinguishable between the mutant and wild-type, as was the final uptake into the seedlings for all tested concentrations (Figure 6a–c). The mean IAA concentration of untreated seedlings at 0 min was approximately 0.1 μM, and the mean internal concentration after 60 min at an external IAA concentration of 0.1 μM therefore represented an approximately 1.4 fold increase. At the higher external IAA concentrations, 1 and 10 μM, the mean internal concentration corresponded to the external concentration after 30 min. Thus uptake alone does not explain the results obtained in transcription measurements.

In a second assay, we measured the polar auxin transport of exogenously applied radioactive auxin in young roots. Acropetal (application at root base) and basipetal (applica-tion at root tip) transport were measured (Figure 7), and all seedlings were PCR-genotyped after the experiment. Polar auxin transport was sensitive to naphthylpthalamic acid (NPA), an inhibitor of auxin efflux catalysed by PIN proteins (Figure 7a,c). We found no statistically significant difference between wild-type and the *abp1/ABP1* mutant in terms of acropetal transport (Figure 7b), although it was slower in the mutant compared to the wild-type. However, in basipetal direction, polar auxin transport was significantly slower in the mutant (Figure 7d).

Gene regulation in *eir1* mutants

The defect in root basipetal auxin transport could be due to mis-regulation of a PIN protein. The localization and inverted polarity of PIN2 in the outer layer of cells in the root tips of *PIN2* mutants (Abas *et al.,* 2006 Wisniewska *et al.,* 2006; Sukumar *et al.,* 2009) suggested that transport activity of this PIN protein could be affected in the *abp1/ABP1* heterozygote. We therefore tested the expression of the same auxin-inducible test genes as for *abp1/ABP1* heterozygotes in the *PIN2* mutant *eir1* (Chen *et al.,* 1998; Luschnig *et al.,* 1998; Müller *et al.,* 1998) in the presence and absence of 1 μM IAA (Figure 8). Of 12 genes tested, three (*IAA2, IAA13* and *ABP1*) were more strongly induced in *eir1* than in the Col wild-type, the regulation of one (*IAA12*) was indistinguishable from that in the Col wild-type, and the regulation of two (*IAA20* and *SAUR9*) was indistinguishable during the first 30 min. In summary, the presence of a non-functional PIN2 protein caused similar consequences as in the heterozygous *abp1/ ABP1* mutant, but with a clearly different 'signature'.

DISCUSSION

Heterozygous *abp1/ABP1* mutants exhibit morphological alterations

Heterozygous *abp1/ABP1* plants were previously described as having a similar phenotype to the wild-type (Chen *et al.,* 2001b). Because the homozygous *abp1/ABP1* mutant is embryo-lethal, only experiments with seed mixtures of 2:1 heterozygous:wild-type seeds are possible, and this may obscure investigations of the morphological

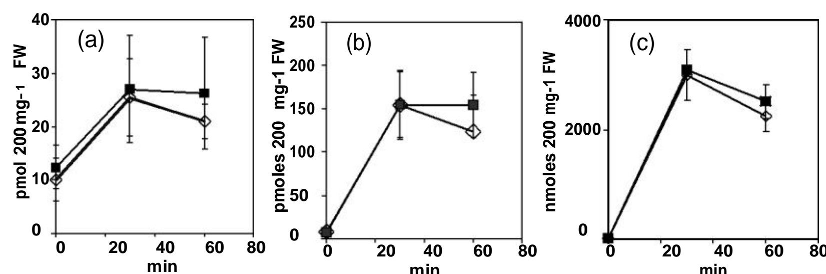

Figure 6. Auxin uptake into wild-type and *abp1/ABP1* seedlings.
Plants were grown, selected, and treated with auxin as described in the legend to Figure 5 and in Experimental Procedures. To stop auxin uptake, plants were quickly rinsed five times with sterile water, dried, and quickly frozen in liquid nitrogen. Analysis of IAA content was performed as described in Experimental Procedures, and the results of three experiments were pooled. Wild-type plants, filled squares; *abp1/ABP1*, open diamonds (n = 4–6). (a) Uptake of 0.1 μM IAA; (b) uptake of 1 μM IAA; (c) uptake of 10 μM IAA.

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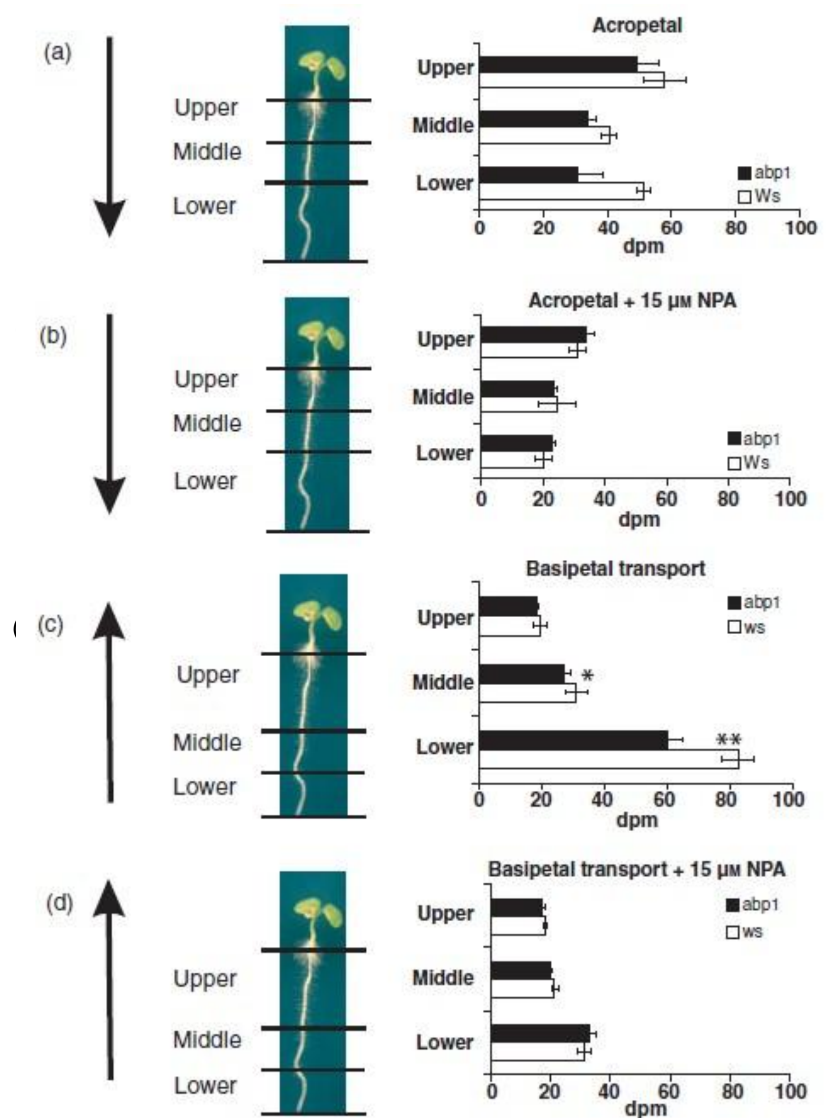

Figure 7. Polar auxin transport in wild-type and *abp1/ABP1* roots.

Two consecutive 5 mm pieces 1 mm away from the source and the residual third root piece were used as indicated on the left. Black bars, *abp1/ ABP1* mutant [n = 18 for (a, d), n = 19 for (b, c)]; white bars, wild-type [n = 12 for (a, c, d), n = 11 for (b)]. Values are means ± SE.

(a, b) Acropetal transport in the presence (a) or absence (b) of 15 μM NPA. There were no statis-tically significant differences.

(c, d) Basipetal transport in the presence (c) or absence (d) of 15 μM NPA. There were no statis-tically significant differences in (d). For (c), there were statistically significant differences from wild-type in the middle segment (*P < 0.05) and the lower segment (**P < 0.001).

phenotype. However, we found that heterozygous seedlings have a waving and slanting root phenotype. Plants with similar phenotypes are often affected in auxin-related processes such as gravity perception or auxin physiology-related processes, or, alternatively, have defects in microtubule-associated proteins (Sedbrook and Kaloriti, 2008). The *abp1/ABP1* mutant had a defect in the gravitropic response of the root, which probably caused the root slanting.

The heterozygous mutants also had longer hypocotyls, which could be related to defective auxin or light signaling (Gray *et al.,* 1998; Ljung *et al.,* 2001; De Grauwe *et al.,* 2005). Additionally, their phototropic response was impaired. In these respects, the *abp1/ABP1* seedlings resemble mutants that are defective in phototropin-triggered phototropism. phototropin1 mutants exhibit long hypoctyls and defective phototropism (Chen *et al.,* 2008), hence an association between the long-hypocotyl phenotype of *abp1/ABP1* mutants and their phototropism defect seems possible.

In addition to altered gravitropism and phototropism and hypocotyl length, apical dominance was decreased in heterozygous plants (Figure 3), resulting in a semi-dwarf stature under long-day conditions. However, under short-day conditions, the size of the adult mutant plants was not different from that of adult wild-type plants, except for a slightly lower number of leaves in the early-flowering heterozygous plants, and the decrease in apical dominance was subtle (Figures 2 and 3). As the major contributor to apical dominance is auxin transport (Ongaro and Leyser, 2008), it is likely that the loss of apical dominance may be explained as a defect related to auxin physiology in the heterozygous *abp1/ABP1* mutant.

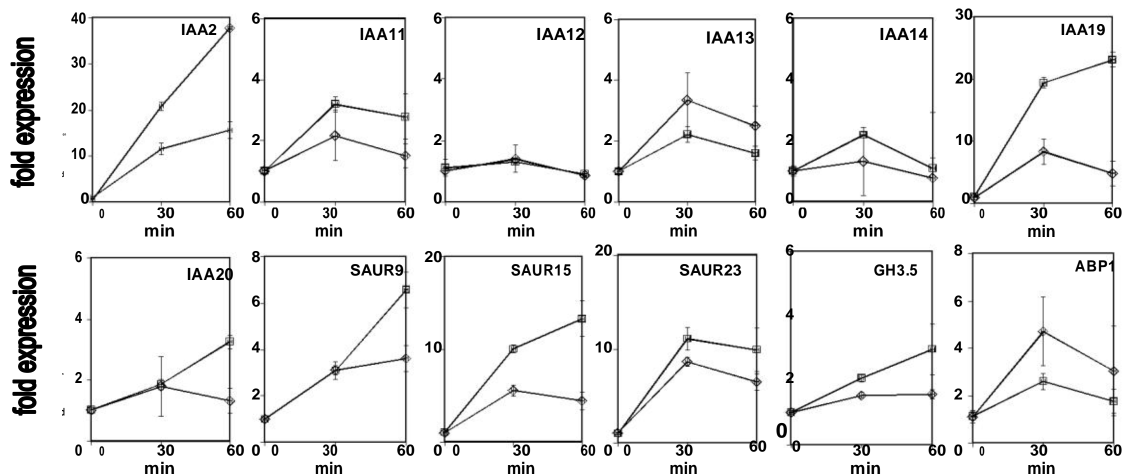

Figure 8. Regulation of early auxin-regulated genes and ABP1 in light-grown wild-type (Col) and *eir1* seedlings.
Wild-type and *eir1* seedlings were grown on half-strength MS agar. After 14 days, seedlings were washed three times in the medium, and transferred to fresh liquid half-strength MS medium and grown for another 5 days. Treatment with 1 μM IAA was performed using fresh medium for the times indicated. For details of RNA extraction and real-time PCR quantification, see Experimental Procedures. The results are from two biological treatments with three technical replicates for each measurement. Statistical analysis was performed as described by Pfaffl and Horgan G.W. (2002). Wild-type, filled squares; *eir1*, open diamonds.

Heterozygous *abp1/ABP1* mutants exhibit defects in gravitropism and phototropism

The physiological phenotype of heterozygous *abp1/ABP1* plants comprises defects in root and hypocotyl gravitropism, hypocotyl phototropism, polar auxin transport and an early-flowering phenotype. The common denominator for gravitropism and phototropism is regulation of polar auxin transport by PIN proteins (Petrásek *et al.,* 2006), and mutants in which both gravitropism and phototropism are defective are comparatively few. The efflux transporters PIN2 and PIN3 have been identified on the basis of the properties of knockout or other mutants as contributing to both gravitropism and phototropism (Müller *et al.,* 1998; Friml *et al.,* 2002). Furthermore, two auxin signaling mutants, namely *arf7* and *iaa19*, have been shown to be both agravitropic and aphototropic (Liscum and Reed, 2002). ARF7 and IAA19 are a transcription factor and a transcrip-tional co-factor, respectively, and the genes regulated by them, or a subset of these genes, must have a critical func-tion in growth in tropisms. All other gravitropic or photo-tropic mutants are mutants in either gravitropism or phototropism alone, and their potential functional links to ABP1 are therefore weaker than proteins with functions in both gravitropism and phototropism. Our data show slower basipetal auxin transport in *abp1/ABP* mutant roots, but acropetal transport was not significantly altered. PIN2-mediated basipetal auxin transport is required for root gravitropism (Wisniewska *et al.,* 2006; Abas *et al.,* 2006; Michniewicz *et al.,* 2007). We therefore suggest that ABP1 acts through the activity changes of PIN proteins on gravitropism and auxin-related functions observed in the heterozygous plants.

The heterozygous *abp1/ABP1* mutant and other mutants of the ABP1 gene strongly influence auxin function, including transcription of early auxin-regulated genes

The most surprising aspect of our findings was that the transcriptional regulation of all early auxin-induced genes tested was lower or slower in the heterozygous *abp1/ABP1* plants than in wild-type seedlings (Figure 5). Summarizing results from treatments with three IAA concentrations, the heterozygous plants can be considered as a partly auxin-insensitive mutant with respect to short-term gene regulation of early auxin genes. The changes in phototropism after 10 h, gravitropism after 24 h and basipetal polar auxin transport in the root after 18 h are relatively immediate responses, and reduced sensitivity was observed in all these tests. By contrast, in the 12-day growth assay in an auxin concentration series, we observed only a slight difference in auxin sensitivity of mutant seedlings compared with wild-type. Thus, ABP1 function is better revealed in studies of short-term regulation of auxin responses rather than tests over a longer period of time, in which a signaling network tends to dampen defects.

Our data on early auxin-induced gene regulation show that all tested *IAA* genes were mis-regulated in the heterozygous *abp1/ABP1* mutant, including *IAA19,* the *SAUR* genes, *GH3.2* and *ABP1* (Figure 5). In an attempt to elucidate the mechanism behind this mis-regulation, we investigated the regulation of these test genes in the PIN2 mutant *eir1* (Chen *et al.,* 1998; Luschnig *et al.,* 1998; Müller *et al.,* 1998). Mis-regulation of PIN2 could be the reason for the observed defect in root basipetal auxin transport (Abas *et al.,* 2006; Wisniewska *et al.,* 2006; Sukumar *et al.,* 2009). Indeed, eight of 12 genes tested were up-regulated to a

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lower extent in the *eir1* background compared with the wild-type, one gene was not differentially expressed, and three were up-regulated by auxin to a greater extent (Figure 8). A defect in *PIN2* could become manifest by defects in regulation of the same set of genes, suggesting that ABP1 and PIN2 occur in largely overlapping regulatory pathways. Our suggestion is that ABP1 and PIN proteins cooperate in a tight regulatory circuit (Figure 9). The differences in the 'signature' of regulation of early auxin genes between *abp1/ABP1* and *eir1* could be explained by participation of additional proteins that regulate cellular auxin concentration. The most likely candidates are other PIN proteins and AUX1 or LAX proteins. Alternatively, mis-expresssion of early auxin genes could be due to a direct effect of ABP1 on TIR1-dependent IAA ubiquitination. However, there is currently no evidence for this second explanation. The mechanisms are not mutually exclusive (Figure 9). Common to all aspects of the *abp1/ABP1* phenotype is that they may be explained by changes in polar auxin transport regulation and local auxin concentration.

Braun *et al.* (2008) the tested transcription of early auxin-regulated genes after a minimum of 8 h of induction of anti-ABP1 antibody expression. Thirteen of 14 *IAA* genes tested were down-regulated transiently or for up to 48 h compared to the non-induced status. Among them, *IAA12* was down-regulated by auxin. These findings are consistent with our results. As we did not need to induce functional down-regulation of ABP1, we were able to monitor changes as soon as 30 min after auxin addition. Braun *et al.* (2008) did not investigate gravitropism and phototropism. In conclusion, our results support the notion that ABP1 is required for early auxin functions.

How can ABP1 'intrude' into the function of TIR1 as receptor regulating the early auxin-regulated genes?

Although auxin binding to ABP1 does undoubtedly occur (Napier *et al.,* 2002), it has gained little acceptance as an auxin receptor. A reason for this might be the lack of knowledge as to how exactly a potential ABP1-induced signal pathway is connected to the cytosol-based regulatory mechanisms of signal transduction. Although a number of such reactions have been shown, the receptor(s) was not unequivocally identified (Scherer and Andre´, 1989; Paul *et al.,* 1998; Tao *et al.,* 2002; Shishova *et al.,* 2007; Lanteri *et al.,* 2008). Furthermore, the postulated docking protein for ABP1 (Klämbt, 1990) needs to be identified for full understanding of ABP1 action. Interestingly, gene dosage effects, such as the haplo-insufficiency for ABP1 reported here, often relate to gene products that interact with other proteins strictly stoichiometrically (Veitia *et al.,* 2008), and are found for various human receptors (Fisher and Scambler, 1994). If ABP1 does indeed require a docking protein for its function, the observed haplo-insufficiency in the heterozygous plants could be due to stoichiometric imbalance.

Direct regulation of early auxin-regulated genes has been shown convincingly (Mockaitis and Estelle, 2008), but can the same genes regulated by TIR1-dependent ubiquitination of IAA proteins also be regulated by a different receptor? Our results show that ABP1 is required for the regulation of early auxin-regulated genes. However, further experiments are required in order to determine whether *ABP1* acts indepen-dently of the function of TIR1 and its homologs.

It has been speculated that the regulation of polar auxin transport by auxin might be independent of $SCF^{TIR1/AFB}$ signalling (Paciorek *et al.,* 2005), suggesting that an auxin receptor other than TIR1 and its homologs is required. We show here that not only is the transcription of early-regulated auxin genes altered in the heterozygous *abp1/ABP1* mutant, but also tropic responses that are commonly associated with regulation of polar auxin transport. These results were corroborated by the finding of reduced basipetal auxin transport in the roots of the heterozygous plants. Therefore, our findings identify ABP1 as a candidate receptor in auxin transport regulation, whereas other potential candidate receptors appear not to be linked to functions investigated here or other known auxin functions (Watanabe and Shimomura, 1998; Shimomura, 2006).

Good examples of auxin functions driven by local auxin accumulation as a result of transport are lateral root formation driven by AUX1, which concentrates auxin in a few pericycle cells initially (Péret et al., 2009), lateral organ formation at the apical meristem (Heisler and Jönsson, 2007), and many other developmental steps (Kleine-Vehn and Friml, 2008). The hypothesis of (very) local auxin concentration differences in auxin transport mutants may be applied to differences in subcellular concentration, as assumed for *pin5* mutants (Mravec *et al.,* 2009). The importance of polar auxin transport during embryo development shown by the requirement for several PIN proteins (Friml *et al.,* 2003) would be a good explanation for the failure of embryo development of *abp1/ABP1* homozygous mutants if ABP1 did indeed have a regulatory role in PIN-dependent auxin transport during embryogenesis (Chen *et al.,* 2001b). However, we found no difference in auxin uptake in *abp1/ABP1* mutants (Figure 6), meaning that no conclusion can be drawn regarding local differences in auxin concentration. We did find reduced basipetal auxin transport in heterozygous roots, explaining the defects in gravitropism and phototropism (Figure 7).

Because *ABP1* itself is an early auxin-regulated gene, regulation of ABP1 transcript levels could be achieved by $SCF^{TIR1/AFB}$-dependent gene regulation, and, vice versa, TIR1 signaling could be regulated by ABP1-dependent regulation of auxin transport and thus, local auxin concentration (Figure 9). This may be the mechanism by which signalling from a sensor for extra-cytoplasmic auxin, suggested to be ABP1, and by the receptor for intra-cytoplasmic auxin, TIR1, is coordinated throughout the plant.

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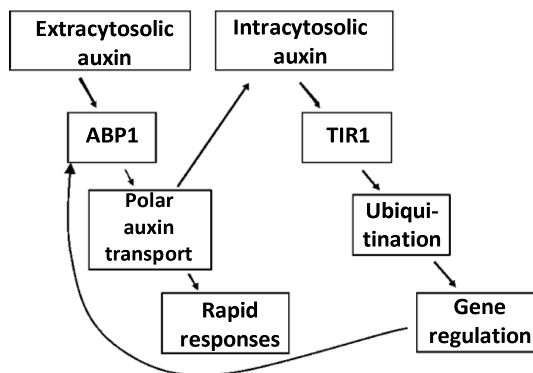

Figure 9 Model of ABP1 action and ABP1–TIR1 interaction.

EXPERIMENTAL PROCEDURES

Plant material

Hetozygous *abp1/ABP1* mutant seeds (stock number N6489) were obtained from the Nottingham Arabidopsis Stock Center (http://arabidopsis.info/), and these proved to be kanamycin-resistant. For long- or short-day experiments, seedlings were not selected on kanamycin agar but were sown directly on peat-based compost soil (Einheitserde, http://www.einheitserde.de/) containing 30% silica sand. The genotypes were determined by PCR.

Seeds from *abp1/ABP1* plants were sown on kanamycin-containing medium and transferred to kanamycin-free medium after 4 days of growth on upright agar plates. Afterwards, they were selected according to their slanting angle. The experiments shown in Figures 1(a,b) and 2 were performed this way on upright agar plates. In the experiments shown in Figure 1(c–e), all seedlings were planted on kanamycin-free medium and the results confirmed the segregation of 2:1 *abp1/ABP1* to wild-type (Chen *et al.,* 2001b). For quantifications, seedlings were scanned using a CanonScan 8800F (resolution of 600 dots per inch; Canon, http://www.canon-europe.com). Root lengths and angles were measured using AxioVisio LE version 4.6 software (Zeiss, http://www.zeiss.com/).

For transcription measurements and auxin uptake experiments (Figures 5 and 6), seedlings were grown in half-strength MS agar medium for 14 days under long-day conditions, the wild-type without kanamycin, and seeds from a kanamycin resistant abp1/ ABP1 plant in medium containing 50 µg ml^{-1} kanamycin. Then resistant *abp1/ABP1* seedlings were selected, washed three times for 5 min in medium without kanamycin, and grown for a further 5 days in half-strength MS liquid medium without kanamycin. Wild-type seedlings were treated the same way, but without kanamycin. For auxin treatment, the medium was removed and replaced by fresh medium without or with the IAA concentration indicated. Seedlings were blotted on filter paper and frozen in liquid nitrogen for further use.

IAA uptake measurements

For auxin uptake experiments, treated seedlings were quickly washed five times in sterile water, blotted and frozen in liquid nitrogen in 200 mg aliquots. To each sample, 1 ml of $CHCl_3/CH_3OH/H_2O$ (1:2:0.3) containing 40 pmol deuterated IAA (d_2-IAA) (Sigma, http://www.sigmaaldrich.com/) was added as an internal standard, and the mixture was shaken for 10 min at 70°C. Following centrifugation (18 000 g, 4°C, 5 min), the supernatant was collected, and the sediment was re-extracted with 0.5 ml $CHCl_3/CH_3OH$ (1:2) and pooled with the previous extract. Phase separation was induced by addition of 0.5 ml H_2O, and, after vortexing for 6 sec, samples were kept at -20°C for 30 min. After brief centrifugation, the upper phase was collected and reduced to approximately 250–300 µl in a Speedvac concentrator (Eppendorf, http://thermoscientific.com). The samples were acidified with 300 µl 0.2% trichlorofluoric acid, and extracted twice with 600 µl ethyl acetate/hexane (3:1) by vigorous vortexing for 30 sec. The organic phases were transferred to a glass vial and reduced to complete dryness in a Speedvac concen-trator. Samples were derivatized using 80 µl N-methyl-N-(trimethylsilyl)trifluoroacetamide (pyridine salt) with 1% trimethyl-chlorosilane (Fluka, http://www.sigmaaldrich.com) (1:1) for 30 min at 90°C then overnight at room temperature. The analysis was performed using a quadrupole GC-MS system (Agilent, http://www.agilent.com) by injection of 1 µl at an injector temperature of 250°C. With a split ratio of 1:1, the sample was loaded onto an HP-5MS column (Agilent) at 1.5 ml min^{-1}. The oven temperature was set to 100°C for 2 min, and gradually increased by 10°C per minute to 160°C, 3°C per minute to 193°C and 12°C per minute to 300°C, and held for 3 min. Identification of IAA and d_2-IAA was based on retention times and fragmentation patterns. Ions were detected by selected ion monitoring and quantified using ions m/z 202 (IAA) and 204 (d_2-IAA). Calculation of the IAA amounts was performed using the CHEMSTATION software (Agilent).

IAA polar transport

Auxin transport assays were performed using with 5-day-old seedlings grown from seeds of a kanamycin-resistant plant so that a 1:2 ratio of wild-type:heterozygous seedlings resulted. To test transport inhibition by NPA, seedlings were transferred to agar plates containing 15 µM NPA 18 h prior to application of radioactive auxin. For measurement of non-inhibited transport, seedlings were transferred to NPA-free plates. The roots were 1.5–2 cm long, and the assays were performed as described by Lewis and Muday (2009), using agar cylinders as a source of radioactive ^{14}C-IAA (Biotrend, http://www.biotrend.com). The final IAA concentration in the agar cylinder was 9 µM, corresponding to 0.5 µCi ml^{-1}. Starting 1 mm away from the source of radioactive auxin, two 5 mm long pieces were cut, and the residual root was used as the third part (see Figure 7). The activity was measured after incubating the samples overnight at 4°C in scintillation fluid. Seedlings were PCR-geno-typed using the hypocotyl and cotyledons.

Nucleic acid analysis

For quantitative RT-PCR, 4 µg of total RNA was prepared using a NucleoSpin® RNA plant kit according to the manufacturer's instructions (Macherey & Nagel, http://www.mn-net.com), and transcribed to first-strand cDNA using a RevertAid™ H Minus first-strand cDNA synthesis kit (Fermentas, http://www.fermentas. com). Primers were selected using PRIMER 3 software (http://www. broad.mit.edu/cgi-bin/primer/primer3_www.cgi) and NETPRIMER software (http://www.premierbiosoft.com/netprimer/netprlaunch/ netprlaunch.html), and checked for primer efficiency and against primer dimer formation. The primers used were 18S rRNA forward (5'-GGCTCGAA GACGATCAGATACC-3'), 18S rRNA reverse (5'-TCGGCATCGTTTA TGGTT-3'), *ABP1* forward (5'-ACGAGAAAATCATACCAATTCGGACTA ACC-3'), *ABP1* reverse (5'-GTATCTACGTAGTGTCACAAAACCTCA AC-3'), *IAA2* forward (5'-GGTTGGCCACCAGTGAGATC-3'), *IAA2* reverse (5'-AGCTCCGTCCATACTCACTTTCA-3'), *IAA11* forward (5'-CCTCCCTTCCCTCACAATCA-3'), *IAA11* reverse (5'-AACCGCCTTCCA TTTTCGA-3'), *IAA12* forward (5'-CGTTGGGTCTAAACGCTCTG-3'), *IAA12* reverse (5'-TTCCGCTCTTGCTG CCTTCA-3'), *IAA13* forward (5'-CACGAAATCAAGAACCAAACGA-3'),

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IAA13 reverse (5'-CACCGTAACGTCGAAAAGAGATC-3'), *IAA14* forward (5'-CCTTCTAAGCCTCCTGCTAAAGCAC-3'), *IAA14* reverse (5'-CCATCCATGGAAACCTTCAC-3'), *IAA19* forward (5'-GGTGACAA-CTGCGAATACGTTACC-3'), *IAA19* reverse (5'-CCCGGTAGCATCCG ATCTTTTCA-3'), *IAA20* forward (5'-CAATATTTCAACGGTGGCTA TGG-3'), *IAA20* reverse (5'-GCCACATATTCCGCATCCTCTA-3'), *GH3.5* forward (5'-AGCCCTAACGAGACCATCCT-3), *GH3.5* reverse (5'-AAGCCATGGATGGTATGAGC-3'), *SAUR9* forward (5'-GACG TGCCAAAAGGTCACTT-3'), *SAUR9* reverse (5'-AGTGAGACCCAT CTCGTGCT-3'), *SAUR15* forward (5'-ATGGCTTTTTTGAGGAGTTTC TTGGG-3), *SAUR15* reverse (5'-TCATTGTATCTGAGATGTGACTGTG-3'), *SAUR23* forward (5'-ATGGCTTTGGTGAGAAGTCTATTGGT-3), and *SAUR23* reverse (5'-TCAATGGAGCCGAGAAGTCA-CATTGA-3'). Quantitative PCR reactions were performed using 1 µl of sixfold diluted cDNA, 200 nM primers and 0.2· Power SYBR® Green PCR master mix (Invitrogen, http://www.invitrogen.com/) in a StepOnePlus™ system (Applied Biosystems, http://www.applied-biosystems.com/). For each pair of primers, the threshold value and PCR efficiency value were determined using cDNA diluted tenfold each time in five dilution steps. For all primer pairs, including the internal standard gene, 18S rRNA, the PCR efficiency was >99%. The specificity of PCR amplification was examined by monitoring the presence of a single peak in the melting curves for quantitative PCR. Amplicons were checked for fragment length on 4% agarose gels. For each determination, two to three biological repeats and three technical replicates for each determination were performed for the subsequent PCR reaction. Relative expression was calculated according to the DDC_t method using the equation: relative expression = $2^{-[\Delta C_{tsample} - \Delta C_{tcontrol}]}$, where $\Delta C_t = C_{t(sample\ gene)} - C_{t(reference\ gene)}$ and C_t refers to the threshold cycle determined for each gene in the early exponential amplification phase (Livak and Schmittgen, 2001). The expression level for the control treatment was set as 1-fold. For statistical analysis, REST 2008 software (Pfaffl *et al.,* 2002) was used.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Seed and seedling morphology of heterozygous *abp1* plants.

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SUPPLEMENTARY INFORMATION

Supporting information of chapter 2, Effendi Y., Rietz S., Fischer U., Scherer G.F.E. 2011

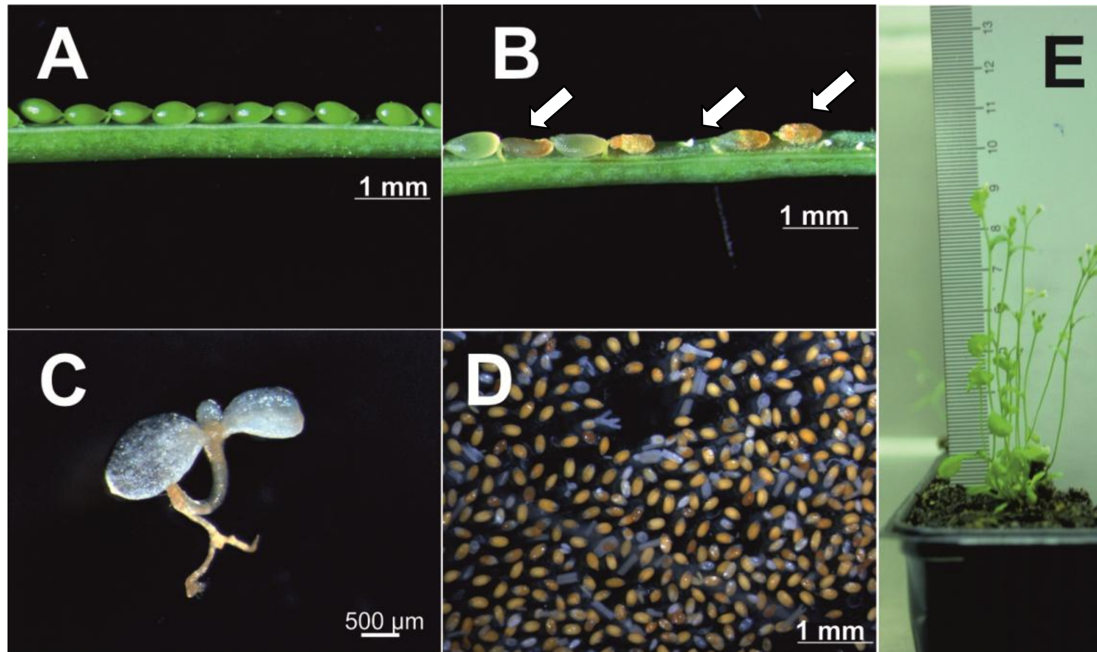

Figure 1S. Seed and seedling morphology of heterozygous *abp1* plants.

(A) Seeds in wild type silique; (B) Seeds in heterozygous *abp1/ABP1* silique. Arrows indicate white seed and dead embryos. Non-developed seeds show only the funiculus (white arrow) on siliques (C) White seeds collected from heterozygous *abp1* plants and germinated did not develop. (D) Survivor seedling grown from a white seed stayed white and non-viable. (E) Single green plant obtained from a white seed. The plant was dwarfish and bushy (8 branches) and died prematurely. No seeds could be obtained.

CHAPTER 3

AUXIN BINDING-PROTEIN1 (ABP1), a receptor to regulate auxin transport and early auxin genes in an interlocking system with PIN proteins and the receptor TIR1

Short Communication

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AUXIN BINDING-PROTEIN1 (ABP1), a receptor to regulate auxin transport and early auxin genes in an interlocking system with PIN proteins and the receptor TIR1

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Key words: AUXIN-BINDING PROTEIN1 (ABP1), auxin transport, early auxin-regulated genes, PIN proteins, TIR1, two-receptor concept

Compared to the past 10 years, a flurry of publications, reviews and experimental papers on ABP1 appeared in the last couple of years. Certainly, the reason is that new methods and conceptual approaches appeared to tackle the questions posed by this enigmatic auxin-binding protein. Part of the enigma is the obvious central importance of ABP1, documented by the embryo-lethal property of the homozygous T-DNA insertion into this gene.[1] At the same time, this very property hindered progress in studying ABP1. Another delaying influence on ABP1 research was the fact that regulation of early auxin genes was fully explained by the mechanism provided by TRI1, the second auxin receptor.[2-4]

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So, what makes a binding protein a receptor? According to Jones and Sussman (reviewed in ref. 5) a receptor binds the ago-nist reversibly, stereospecifically, selectively, and in a defined stoichiometry. The K_D should correspond to concentrations which are able to induce responses when applied in physiological experi-ments. Ideally, mutants of the prospective receptor should explain why they are critical to the action of the receptor. The first action of the receptor upon agonist binding should be to undergo a conformational change and trigger a change of activity in the next protein in a signal network. The 3-dimensional structure of TIR1 binding a fragment of IAA17 and auxin explains the action of TIR1 even though an induced conformational change was not directly proven.[4] Recently, for the C-terminus of ABP1 a flip-flop-type of movement upon binding auxin was predicted by modeling,[6] filling a long-standing gap of knowledge, even though the mobility of the C-terminus was indicated.[7] The weakness of ABP1 research is that a mechanism of action cannot be derived from its structure as it is a small dimeric glycoprotein binding to the extracytosolic side of membranes.[8] For signal transduction, a "docking protein" is required traversing the membrane so that the signal can induce actions on the other, the cytosolic side.[9]

Recent publications provide new answers to long-standing questions on ABP1 as being a receptor.[10-13] First, plants suppress-ing ABP1 protein by ethanol-induced expression of anti-ABP1 FAB fragments of specific antibodies were developed. These plants exhibited a number of auxin physiology-related defects so that, for the first time, viable inducible *abp1* mutants were generated.[10] Robert et al. extended research on earlier findings that auxin rapidly inhibits endocytosis of PIN1 and PIN2 with the consequence that efflux transport of auxin catalysed by PIN proteins is enhanced. Inhibition of auxin efflux transport by auxin was too rapid to be explained by transcription and translation of new PIN proteins but required an auxin receptor which could not be TIR1.[14] Robert et al. showed explicitly that endocytosis inhibition of PIN proteins is insensitive to cycloheximide so that auxin efflux enhancement does not depend on newly synthe-sized PIN proteins. They showed that endocytosis inhibition by auxin is independent on TIR1-like receptors in quadruple *tir1/ afb* mutants but dependent on intact ABP1, evidenced by using mutants for both receptors. This strongly ties ABP1 to polar auxin transport regulation. Xu et al. investigated auxin-induced small G-protein signaling in epidermal cell pattern formation as another response, too rapid to be explained by TIR1. They showed that ABP1 is the receptor for this G protein-binding response that was detectable after 1 min. Again, using the com-parison of wild type to *abp1* mutants lead to the conclusion that ABP1 is the receptor for this response. Similarly, tip-growing root hairs require binding of small G-proteins coupled to the receptor kinase *FERONIA* for auxin-induced tip growth of root hairs.[15] Thus, auxin signaling uses a two-receptor system which is the best known among several others in plants.[16]

Our own work discovered that a heterozygous *abp1/ABP1* plant is an auxin mutant in its own right.[13] Heterozygous *abp1/ABP1* plants are defect in a range of typical auxin responses requiring polar transport: phototropism, gravitropism, apical dominance and basipetal auxin transport in the root. Most importantly, all 12 early auxin genes that we investigated were upregulated less efficiently in the mutant than in wild type 30 min after NAA application. Moreover, *ABP1* itself was found to be an early auxin-regulated gene. Early auxin gene regulation was also tested in the *eir1/pin2* background, *eir1* being a loss-of-function allele of the *PIN2* gene. PIN2 is a major regulator of polar auxin transport in phototropism and gravitropism.[17,18] Again, in *eir1* almost all 12

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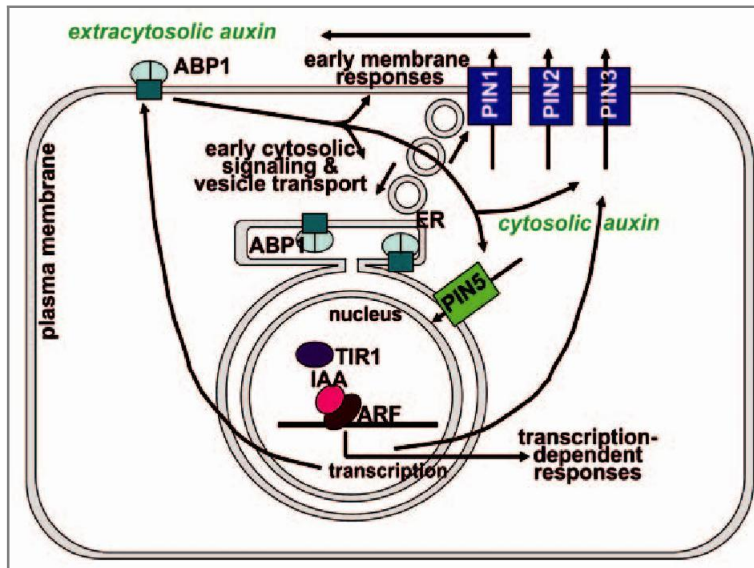

Figure 1. model of auxin signal transduction. the receptor ABP1 is depicted as a dimer in complex with a transmembrane "docking protein." ABP1 triggers a number of typical signaling pathways in the cytosol (reviewed in ref. 16). these responses include the phosphorylation status of Pin proteins (not shown in drawing) and control of endocytosis/exocytosis balance.[21] Several Pin proteins, including Pin1, Pin2 and Pin3 are integrated into cell polarity and auxin efflux transport to regulate extracytosolic auxin concentration and, thereby, polar auxin transport and tropisms. indirectly, they may also regulate cytosolic auxin concentration as is assumed for Pin5 which is localized to the Er. this localization is postulated to increase nuclear auxin concentration where it is sensed by the receptor tir1. Formation of the ternary complex [tir1 x auxin x iAA] leads to ubiquitination of iAA proteins and their hydrolysis by the proteasome. At least Pin2 and Pin3 are regulated by phosphoryation and rapid transcriptional responses. tir1 is also assumed to be the relevant receptor for *ABP1* transcriptional regulation so that ABP1, Pins and tir1 are a completely interlocking system of two receptors linked by auxin transport. other systems of two or more receptors for one signal where one receptor is closely associated to regulation of proteasomal activity are known or likely in plants.[18]

is supposed to enhance auxin concentration in the nucleus although auxin diffusion through the inner nuclear membrane was not explained[20] whereas PIN1- and PIN2-directed auxin efflux would enhance auxin concentration in the cell wall,[14] sensed there by ABP.[11-13] TIR1, by transcriptionally regulating *ABP1*, would be coupled to ABP1. Regulation at the post-translational level of several PIN proteins is also described which will make the whole system even more complex.[21] The coupling to receptors of other auxin transport regulating proteins, AUX1 and LAX, remains to be defined but, undoubtedly, they contribute to auxin concentration regulation.

Previously, we had not tested expression of *PIN* genes as potential early auxin genes. *PIN2* and *PIN3* are rapidly upregulated by auxin whereas *PIN1* is only weakly upregulated during 1 h in wild type seedlings (**Fig. 2**). In heterozygous *abp1/ABP1* and in *eir1* mutant plants these genes are not upregulated within 1 h after NAA application or less upregulated like *PIN2* in *abp1/ABP1* (**Fig. 2B and C**). This transcriptional response is aberrantly slow as compared to the respective wild types so that *abp1/ABP1* and *eir1* plants are both multiply damaged in auxin transport. *PIN5* was downregulated by about 50% by auxin in the Ws wild type but not in the Col wild type but the respective mutants *abp1/ABP1* (Ws) and *eir1* (Col) did not differ significantly from wild types in *PIN5* transcription in the presence of auxin (**Fig. 2D and H**). Our results on transcription generally agree with previous ones.[20]

How slight downregulation of PIN5 at the ER genes of the genes tested were mis-regulated demonstrating that mis-regulation of polar auxin transport leads to changes in auxin-induced transcription. Transcription regulation is the function of TIR1 and its AFB homolog. Quite fittingly, when the mutant *tir1* was discovered it was named "TRANSPORT INHIBITOR RESISTANT1" because it was screened as resistant to inhibitors of PIN-dependent auxin transport.[19] Thus, we showed that mutations in *ABP1* and *PIN2* have similar consequences on gene regulation as a rapid response. This suggests that they are operating in the same signaling chain or network in a closely linked logical position in that network. In fact, there could be several auxin transport proteins contributing to regulation of signal strength. The general agreement is that regulation of early auxin genes is executed by TIR1 and the homologous AFB proteins by inducing proteolysis of IAA proteins, co-repressors of early auxin gene regulation. Most likely therefore, besides PIN2 other PIN proteins are mediators between ABP1 and TIR1, as indicated in the scheme in **Figure 1**. Noteworthy is PIN5 which is localized to the ER membranes and hypothesized to concentrate auxin first in the perinuclear ER and somehow direct auxin into the nuclear cytosol,[20] where it can be sensed by TIR1. This PIN5 localisation

and upregulation of PIN2 and PIN3 at the plasma membrane is coordinating extracytosolic and cytosolic auxin concentration remains open. PIN2 and PIN3 are of major importance for directing polar auxin transport in tropisms and PIN5 may regulate nuclear auxin concentration more directly. Together however, this underscores how tightly interwoven the regulatory circuit of ABP1, PINs and TIR1/AFBs is as a network where none of the components is independent of the other (**Fig. 1**). Coupling and interlocking of these components happens at the transcriptional level, protein phosphorylation level, PIN protein transport in membrane vesicles by endocytosis and exocytosis, and coordinated polar auxin transport throughout the plant body.[13,21]

In addition to regulation of auxin fluxes or auxin concentration in compartments, coupling of ABP1 to TIR1 could be achieved by mechanisms of biochemical signal transduction to regulate the enzymatic activity of TIR1 by a post-translational mechanism. Presently, such a possibility remains completely vague and is not supported by data. However, both ways to regulate genes via TIR1-mediated mechanism would not mutually exclude each other and need a receptor.[14] The function of ABP1 is presumed to trigger biochemical (post-translational) mechanisms

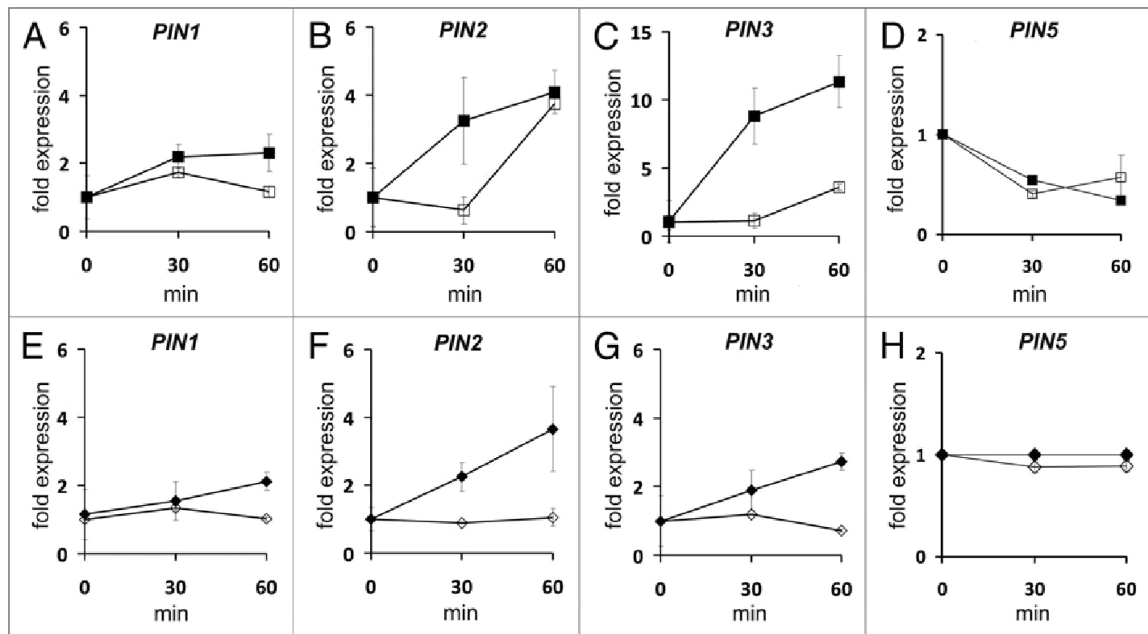

Figure 2. rapid regulation of Pin genes by auxin in the heterozygous *abp1/ABP1* receptor mutant and the *PIN2* mutant *eir1*. (A–D) Quantification by real time PCr of transcription in wild type Ws (filled squares) and *abp1/ABP1* seedlings (open squares). (E–h) Quantification by real time PCr of tran-scription in wild type Col (filled diamonds) and *eir1* seedlings (open diamonds). methods were described in reference 16. two biological treatments with three technical repeats each were used for calculation of average and SD. List of primers: Pin1-forw: GGA GAC ttA AGt AGG AGC tCA GCA; Pin1-rev: CCA AAA GAG GAA ACA CGA AtG; Pin2-forw: tAt CAA CAC tGC CtA ACA CG; Pin2-rev: GAA GAG AtC Att GAt GAG GC; Pin3-forw: GAG ttA CCC GAA CCt AAt CA; Pin3-rev: ttA CtG CGt GtC GCt AtA Gt; Pin5-forw: ACC CtG CCG CtC ttC ACC A; Pin5-rev: GCC CAC AAC GCt AAG ACC G.

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of protein activity-regulation on several signaling pathways and the function of TIR1 is to regulate gene activity. This model of a two-receptor system for one signal is apparently also found to be realized for other plant-typical signals.[16]

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CHAPTER 4

abp1/ABP1 and *abp1-5* are both auxin and red light signaling mutants

abp1/ABP1 and *abp1-5* are both auxin and red light signaling mutants

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Abstract

At present, there are two classes of auxin receptors, one typified by AUXIN-BINDING PROTEIN 1 (ABP1) and the other by TRANSPORT INHIBITOR RESISTANT 1 (TIR1). TIR1 indirectly mediates auxin-regulated gene expression while ABP1 directly regulates plasma membrane activities. At least one ABP1 function is to regulate the trafficking of PIN proteins, facilitators of auxin transport was identified. However, ABP1 control of auxin transport at the whole plant level is less understood. Therefore, we determined if physiological activities that are dependent on auxin transport changes were altered in *abp1* mutants. We use physiological methods and qPCR to measure gene activity. The weak allele *abp1-5* mutant had reduced gravitropism consistent with altered auxin transport. Probably as a consequence of altered auxin transport, auxin regulated gene expression in far red enriched light in *abp1-5* was generally slowed down and hypocotyl lengths were taller in *abp1/ABP1, abp1-5* but not in *tir1*. The phenotypes of the *abp1* mutants provide indirect evidence that cellular action of ABP1 on PIN trafficking is a mechanism to control auxin transport and physiological responses that utilize on auxin gradients.

Keywords:

AUXIN-BINDING PROTEIN1 (ABP1), early auxin-regulated genes, gravitropism, growth regulation, phototropism, phytochrome, TIR1, shade avoidance

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INTRODUCTION

Auxin initiates responses by two different receptors, ABP1 and TIR1 (Tromas et al., 2010; Scherer, 2011). TIR1 mediates auxin effects on gene expression (Mockaitis & Estelle, 2008), while ABP1 mediates auxin effects at the plasma membrane (Jones, 1994; Napier et al., 2002;

Robert et al., 2010; Xu et al., 2010) . ABP1 is essential for development and many rapid cellular changes (Jones et al., 1998; Chen et al., 2001a/b). ABP1-mediated rapid responses such as membrane hyperpolarisation (Barbier-Brygoo et al., 1989), channel regulation (Thiel et al., 1993; Blatt and Thiel 1994), proton extrusion (Rück et al., 1993), phospholipase A activation (Scherer and Andrè, 1989; Paul et al., 1998), PLD activation (Lanteri et al., 2008), and transient increase in cytosolic calcium (Shishova et al., 2007; Monshausen et al., 2011) are too rapid to be reconciled with TIR1 as the only auxin receptor type, assuming that TIR1 sole function is mediating changes in gene transcription (Badescu and Napier, 2006; Scherer, 2011).

ABP1 is a small glycoprotein localized at the extracytosolic side of the ER and, to a smaller extent, at the extracytosolic side of the plasma membrane (Napier et al., 2002). The *ABP1* expression pattern is strongly overlapping with that of the artificial auxin-activated *DR5* promoter coupled to the *uidA* gene (Klode et al., 2011) suggesting a causal relationship between ABP1 action and auxin concentrations, consistent with the observation that auxin regulates *ABP1* transcription (Hou et al., 2006; Effendi et al., 2011). In order to transmit signaling to cytosolic proteins, a transmembrane protein, “docking protein” or binding protein for ABP1 was postulated (Klämbt, 1990). A critical feature of hormone receptors is that the activated pool size limits the amplitude and/or rate of signal transduction at physiological concentrations of cognate hormone (Levitzki, 1981; Kenakin, 2004). Consistent with ABP1 number being rate limiting for auxin responses, heterozygous *abp1/ABP1* mutant displays auxin-signaling defects (Effendi et al., 2011). We speculated that proper stoichiometry of ABP1 and the hypothetical binding protein was rate limiting for signal output and any disturbance of stoichiometry caused a mutant phenotype. This gene dosage effect or haploinsufficiency (Veitia et al., 2008) is common for receptors in humans (Fisher and Scambler, 1994). A dosage effect for ABP1 function was also demonstrated using conditional deletion by expressing a recombinant antibody fragment directed against ABP1, a line designated *abp1-SS12K* (Braun et al., 2008).

The point mutation *abp1-5* (H59>Y) is a weak allele that confers phenotypes that can not be explained by haploinsufficiency (Robert et al., 2010; Xu et al., 2010). Most, if not all, phenotypes associated with ABP1 are linked to misfunction of polar auxin transport conducted or regulated by PIN proteins (Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011; Effendi and Scherer, 2011). PIN1 proteins are located on the plasma membranes along

the tips of epidermal cell lobes and are linked to the expansion of lobes in an auxin signaling pathway that uses ABP1 as a receptor and small G proteins as intermediates (Xu et al., 2010). At these positions, the level of auxin is critical for proper development of pavement cells (Xu et al., 2010). Robert *et al.* (2010) showed that ABP1 is the receptor for the auxin-inhibition of endocytosis of PIN proteins. As a consequence, the efflux transport by these PIN proteins is enhanced (Paciorek et al., 2005). Another example of a possible link between ABP1 and polar auxin transport is the correlation of ABP1, auxin concentration, and H^+-ATPase localisation in embryo development (et al., 2010). We showed, in particular, that the heterozygous T-DNA insertion mutant *abp1/ABP1* had defects in root and hypocotyl gravitropism, in basipetal auxin transport in the root, in apical dominance, and regulation of early auxin-activated genes (Effendi et al., 2011). In our model, we linked these functions to the regulation of auxin transport which, in turn, regulates the auxin concentrations perceived by the extracytosolic ABP1 receptor and the nuclear receptor TIR1 (Effendi et al., 2011; Effendi and Scherer, 2011).

Another property, the early flowering phenotype of *abp1/ABP1* mutants was not immediately explained by a defect in polar auxin transport. This and the slightly longer hypocotyls of *abp1/ABP1* seedlings indicated a potential link of ABP1 to light responses on auxin transport. Earlier reports on red and blue light on auxin transport showed interaction between light and auxin transport and auxin-binding activity (Shinkle and Jones, 1988; Jones et al., 1991; Shinkle et al., 1992; Barker-Bridges et al., 1997; Shinkle et al., 1998) but few facts about ABP1 were known at that time and even less known about PIN proteins prior to their first molecular description (Gälweiler et al. 1998). One possibility is that early flowering in short days is related to phytochrome action because *phyB* mutants flower early in short days (Reed et al., 1994). We found that hypocotyl elongation and induction of light-regulated genes in response to white light supplemented with a low ratio of R:FR was changed in *abp1-5*. In *tir1-1* no elongation response was found and changes in regulation of light-regulated genes were dissimilar to Col-0 and *abp1-5*.

RESULTS

The mutant *abp1-5* containing a H59>Y point mutation was isolated by the TILLING method (Robert et al., 2010); this mutant has near-normal morphology (Fig. 1S & 2S and data not shown). Moreover, the root gravitropic response in roots was less active than in wild type (Fig. 1 b). The gravitropic response of hypocotyls and the phototropic response to laterally applied blue light of hypocotyls of *abp1-5,* grown in the dark, were not significantly different indistinguishable from wild type (Fig. 1 a and c).

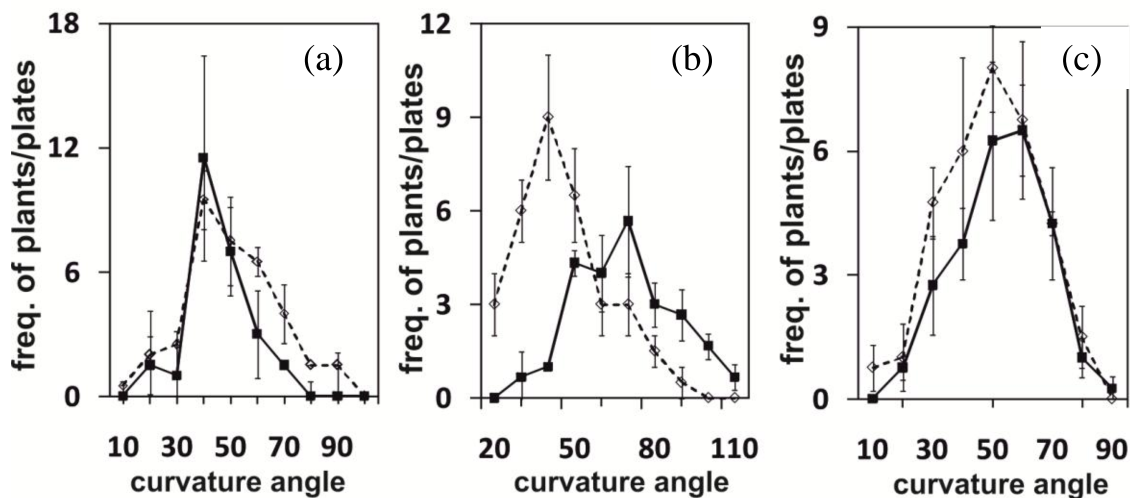

Figure 1. Gravitropic and phototropic responses in 3 days old dark grown Col-0 (black squares) and *abp1-5* (diamonds) seedlings.
(a) Gravitropic bending angles of hypocotyls after 24h tilting by 90° (mean Col-0: 44.8°; n=57; mean *abp1-5*: 46.7°; p<0,54 difference not significant). (b) Gravitropic bending angles of roots after 24h tilting by 90° (mean Col-0: 65.3°; n=71; mean *abp1-5*: 41.1°; n=65 p<0.001). (c) Phototropic bending angles of hypocotyls after 8h lateral blue light (10 μmoles.m^{-2}sec^{-1}) (mean Col-0: 48.9°; n=135. mean *abp1-5*: 45.7°; n=102; p< 0,114; difference not significant). For each panel 3-4 agar plates containing about 30 seedlings were evaluated. Data points represent means of each angle size group and S.E.

As shown in Fig 2S, both flowering time and the number of rosette leaves at the beginning of flowering were nearly identical in *abp1-5* and in wild type. Flowering time in long days was almost identical to the respective wild type in *abp1/ABP1* (Effendi et al., 2011) so that we did not pursue this further with *abp1-5.*

Expression of early, auxin up-regulated genes in the heterozygous *abp1/ABP1* mutant is slower and less sensitive to the addition of auxin (Effendi et al., 2011). Most auxin-regulateed genes tested in *abp1-5* were regulated more slowly that in wild type (Fig. 2a, p values indicated). Seven genes were up-regulated slower (*IAA2, IAA19*, *IAA20, GH3.5, SAUR23,*

PIN3, ABP1), two were up-regulated stronger after 60 min (*IAA11, SAUR15*) the other six showed the same regulatory pattern as in wild type seedlings. Basal transcription of all genes tested in *abp1-5* did not differ significantly from wild type levels (Fig. 1S, 2S, Fig. 2b).

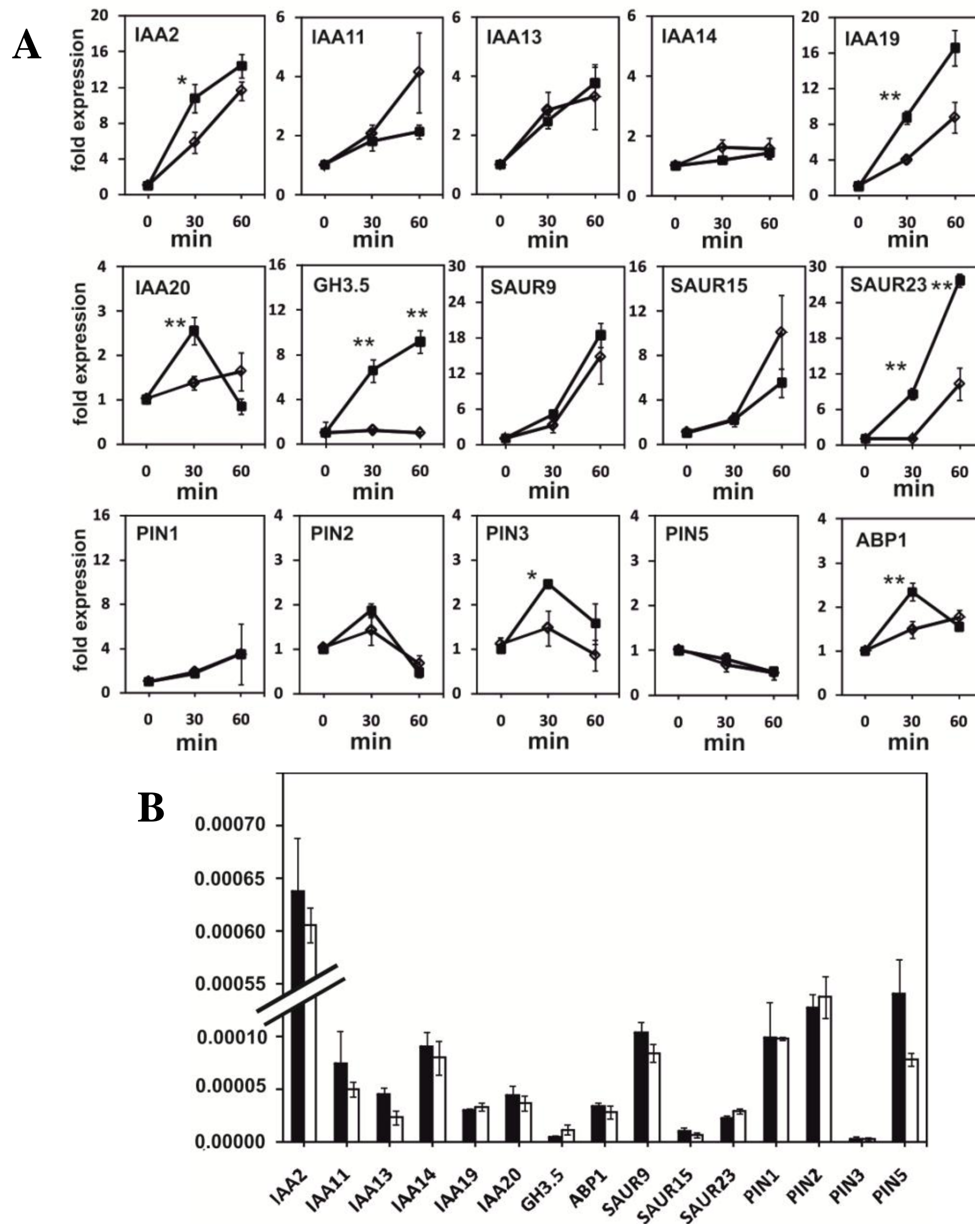

Figure 2. Rapid regulation of early auxin genes by 10 µM 1-NAA in Col-0 wild type and *abp1-5* mutant seedlings.

(a) Seedlings were grown for 14 days in white light of about 24.5 µmoles.$m^{-2}sec^{-1}$. Expression was normalized to t=0 set as 1-fold for either genotype. Black squares: wild type Col-0; diamonds: *abp1-5*. Significance levels in (a): *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. (b) Comparison of "basal" expression levels in *abp1-5* to Col-0 at t=0. Expression of the reference gene 18S in wild type Col-0 was set as 1-fold. For all genes in (b) the means were statistically identical ($p<0.05$ at least).

Early flowering, leaf hyponasty, reduced cotyledon expansion, and accelerated hypocotyl and petiole elongation are characteristics of the shade avoidance response in plants. In both *abp1-*

5 and in *abp1/ABP1,* the response to shade was tested and compared to the response in *tir1-1* and *tir1-9* alleles. Plants were grown first in white light for 3 days and either continued with a high R:FR ratio or at a low R:FR ratio for another 5 days (Wang et al., 2011). Both *abp1* mutants showed clear hypersensitivity in hypocotyl elongation in light enriched in far red (low R:FR ratio) and were taller than the respective wild types (Fig. 3). A wild type-like response in *abp1* mutants was found in red light (high ratio R:FR). TIR1 is the best characterized auxin receptor known to regulate early auxin genes by auxin stimulated ubiquitination of IAA proteins, negative co-transcription factors for these genes (Ruegger et al., 1998; Gray et al., 1999; Mockaitis and Estelle, 2008).

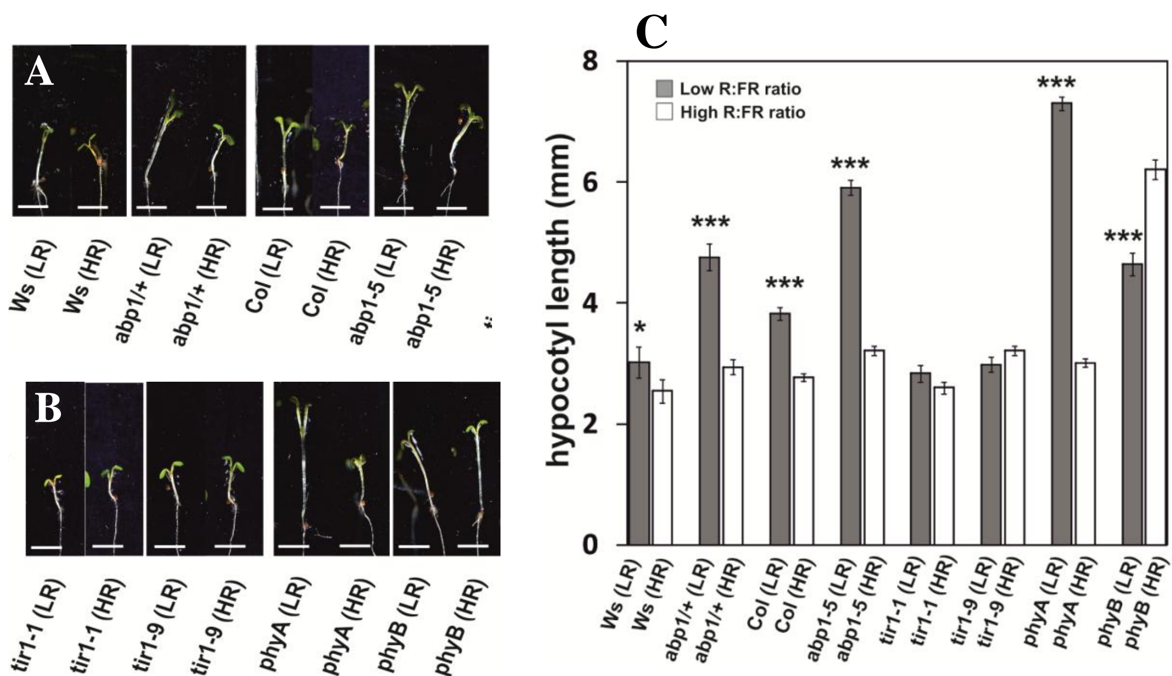

Figure 3. Shade avoidance responses in *abp1-5* and *abp1/ABP1* in comparison to Col-0, *phyA* and *phyB*.

Shade avoidance was tested by growing seedlings for 3 d in white light and 5 more days in white plus added low R:FR ratios (LR) or high ratios of R:FR (HR). Seedlings from seeds from an *abp1/ABP1* plant were verified by PCR-genotyping as either Ws wild type or *abp1/ABP1* mutant (Effendi et al., 2011). For comparison, *phyA* and *phyB* mutants were added to the tests. (A) Representative seedlings of every line used were grown in low or high ratio of FR:R. Bar = 5 mm. (B) Hypocotyl lengths of seedlings grown in low (dark bars) or high ratio (white bars) of R:FR. Hypocotyl lengths of seedlings were evaluated. LR and HR treatments were statistically different except for the *tir1* alleles. Significance levels in (B): *: $p<0.05$; **:$p<0.01$; ***: $p<0.001$; (n=55-90; S.E.).

Therefore, we also tested two *tir1* alleles, *tir1-1* and *tir1-9*, in their response to shade conditions (Fig. 3). We found little differences in response to both light conditions, high ratio

of R:FR and low ratio of R:FR in both *tir1* alleles. The hypocotyl elongation response to shade was slightly smaller than in Col-0 wild type (Fig. 3).

The hypersensitive shade response of *abp1-5* mutants prompted quantitation of expression of several light-regulated genes in the shade response (*ATHB2, PIL1, PIF5*, *HFR1*) and of both auxin and light-regulated genes (*IAA19, IAA29, PIN3*) (Devlin et al., 2003; Salter et al., 2003; Sessa et al., 2005; Roig-Villanova et al., 2006; Tepperman et al., 2006; Hornitschek et al., 2009; Keuskamp et al., 2010a; Keuskamp et al., 2011; Kunihiro et al., 2010). After 3 d in white light seedlings were treated for 1h continuing white light plus added far red (low ratio R:FR or shade) (Fig. 4). As a control seedlings treated with white light only were set as 1-fold expression. After 1h light with added far red the tested genes were, in general, induced highest, consistent with Tepperman *et al.* (2006). In *abp1-5* induction by shade was about four-fold lower than in Col-0, and in *phyB* even roughly eight-fold lower but the "peak pattern" was similar for all three. This can be interpreted as a decrease of PHYB control of repressing genes (Jiao et al., 2007). Induction of *ATHB2* in *tir1-1* was low and induction of *IAA29* was highest in all genotypes. In *phyA, ATHB2* induction was high and that of *IAA29* was modest and only these two genes were induced noticeably in *phyA*. So that the expression pattern of the tested genes was dissimilar to *abp1-5* or Col-0. In white light with high ratio R:FR light added, *ATHB2* expression was high, thus correlating with tall hypocotyls but low in Col-0, *abp1-5. IAA29* was modestly induced in phyB and Col-0 but strongly in *abp1-5* and not in *tir1-1*. *ATHB2* and *IAA29* were also induced by low R:FR light in *tir1-1* so that the overall pattern in *tir1-1* was somewhat similar to that in *phyA*. In any case, regulation of light-regulated genes was clearly disturbed in *abp1-5* and *tir1-1*.

DISCUSSION

Shade avoidance is a complex trait involving inputs from light and hormones, especially auxin. Based on published observations (Shinkle and Jones, 1988; Jones et al., 1991; Shinkle et al., 1992; Barker-Bridges et al., 1997; Shinkle et al., 1998; Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011) and the data presented here, we propose that a nexus in this trait is ABP1. The shade avoidance elongation response is induced in plants by sensing a low R:FR ratio in white background light (Ballaré et al., 1991; Keuskamp et al., 2010b). Shade avoidance response is primarily sensed by PHYB setting it into a low signal output mode (Reed et al., 1993) induced by a low R:FR ratio but PHYD and PHYE participate in sensing

(Aukermann et al., 1997; Devlin et al., 1998; Devlin et al., 1999). Low signaling activity of CRY1 in low blue light also contributes to shade avoidance response (Ballaré et al., 1991; Djakovic-Petrovic et al., 2007, Kunihiro et al., 2010; reviews: Ballaré, 2009; Keuskamp et al., 2010b).

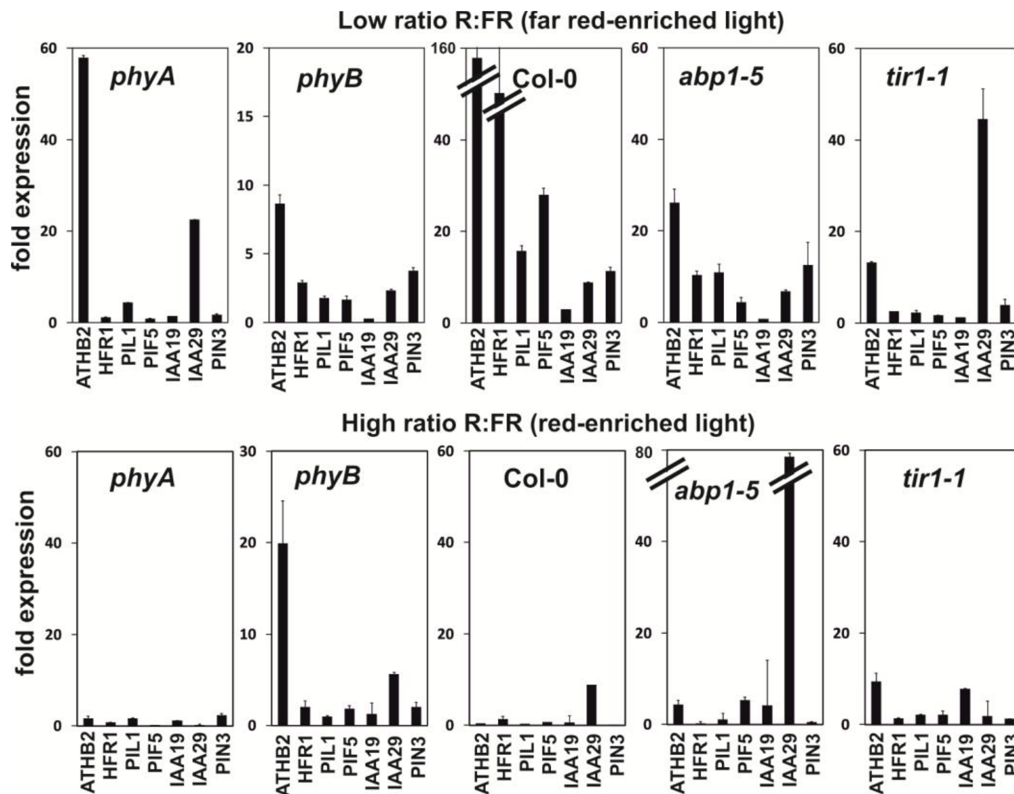

Figure 4. Comparison of regulation of genes by low ratio R:FR and high ratio R:FR in Col-0, *phyA, phyB, abp1-5* and *tir1-1*.

Seedlings were tested after growing for 3 d in white light and 1 h in white light or white plus added low R:FR ratios or high ratios of R:FR. Expression was normalized to t=0 in white light only and set as 1-fold for either genotype. Error bars were calculated according to Pfaffl *et al*. (2002) and are significant when not overlapping ($p<0.05$ at least). Note the lower scales in the *phyB* panel.

Major elements of shade avoidance responses are hypocotyl and shoot elongation, leaf shape, long petioles, and hyponasty of leaves. A late developmental response to shade is early flowering (Franklin, 2008; Lau and Deng, 2010; Stamm and Kumar, 2010). The properties of the *abp1* mutants identify ABP1 as a new player in the genetic interaction with PHYB. Both *abp1* mutants were hypersensitive to shade conditions in elongation (Fig. 3) and *abp1/ABP1* flowered early (Effendi et al., 2011). The known auxin input into the shade avoidance response is an increase of auxin signal strength by shade-dependent induction of *TAA1,* an auxin biosynthesis gene (Tao et al., 2008), but it is still unclear how exactly auxin signaling

makes its input into shade responses (Franklin, 2008; Stamm and Kumar, 2010; Keller et al., 2011; Nozue et al., 2011).

A potential common intermediate of auxin and PHYB signaling in shade avoidance syndrome could also be PIN3 (perhaps also other PIN proteins) (Keuskamp et al., 2010a; Keuskamp et al., 2011) placing an emphasis on changes in polar auxin transport. These were postulated early to play at least an important if is not decisive role (Morelli and Ruberti, 2000, 2002). A number of auxin-induced genes were described also as shade-induced, (*IAA3, IAA19, IAA29, PIN3*) (Devlin et al., 2003; Tao et al., 2008; Keuskamp et al., 2010a; Kunihiro et al., 2010; 2011). Keuskamp *et al.* (2010a) postulated that besides transcriptional regulation, PIN3 relocalisation in shade to plasma membrane in lateral (tangential) cell walls was important for elongation. However, of particular note is that by auxin in *abp1-5* and *abp1/ABP1* mutant *PIN3* and *IAA19* were up-regulated by auxin more slowly than in wild type (Fig. 2a; Effendi et al., 2011; Effendi and Scherer, 2011). Among the auxin and shade-regulated genes only the encoded proteins IAA3 and IAA19 are ubiquitinated by TIR1 in an auxin-dependent mechanism (Dreher et al., 2006; Maraschin et al., 2009) so that genes like *PIN3* and *IAA29* could be more activated by shade-induced auxin biosynthesis e.g. *TAA1* (Tao et al., 2008), esüecially, after long treatments (Keuskamp et al., 2011).

We show here that ABP1, but not TIR1, participates in the shade avoidance response, although we do can exclude other members of the TIR/AFB family. The failure of the *tir1* mutants to show a response to shade could be part of a mechanism relying on genes which are co-regulated by auxin and light and only some of the encoded proteins are ubiquitinated by TIR1. Because ABP1 is extracytosolic, any direct interaction with PHYB is not expected; therefore we favor to propose an indirect role of ABP1 on gene expression in the shade avoidance response.

We concluded that ABP1 controls polar auxin transport, either its flux, rate, or direction (Effendi et al., 2011). Specifically, regulation of *PIN3* and perhaps other *PIN* genes could be part of the common intermediates between ABP1 and PHYB. An interaction of ABP1 binding auxin (Jones et al., 1989; Steffens et al., 2001), red light, and auxin transport, is well established for cell expansion (Shinkle and Jones, 1988; Zhou et al., 1990; Shinkle et al., 1992; Jones et al. 1991; Barker-Bridges et al., 1997; Shinkle et al., 1998). Regulation may occur at the protein activity level, PIN protein subcellular distribution, and/or at the

transcriptional level. Changes in auxin transport patterns as part of the shade avoidance responses were proposed in a visionary paper by Morelli and Roberti (2002). It was suggested that in shade, PIN3 diverts the polar auxin transport laterally to the outer cells of the hypocotyl leading to enhanced elongation (Morelli and Ruberti, 2002; Keuskamp et al., 2010a). ABP1 regulates polar auxin transport at the organ level (Effendi et al., 2011), by regulation of *PIN3* expression (Effendi and Scherer, 2011), and by shifting the balance of endo- and exocytosis of PIN proteins and, thus, their subcellular localisation as means of transport activity regulation (Robert et al., 2010). Translocation of PIN3 by blue light in phototropism was shown recently (Ding et al., 2011). Expression as well as localization of PIN2 strongly depends on light (Laxmi et al., 2008). ABP1 is involved in regulation of polar auxin transport and of regulation of early auxin genes so that we proposed a regulatory nexus consisting of ABP1, TIR1, and PIN's (Effendi et al., 2011; Effendi and Scherer, 2011) so that involvement of ABP1 as part of this nexus also involves PIN proteins which, in turn, may co-regulate shade avoidance responses. TIR1 likely either executes regulation of early auxin genes according to the nuclear auxin concentration or one has to postulate an as yet undefined regulatory input onto TIR1 activity independent of auxin concentration via a cytosolic PHYB signaling pathway (Rösler et al., 2010). Since regulation of TIR1 activity by PHYB is totally speculative, we favor the former possibility to explain why *tir1* mutants did not show an obvious response to shade. All in all, the data make clear that *abp1-5* and *tir1-1* are light mutants, not just auxin mutants. Our work is the beginning of not only defining light dependency of auxin physiology-related genes but defining prominent auxin mutants as light mutants with aberrant regulation of red light-induced genes.

MATERIAL AND METHOD

Plant material and Growth Conditions

Heterozygous kanamycin resistant *abp1/ABP1* mutant seeds (stock number N6489) were obtained from the Nottingham *Arabidopsis*Stock Center and are in Ws background (NASC) England and genotypes verified as before (Effendi et al. 2011). Isolation of *abp1-5* (Col-0 background) was described (ABRC stock center (# CS91358)) and *abp1-5* contained a change of a conserved histidine to a tyrosine (H59>Y) (Robert et al., 2010) in the auxin-binding pocket of ABP1 (Woo et al., 2002). *phyA* and *phyB* are in Col-0 background and were obtained from M. Zeidler.

For gravitropism and phototropism experiments, seeds were stratified for 4d, treated for 4h with white light and grown for 3 days on upright 0.5 x MS agar plates in the dark at 22.5°C. For testing gravitropism, plants were turned 90° for 24h and then scanned. For phototropism 10 µmoles m^{-2} sec^{-1} lateral blue light was applied and plants were scanned after 8 hours (CanonScan 8800F; resolution 600 dot per inch). For testing shade avoidance, seeds were stratified for 4 d, treated with white light for 4 h, and then kept in the dark for 24 h. Then for 3 days white light (24.5 µmoles m^{-2} sec^{-1}) was applied, after that red and far-red either with a high R:FR ratio (2.1) or a low R:FR ratio (0.098) were applied in an LED box at 22.5 °C for hypocotyl length experiments (CLF, Plant Climatics) for another 3 days at 22.5 °C (for spectra see supplemental Fig. 3). Lengths or angles were measured using AxioVision LE Ver.4.6 software (Zeiss-Germany). For flowering experiments, plants grew in a growth chamber 1 at 22.5 °C in 8/16 (L/D). Each experiment was done at least two times.

Nucleic acid Analysis

For transcription measurements, seedlings were grown in 0.5x MS agar-medium for 14 days in long days. For the auxin treatment, the medium was removed and replaced by fresh medium containing 10 µM 1-NAA. Seedlings were blotted on filter paper and frozen in liquid nitrogen for further use. For light treatments, plants were grown for 7 days in white light, then far red or red was added for 1 hr (spectra in Supplement Fig. 3) and expression compared to plants from white light only. For quantitative RT-PCR, 4 µg of total RNA was prepared with the NucleoSpin® RNA Plant kit according to the manufacturer's instructions (Macherey and Nagel) and transcribed to first strand cDNA with RevertAidTM 10 H Minus First Strand cDNA Synthesis kit (Fermentas). Primers and methods were as described (Effendi et al., 2011; Effendi and Scherer, 2011). For each data point, two to five biological repeats and three technical replicates for each determination were done in the subsequent PCR reaction. Relative expression was calculated according to the ΔΔCt method using the equation: relative expression = 2-[ΔCt sample - ΔCt control], with ΔCt = Ct(sample gene) - Ct(reference gene), where Ct refers to the threshold cycle determined for each gene in the early exponential amplification phase (Livak and Schmittgen, 2001). The control treatment at t=0 min was set as 1-fold expression level. For statistical analysis the REST 2008 software (Pfaffl et al., 2002) was used.

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SUPPORTING INFORMATION

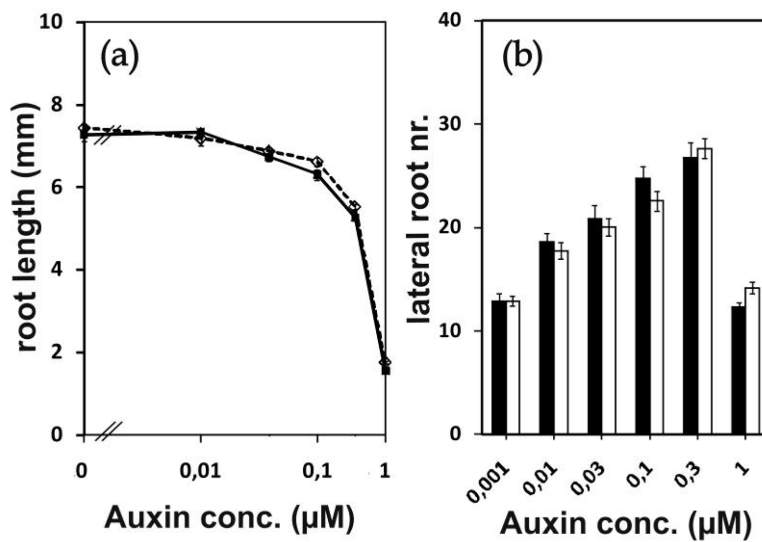


Fig. 1S. Auxin sensitivity of *abp1-5*. (a) Root length of 10 days old light-grown Col-0 (black squares) and *abp1-5* (diamonds) seedlings. (S.E., n=30). (b) Lateral root formation in response to 1-NAA in 10 days old light-grown Col-0 (black bars) and *abp1-5* (white bars) seedlings. (S.E., n=30).

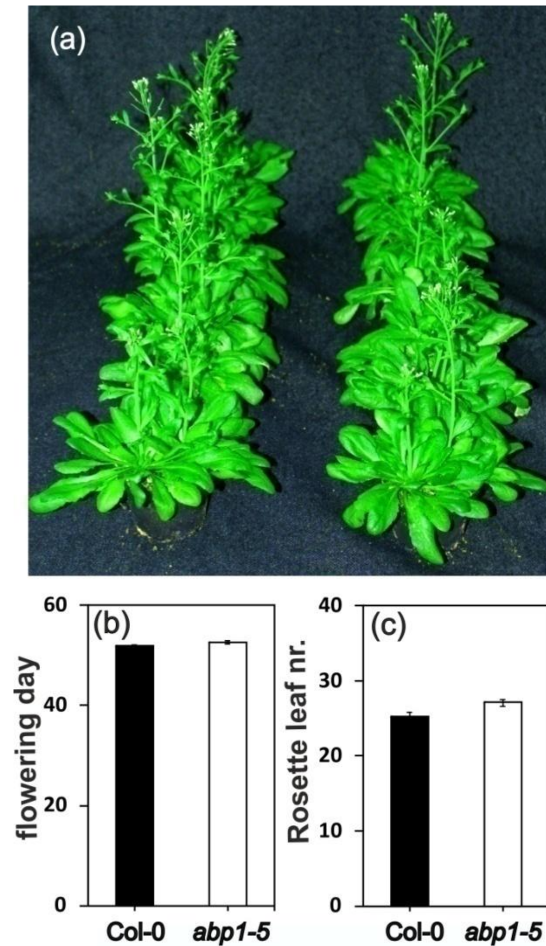
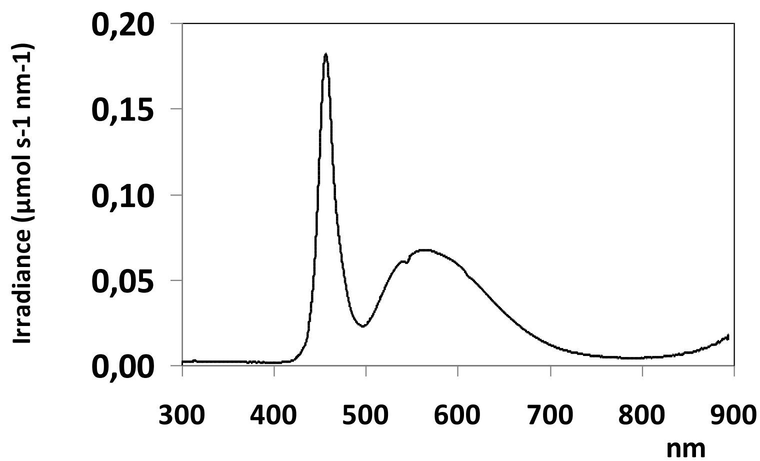


Fig. 2S. Flowering date in Col-0 and *abp1-5* plants grown in short days (16/8 L/D).

(a) Representative groups of plants at day 52. Left: Col-0; right: *abp1-5*. (b) Day of appearance of first flower. (c) Number of rosette leaves at the day of flowering. (S.E.; n=33).

(a)



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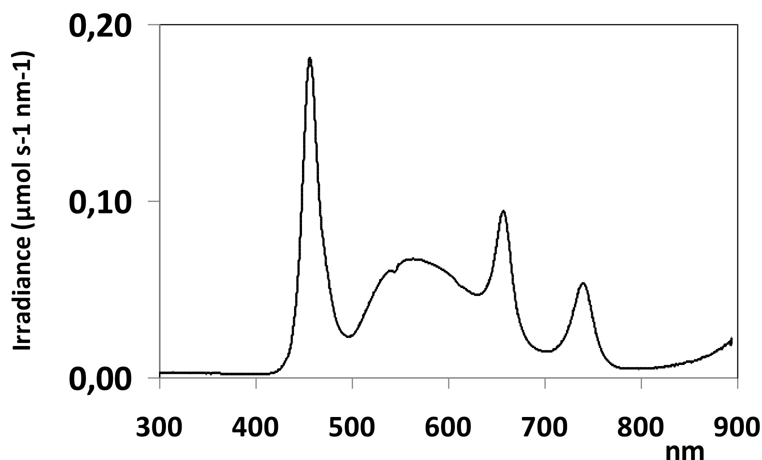

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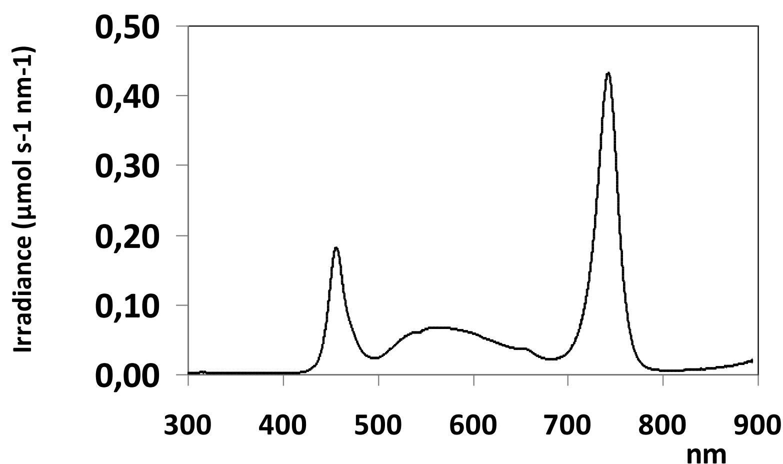

Fig. 3S. Spectra used in the shade avoidance experiments.

(a) White LEDs only. (b) White LEDs with added red and far red LEDs to generate a high ratio of R:FR of 1.1 (c) White LEDs with added red and far red LEDs to generate a low ratio R:FR of 0,098. (ratio: 665-675nm/725-735nm).
Spectra were measured with a spectrometer Ocean Optics "USB4000" equipped with Cosinus corrector and the software Spectra Suite.

Supplemental Table 1.

Primer for light experiment	**Sequence (5' – 3')**
*ATHB2*_F	GAG GTA GAC TGC GAG TTC TTA CG
*ATHB2*_R	GCA TGT AGA ACT GAG GAG AGA GC
*HFR1*_F	CAC AAG ACG GAC AAG GTT TCG
*HFR1*_R	GTC AGC ATG TGG TTG TGC ATT C
*PIL1*_F	TGG TGC CTT CGT GTG TTT CTC A
*PIL1*_R	GGA CGC AGA CTT TGG GAA TTG
*PIF5*_F	GAT GCA GAC CGT GCA ACA AC
*PIF5*_R	CTT TTA TGC TTG CTT AGG CG
IAA19 forw	GGT GAC AAC TGC GAA TAC GTT ACC
IAA19 rev	CCC GGT AGC ATC CGA TCT TTT CA
*IAA29*for	TCC TCT GGA ATC CGA GTC TTC
*IAA29*rev	GGT GGC CAT CCA ACA ACT T
PIN3-forw	GAG TTA CCC GAA CCT AAT CA
PIN3-rev	TTA CTG CGT GTC GCT ATA GT

Supplemental Table 2.

Primer for auxin experiment	**Sequence (5' – 3')**
18S rRNA forw	GGC TCG AAG ACG ATC AGA TAC C
18S rRNA rev	TCG GCA TCG TTT ATG GTT
ABP1 forw	ACG AGA AAA TCA TAC CAA TTC GGA CTA ACC
ABP1 rev	GTA TCT ACG TAG TGT CAC AAA ACC TCA AC
IAA2 forw	GGT TGG CCA CCA GTG AGA TC
IAA2 rev	AGC TCC GTC CAT ACT CAC TTT CA
IAA11 forw	CCT CCC TTC CCT CAC AAT CA
IAA11 rev	AAC CGC CTT CCA TTT TCG A
IAA12 forw	CGT TGG GTC TAA ACG CTC TG
IAA12 rev	TTC CGC TCT TGC TGC CTT CA
IAA14 forw	CCT TCT AAG CCT CCT GCT AAA GCA C
IAA14 rev	CCA TCC ATG GAA ACC TTC AC
IAA19 forw	GGT GAC AAC TGC GAA TAC GTT ACC
IAA19 rev	CCC GGT AGC ATC CGA TCT TTT CA
IAA20 forw	CAATATTTCAACGGTGGCTATGG
IAA20 rev	GCC ACA TAT TCC GCA TCC TCT
GH3.5 forw	AGC CCT AAC GAG ACC ATC CT
GH3.5 rev	AAG CCA TGG ATG GTA TGA GC
SAUR9 forw	GAC GTG CCA AAA GGT CAC TT
SAUR9 rev	AGT GAG ACC CAT CTC GTG CT
SAUR15 forw	ATG GCT TTT TTG AGG AGT TTC TTG GG
SAUR15 rev	TCA TTG TAT CTG AGA TGT GAC TGT G
SAUR23 forw	ATG GCT TTG GTG AGA AGT CTA TTG GT

SAUR23 rev	TCA ATG GAG CCG AGA AGT CAC ATT GA
PIN1-forw	GGA GAC TTA AGT AGG AGC TCA GCA
PIN1-rev	CCA AAA GAG GAA ACA CGA ATG
PIN2-forw	TAT CAA CAC TGC CTA ACA CG
PIN2-rev	GAA GAG ATC ATT GAT GAG GC
PIN3-forw	GAG TTA CCC GAA CCT AAT CA
PIN3-rev	TTA CTG CGT GTC GCT ATA GT
PIN5-forw	ACC CTG CCG CTC TTC ACC A
PIN5-rev	GCC CAC AAC GCT AAG ACC G

CHAPTER 5

New in-vitro generated *abp1* mutants show impairment of auxin-related functions and are defect in red light responses

New in-vitro generated *abp1* mutants show impairment of auxin-related functions and are defect in red light responses

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ABSTRACT

Auxin Binding Protein1 (ABP1) has been proposed as membrane-bound auxin receptor in plants based on early studies on this protein. Recent progress in ABP1 research suggested that ABP1 could be an auxin receptor for rapid auxin-related processes. A problem facing ABP1 research is lethality in the embryo stage of *abp1* null mutant, which indicates *ABP1* is an essential gene in the plant although, even at the same time this hinders post-embryonic investigation of this protein. We designed and characterized in-vitro generated *abp1* mutants containing a point mutation in the presumed auxin binding site of ABP1. The *abp1* mutants showed defects in auxin-related functions such as in gravitropic and phototropic responses, early flowering, and reduced transcript levels of early auxin responsive genes. Additionally, *abp1* mutants showed insensitivity to red and far-red light in hypocotyl elongation. Morphology and responses to far red-enriched white light indicate hypersensitivity to shade in these mutants. Transcriptional expression of some shade-induced genes in response to far red- and red-enriched white light was altered in all *abp1* mutants. This provides initial evidence of a regulatory link between auxin and phyB-mediated light responses via ABP1 action. Taken together, the new *abp1* mutants showed mutant properties not only as auxin mutants but also light mutants.

Keywords: AUXIN BINDING PROTEIN1, auxin-regulated genes, red light-regulated genes, engineered *abp1* mutants, shade

INTRODUCTION

Auxin is a phytohormone that it is known to regulate many physiological processes of growth and development in plants such as morphogenesis, organogenesis, reproduction, secondary growth, apical dominance, cell elongation and division, and tropic response of root and shoot in response to external stimuli such as light and gravity (Davies 1995; Leyser, 2006; Benjamins and Scheres, 2008; Mockaitis and Estelle, 2008; Chapman and Estelle, 2009). At the molecular level, many genes are known to be regulated by auxin (Hagen and Guilfoyle, 1985; McClure and Guilfoyle, 1987; Abel and Theologis, 1996; Remington et al., 2004; Okushima et al., 2005; Overvoorde et al., 2005).

In contrast, despite a great body of information has been collected on the auxin function, how this small molecule is perceived by receptor(s) and can influence all of these processes remains largely unclear at the molecular level. Since auxin was identified as indolyl-3 acetic acid (IAA), many efforts have been dedicated to understand auxin signaling pathways. Perception of auxin by potential receptors in plants has been investigated since the eightieths. The first putative auxin receptor that was identified is AUXIN BINDING PROTEIN1 (ABP1) based on biochemical, pharmacological, electrophysiological as well as molecular genetic data (Jones, 1994; Napier 1995; Napier and Venis 1995). However, as a receptor ABP1 it remained in debate due to still unclear information on the molecular function of this protein. The discovery of a soluble nuclear auxin binding receptor, TRANPORT INHIBITOR RESISTANT1 (TIR1) and homologous AFB proteins, is considered as a breakthrough in auxin receptor research. TIR1/AFB mediates transcriptional regulation of many of auxin-induced genes via ubiquitinylation and degradation of transcriptional repressor proteins, the IAA proteins (Kepinski and Leyser, 2005; Dharmasiri et al., 2005; Mockaitis and Estelle, 2008; Lee et al., 2009). As yet TIR1/AFB is known as the auxin receptor to mediate gene regulation. However, some rapid auxin-dependent processes such as activation of phospholipase A, changes in membrane K^+ currents, membrane hyperpolarisation, activation of proton pumping, MAP kinase, and inhibition of endocytosis are unlikely to be associated with TIR1-based signaling (Scherer and Andre, 1989; Rück et al., 1993; Hager, 2003; Mizoguchi et al., 1994; Paciorek et al., 2005; reviewed in Badescu and Napier, 2006; Scherer, 2011).

ABP1 was considered as a potential candidate for an auxin receptor (Jones, 1994) due to its specific binding to auxin with physiologically relevant affinity (Rück et al., 1993; Jones et al., 1998; Steffens et al., 2001). Supporting evidence of ABP1 as a putative auxin receptor mostly was obtained from physiological and biochemical experiments such as inhibition of plasma membrane hyperpolarization in mesophyll protoplast using antibodies raised against tobacco ABP1 (Barbier-Brygoo et al., 1989, 1991; Leblanc et al., 1999), enhancement of H^+-ATPase activity in maize coleoptile protoplasts (Rück et al., 1993) and agonist activity in the hyperpolarization response after treatment with D16 peptide antibodies against the putative auxin-binding site (Rück et al., 1993). ABP1 contains the ER retention signal KDEL so that ABP1 is predominantly localized in the ER, where the pH is ineffective for auxin binding (Jones, 1994). However, a few percent ABP1 of the total could be sufficient to elicit known plasma membrane-localized responses. ABP1 lacks a transmembrane domain suggesting it

requires a 'docking' protein for anchoring to the plasma membrane (Klämbt, 1990). Furthermore, there is not much direct evidence for signaling events downstream of ABP1, leading to long-term responses like gene regulation. Thus, as an auxin receptor, ABP1 is still not fully recognized.

Recent experiments using *abp1* mutants and biotechnological methods to regulate *ABP1* activity are considered as new evidence that revitalized the old hypothesis that ABP1 is an auxin receptor (David et al., 2007; Braun et al., 2008; Tromas et al., 2009; Xu et al., 2010; Robert et al., 2010; Effendi et al., 2011). Using conditional expression of antibodies against ABP1 expression in the apoplast in *Arabidopsis,* the transcript level of a large spectrum of *IAA* genes was shown to decrease after eight hours (Braun et al., 2008; Tromas et al., 2009). A similar result was obtained with the heterozygous *abp1/+* mutant that expressed lower levels of early auxin-induced genes in comparison to wild type already after 30 min auxin application (Effendi et al., 2011; Effendi and Scherer, 2011). Although no direct experimental study has shown how exactly the functional mechanism of ABP1 on gene regulation is executed, these results raise the question regarding functional links between TIR1/AFB and ABP1. Moreover, loss of function of ABP1 leads to embryo lethality in *Arabidopsis.* This indicates *ABP1* is an essential gene, at least at the embryo developmental stage (Chen et al., 2001). However, at the same time this lethality property thwarts the further investigation of the mechanisms underlying the action of ABP1 (Scherer, 2011).

Recent reports added substantial evidence to the role of ABP1 as an auxin receptor (Xu et al., 2010; Robert et al., 2010; Effendi et al., 2011). Xu *et al.* (2010) provided interesting evidence of a signaling pathway by ABP1 at the plasma membrane which rapidly activates the small G proteins ROP2 and ROP6. Following the activation of ROP2 and ROP6, regulation of planar morphogenesis and lobe formation of pavement cells was induced. This rapid activation of ROP2 and ROP6 pathway was not detected in the *abp1-5* mutant (H59>Y59). PIN1 internalization was increased significantly in *abp1-5* mutant and, at the same time, lobe formation was abolished in pavement cells. Robert *et al.* (2010) showed that ABP1 mediated internalization of PIN proteins by acting as a positive factor in clathrin recruitment to plasma membrane. ABP1 promoted endocytosis and, thus, regulated the cycling of PIN proteins from plasma membrane to endosomes. The authors also demonstrated that the cycling of PIN proteins is facilitated by signaling mechanism which did not depend on transcription and was not associated with TIR1-based signaling. Effendi *et al.* (2011) demonstrated that even

heterozygous *abp1/+* plants showed defects in many auxin response-phenotypes, having agravitropic roots and hypocotyls, aphototropic hypocotyls, decreased apical dominance, and decreased basipetal auxin transport in the root. Moreover, transcriptional regulation of early auxin-induced gene families (*IAA*s*, GH3* and *SAUR*s) was found defect in *abp1/+*, thus transcript level of these genes were lower than in wild type 30 minutes after auxin application. Taken together, it is now obvious that *abp1* mutants are important tools for establishing further research on ABP1. Unfortunately, the collection of *abp1* mutant so far is limited in number and types (Chen et al., 2001, Braun et al., 2008; Tromas et al., 2009; Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011).

The cross-talk between auxin and light in the growth regulation of the plants has been intensively investigated (Behringer and Davies, 1992; de Lucas et al., 2008), especially in response to shade avoidance (Morelli and Ruberti, 2002; Tanaka et al., 2002; Lorrain et al., 2008; Sorin et al., 2009; Kozuka et al., 2010; Keuskamp et al., 2010). Auxin-responsive functions were reported to be affected by light, such as gravitropism (Haga and Iino, 2006), hypocotyl elongation (Steindler, 1999; Tao et al., 2008), petiole elongation (Tao et al., 2008; Kozuka et al., 2010), auxin polar transport (Kanyuka et al., 2003; Keuskamp et al., 2010; Liu et al., 2011) and auxin biosynthesis (Tao et al., 2008). The effect of auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) in the reduction of hypocotyl shade avoidance response was also demonstrated (Steindler et al., 1999; Pierik et al., 2009). Moreover, global expression profiling revealed that some early auxin-responsive genes are also induced by low ratio red:far red (R/FR) (Devlin et al., 2003; Sessa et al., 2005; Tao et al., 2008). This all indicates a close regulatory link between auxin and light responses.

In this study, we designed a new class of *abp1* mutants and examined their functional roles in responses to auxin. Several auxin-induced physiological functions and auxin-responsive transcription were investigated. Furthermore, cross-talk between auxin and light pathway was investigated in the *abp1* mutants by characterizing their auxin-mediated responses as well as transcriptional levels of light-responsive genes. Here, we show that *abp1* mutants have defects in auxin-related physiological functions such as root and hypocotyl gravitropic response, phototropic response, sensitivity to auxin of root growth and a lower transcription level in auxin-responsive genes. We also observed that a defect had lead to insensitivity in red light responses as well as a hypersensitive shade avoidance response as a consequence. Furthermore, alteration in expression of light-induced genes induced by far red or red added

to white light in the *abp1* mutants was observed. Thus, taken together this study provides evidence that *ABP1* is necessary for crosstalk of auxin and phytochrome signaling.

RESULTS

Auxin-related functions in *abp1* mutants are defect

We designed and developed new *abp1* mutants of *Arabidopsis* by transforming *ABP1* cDNA containing mutations in the putative auxin binding sites of ABP1 (Woo et al., 2002) into heterozygous T-DNA insertion *abp1/+* mutant (Chen et al., 2001).

We wanted to eliminate wild type ABP1 protein from these plants by selecting lines homozygous for the insertion so that effects of loss of function in viable plants might be observed. However, it previously has been reported by Chen *et al.* (2001) homozygous null *ABP1* mutant plants are lethal in the embryo stage, thus complete null *ABP1* plants were never present. The expression of the recombinant ABP1 cDNA was under control of 35S promoter. At C-terminal position, a strep II tag and a flag tag were inserted before the C-terminal KDEL.

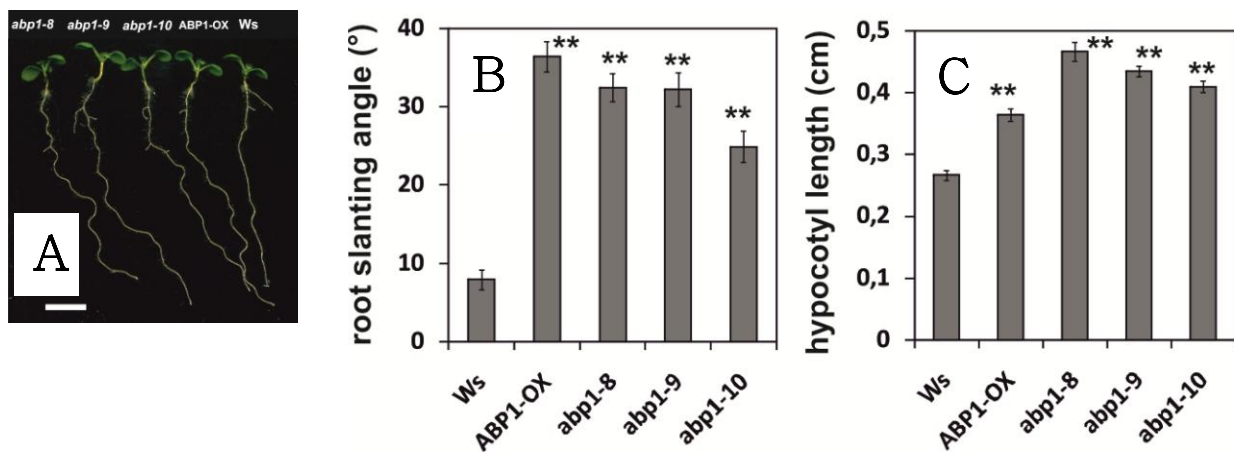

Figure 1. Slanting and waving of roots and elongated hypocotyls in *abp1* mutants. (A) Representative images of 7 days light grown seedlings Ws, *ABP1-OX* and *abp1* mutants. Scale bar = 0.5 cm (B) Seedlings were grown on ½ MS media containing 1% sucrose and 0.5% Gelrite (Duchofa-Biochemie). After 7 days growth under 8h/16h white light condition, slanting angles were quantified. (C) Hypocotyl length of 10 days light growth seedlings. For both experiments, data were collected from three independent experiments, each replication contains n > 25 seedlings for each lines. Values are means ± S.E. (p < 0.001 for in-vitro *abp1* mutants versus Ws). Bar = 5 mm.

Through double selection of transformed progeny on BASTA and kanamycin containing agar, followed by PCR genotyping with primers against the insertion allele of *ABP1* (Chen et al., 2001), we were able to isolate three stabile *abp11* mutants, *abp1-8 (*Thr54>Ile54), *abp1-9* (Leu25>Tyr25) and *abp1-10* (His106>Asn106).

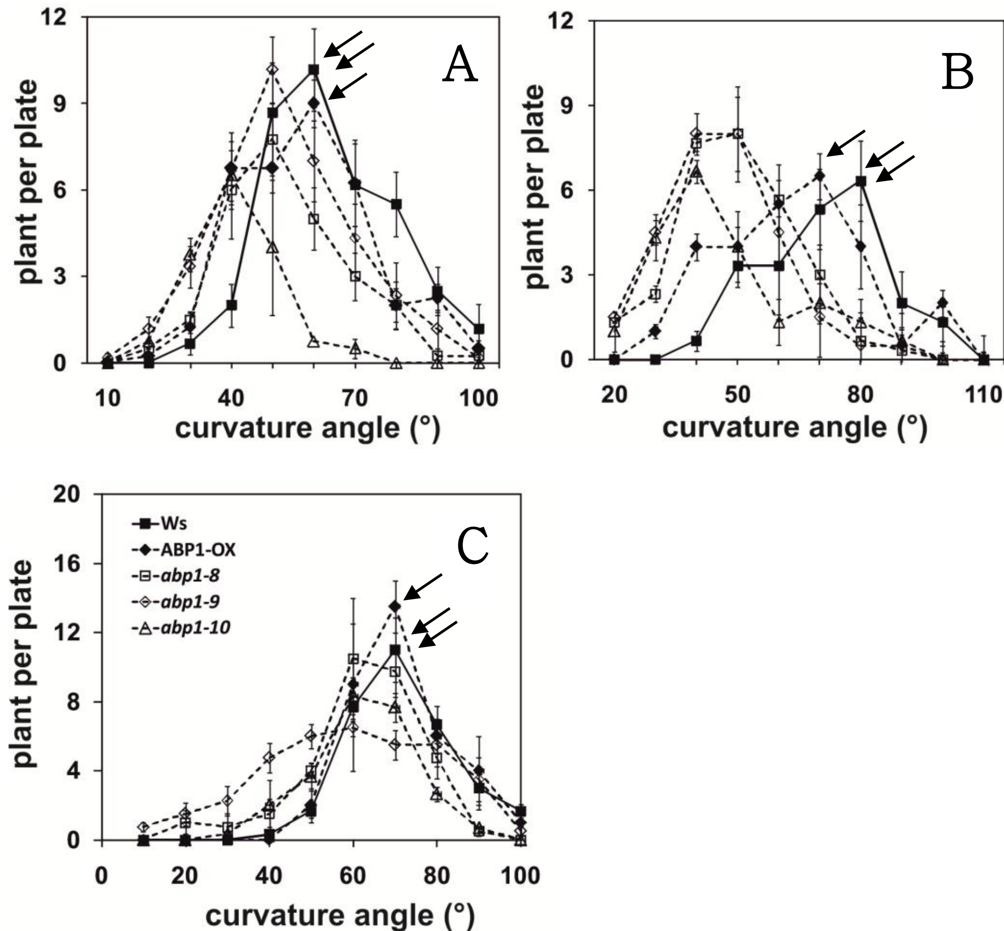

Figure 2. Response to gravity and lateral blue light in Ws, *ABP1-OX* and *abp1* mutants.

(A) Gravitropic responses of the hypocotyls of 3 days old dark-grown seedlings. Seedlings were grown vertically for 4 days in the dark, tilted by 90° for 24 hours and gravitropic angles were determined. Data were obtained from five plates for each genotype with n > 150 total of seedlings. Data are means (± S.E.) of seedling per plate (B) Gravitropic responses of the roots of 3 days-old dark-grown seedlings after 24 h. Growth and quantification were performed as described in (A). Five plates per genotypes with total > 100 seedlings were used for generating the graph (B). Values are means ± S.E. (C) Phototropic responses of hypocotyls of 4-days dark grown seedlings. Growth conditions and quantification were performed as described in (A). Four plates per genotype with total > 96 seedlings for Ws and > 75 seedlings for each *abp1* mutants we counted. Values are means ± S.E. Phototropism was induced by lateral blue light (10 µmol m^{-1} s^{-2}) for 8 hours from LED light (CFL, PlantClimatic, GmbH, http://www.plantclimatics.de). One arrow = *ABP1-OX*, two arrows = Ws.

These *abp1* mutants express no longer the *ABP1* wild type allele and were made homozygous for the mutant allele. We transformed also the 35S::*ABP1* wild type cDNA with tags constructed into heterozygous *abp1/+*, termed *ABP1-OX*.

abp1 mutants showed mutant properties when grown in white light and also in certain light conditions. *abp1* mutants as well as *ABP1-OX* showed a wavy pattern and slanting root growth (Fig.1 A-B), and had longer hypocotyls (Fig.1 C) in comparison to wild-type. Hypocotyls and roots of *abp1* mutants were less responsive to gravity (Fig. 2 A). The hypocotyls of wild type seedlings showed a dominant single peak at 60° bending which was also observed for *ABP1-OX* plants whereas *abp1-8* and *abp1-9* produced a single peak at 50° and *abp1-10* had a peak at 40°. Similar results were obtained for gravitropic responses of roots where seedlings of *abp1-8*, *abp1-9* and *abp1-10* showed a weaker response by producing a peak in bending angles at 50° in *abp1-8* and *abp1-9*, and 40° in *abp1-10*. Wild type and *ABP1-OX* displayed a peak at 80° and 70° bending angle respectively (Fig. 2 B). We analyzed phototropism in *abp1* mutants by exposing 4 days old dark grown seedlings to lateral 10 µmol m^{-1} s^{-2} blue light for 8 h. We found hypocotyls of *abp1* mutants were less sensitive to blue light as compared to wild type and *ABP1-OX*, and bending angles were approximately 60° in *abp1* mutants and 70° in wild type and *ABP1-OX* (Fig. 2 C). From these data, it is obvious that all *abp1* mutants are less sensitive in gravitropism and phototropism in comparison to wild type whereas wild type cDNA overexpressing *ABP1-OX* showed no insensitivity.

***abp1* mutants exhibit insensitivity to auxin**

We tested sensitivity of *abp1* mutant to auxin by growing seedlings on agar media containing increasing auxin concentrations. Root length and lateral root number were analyzed. There were only small differences in main root length between wild type and *abp1* mutants observed at auxin concentrations of 0.01 µM – 0.05 µM, except *ABP1-OX* which showed slightly longer roots than wild-type at 0.01 µM – 0.1 µM (Fig. 3 A).

A significant decrease was found in lateral root numbers in all *abp1* mutants, particularly in response to auxin higher than 0.03 µM (Fig. 3 B). Wild type and *ABP1-OX* had more lateral roots in comparison to *abp1* mutants at these auxin concentrations. These data indicate lower auxin sensitivity in *abp1* mutants in comparison to wild type and *ABP1-OX* plants.

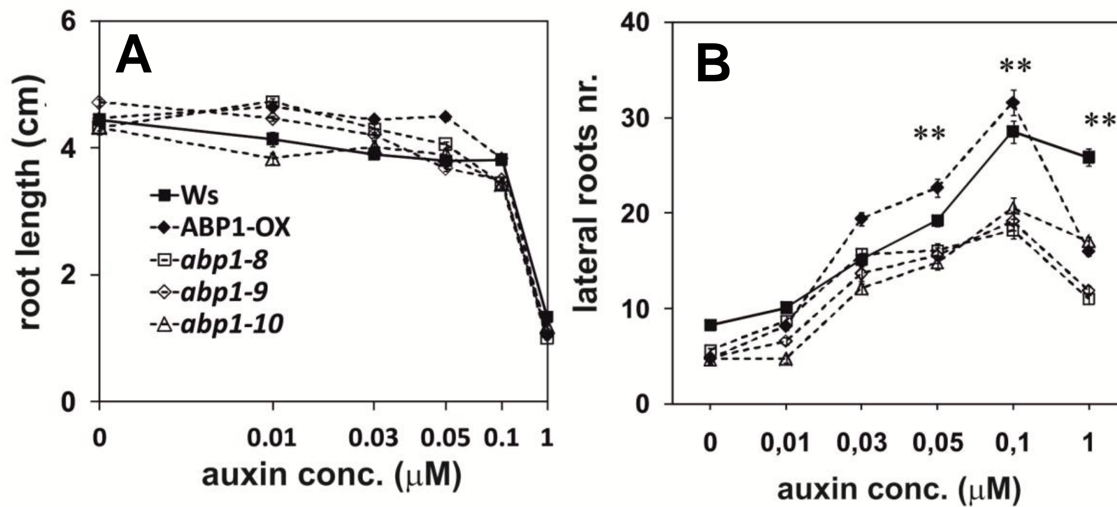

Figure 3. Auxin sensitivity of root response of Ws, *ABP1-OX* and *abp1* mutant seedlings.

Seedlings of Ws, *ABP1-OX* and *abp1* mutants were grown on vertical agar media without auxin for 4 days, then transferred to plates containing increasing auxin concentration for 6 more days before main root length was determined (A) and lateral root number (B). Data for each genotype were obtained from three plates with total n = 30. Experiments were repeated two times independently. Values are means with S.E. (differences were $p < 0.001$ for Ws and *ABP1-OX* versus *abp1* mutants indicating by **)

Lower auxin sensitivity is revealed in gene expression in the *abp1* mutants

Several previous studies have shown that most of auxin-related mutants confer severely defective phenotypes and are also impaired in auxin-induced gene expression (Park et al., 2002; Braun et al., 2008; Effendi et al., 2011). Expression of seven early auxin-induced genes (*IAA2, IAA11, IAA14, IAA19, SAUR9, SAUR23, GH3.5,* and *ABP1*) and four PIN genes (*PIN1, PIN2, PIN3,* and *PIN5*) in *abp1* mutants, wild type and *ABP1-OX* was tested. We focused to measure expression level these genes at 0 min, 30 min and 1 hour after auxin treatment (Effendi et al., 2011).

At 30 minutes after treatment with 1 μM 1-NAA, seven (*IAA2, IAA11, IAA19, SAUR9, SAUR23, GH3.5,* and *ABP1*) of eight early auxin-responsive genes showed up-regulation in wild-type seedlings by approximately two to fivefold (Fig. 4). *abp1* mutants showed almost no up-regulation in most early auxin-responsive genes, a slight up-regulation less than twofold was found for *IAA2* and *IAA19* in all *abp1* mutants.

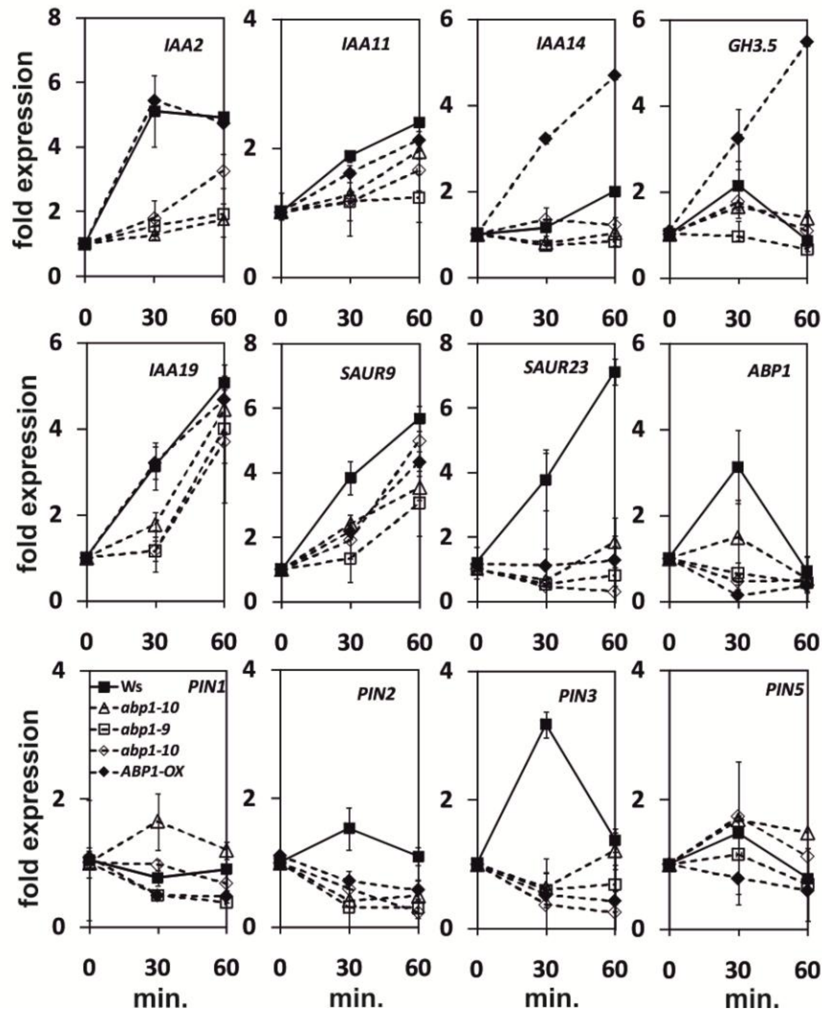

Figure 4. Transcriptional expression of early auxin genes and some PIN genes in light grown Ws, *ABP1-OX* and *abp1* mutants.

Seedlings were grown on ½ MS media agar on 8h/16h white condition for 14 days. Seedlings were then incubate on ½ MS liquid media for acclimatisation for 2 hours, then transferred and incubated in fresh ½ MS liquid media containing 1 µM 1-NAA for 0 min, 30 min and 60 min. Seedlings were dried as quickly as possible with tissue paper and frozen in liquid nitrogen. For detail of RNA extraction and cDNA synthesis, see Experimental Procedures. Quantitative Real Time PCR (qRT-PCR) data were obtained from three biological replications with three technical replications for each target gene. Statistical analysis was performed as described by Livak and Schmittgen (2001) and verified using the method as described by Pfaffl *et al.* (2002).

A slight up-regulation in *SAUR9* was observed in *abp1-10*, but lower in comparison to wild type. *ABP1-OX* showed up-regulation in five genes (*IAA2, IAA11, IAA14, IAA19,* and *GH3.5*) with similar fold expression as in wild type. However, no up-regulation was found in *SAUR9, SAUR23* and *ABP1* in the *ABP1-OX*. We noticed that *abp1-8* and *abp1-9* as well as *ABP1-OX* seedlings showed down-regulation in *SAUR23* and *ABP1* genes after 30 minutes auxin

treatment, while wild type showed up-regulation of these genes. In comparison to wild type, expression of *PIN2* and *PIN3* was down-regulated in *abp1* mutants and *ABP1-OX* seedlings, while in wild type these genes were up-regulated after 30 minutes auxin treatment. *PIN1* and *PIN5* expression was near identical in all genotypes and little or not at all influenced by auxin (Fig. 4).

Taken together, the lower transcription of early auxin-responsive genes and *PIN* genes in *abp1* mutants clearly indicate insensitivity to auxin in *abp1* mutants in comparison to wild type and in comparison to *ABP1-OX*.

Response to monochromatic continuous red and far-red lights in *abp1* mutants

Cross-talk between auxin and light in plant growth regulation has been intensively investigated, particularly, responses to shade light (Devlin et al., 2003; Vandenbusche et al., 2003; Roig-Villanova et al., 2007; Hortnischek et al., 2009; Sorin et al., 2009; Keuskamp et al., 2010). Since *abp1* mutant seedlings had longer hypocotyls under white light condition (Fig. 1 C) this suggested that *abp1* mutants could have defects in light responses. We investigated responses of *abp1* mutants to different monochromic light by growing seedlings for 1 day in the dark and for 3 days either in continuous 1 µmol m^{-1} s^{-2} red light or 1 µmol m^{-1} s^{-2} far-red light. Under red light, *abp1-8, abp1-9* and *abp1-10* seedlings showed significantly longer hypocotyls than wild type and *ABP1-OX* seedlings (Fig. 5 A,B) and the hypocotyl growth direction was more or less random in this light condition (Fig. 5 C). Interestingly, in *abp1-8* and *abp1-9*, the hypocotyls were even longer than in *phyB-9* mutant seedlings, while *abp1-10* showed a length similar to *phyB-9* mutant seedlings (Fig. 5 A,B).

Similar to the responses in red light, all *abp1* mutants seedlings displayed longer hypocotyls in continuous far red in comparison to wild type but shorter in comparison to *ABP1-OX* and *phyA-211* mutant seedlings (Fig. 5 D and E). Since hypocotyl elongation is inhibited by continuous far-red light in a fluence-dependent manner (Whitelam et al., 1993) except in *phyA* mutants, long hypocotyls in *abp1* mutants under far red light condition suggested that *abp1* mutants might convey defective PHYA-mediated responses. However, not all *phyA* deficiency responses in de-etiolated seedlings were observed in *abp1-8*, *abp1-9* and *abp1-10*. For examples, the *abp1* mutants and *ABP1-OX* displayed no apical hook and opened and expanded cotyledons like in wild type (Fig. 5 F). Moreover, red and far-red light are known to reduce gravitropism in hypocotyls leading to randomization of hypocotyl direction (Robson and Smith, 1996; Kim et al., 2011).

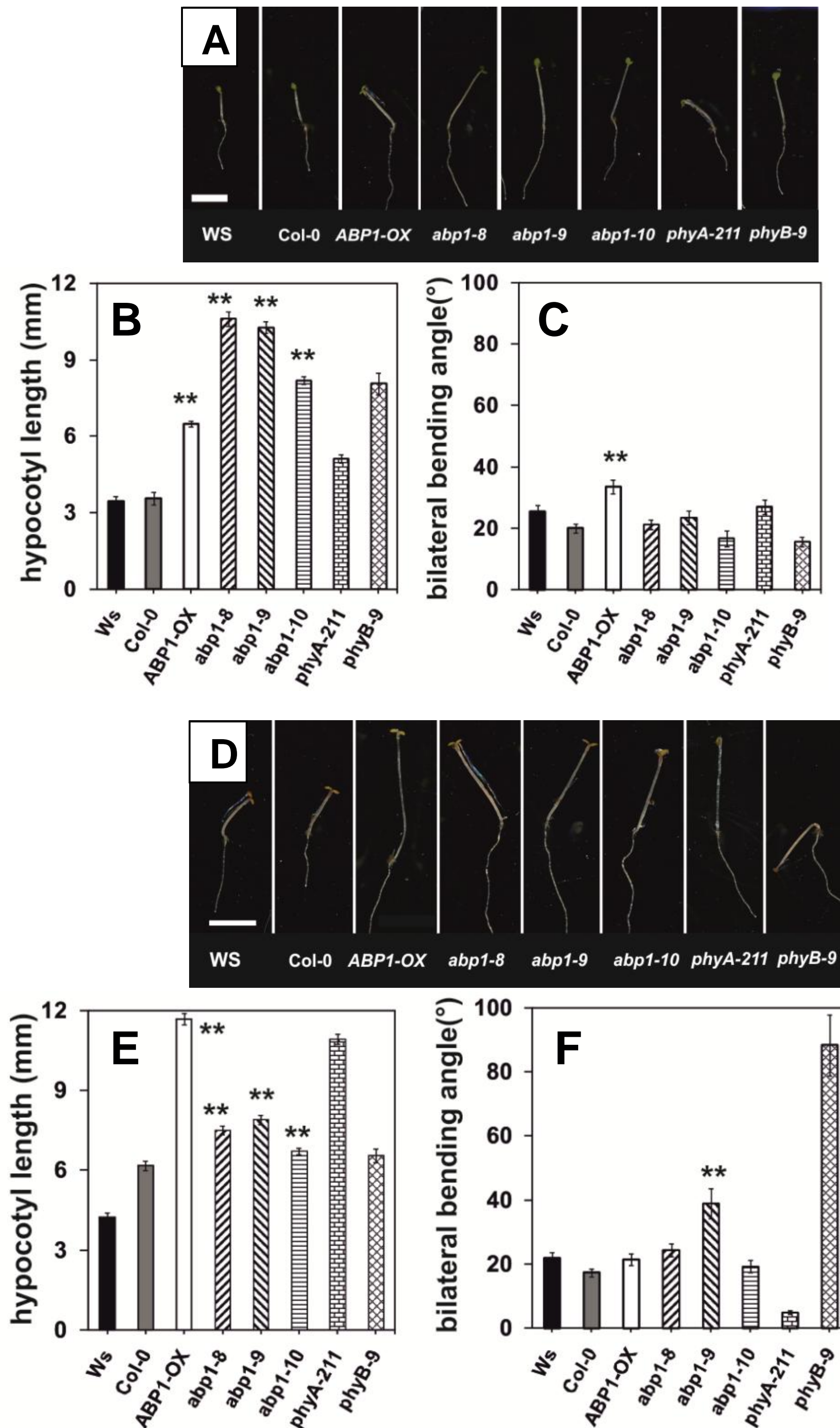

Figure 5. Responses of hypocotyl to monochromatic continuous red or far-red in Ws, *ABP1-OX*, *abp1* mutants, *phyA-211* and *phyB-9*. Representative images of red light-grown seedlings (A) and far-red-grown seedlings (D). One day dark-grown seedlings were grown further under either with 1 µmol m^{-1} s^{-2} of red light or 1 µmol m^{-1} s^{-2} far-red light for 3 days on half strength MS Gelrite (Duchefa-Biochemie) media containing 1% sugar. Quantifications of hypocotyl length under red light and far-red light are shown in (B) and (E) respectively. The growth direction of hypocotyl under the same light condition was measured (red light, C) and (far-red, F). Experiments were repeated three times independently and each replication contains > 30 seedlings for each genotype. Values are means with ± S.E. ($p < 0.001$). Bar = 5 mm.

The data on growth direction of hypocotyls in *abp1* mutants again showed that not all *phyA* mutant properties are present in the *abp1* mutants. We found that hypocotyls of *abp1* mutants showed some randomization of growth direction in comparison to completely upright *phyA* mutant seedlings in far-red (Fig. 5 F) indicating only partialy insensitivity to far-red inhibition of gravitropism (Liscum and Hangarter, 1993; Robson and Smith, 1996) in *abp1* mutants. Thus, we suggest that *ABP1* is required only partially for far-red responses.

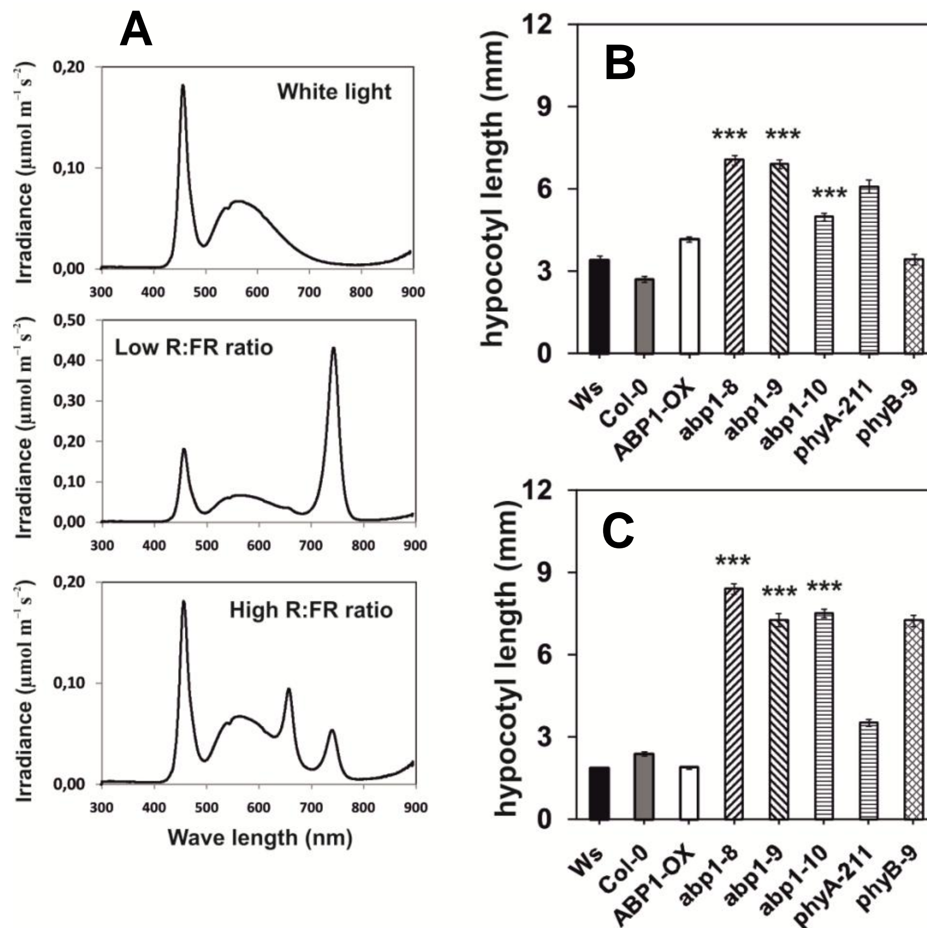

Figure 6. Responses of hypocotyl elongation in Ws, *ABP1-OX* and *abp1* mutants to far-red enriched light (low R:FR ratio) and red enriched light (high R:FR ratio).

(A) Light spectrum that were used in the experiments were measured using spectrometer USB4000 (Ocean Optic) and analyzed using software Spectrasuite (Ocean Optic). (B,C) Hypocotyl elongation in responses to low R:FR ratio or high R:FR ratio light. Seedlings were grown vertically on ½ MS agar media under constant white light (24.5 µmol m^{-1} s^{-2}) for 3 days, then added either with low R:FR ratio (0.098) (B) or with high R:FR ratio (2.1) (C) for 3 days more. Data were obtained from n > 120 seedlings for each genotype. Experiments were repeated independently 3 times with similar results and graphics were presented here represent one of three replications. Values are means with S.E. ($p < 0.001$).

***abp1* mutants are hypersensitive in response to shade-simulated light and flower early**

Changes in the ratio of red and far-red light are main cues for plants to pursue a strategy to avoid or tolerate this neighbor-induced light condition (Robson et al., 2010). The most dramatic response to shade light is hypocotyl elongation which can be remarkably rapid and start in only a few minutes (Ruberti et al., 2011). To investigate the response of *abp1* mutants to shade light, we grew *abp1* mutants seedlings under far red-rich light (low R:FR ratio) and red-rich light (high R:FR ratio) and analyzed the hypocotyl length. *Arabidopsis* seedlings were grown for 3 days under continuous white light (24,5 µmol m^{-1} s^{-2}) before a mixture of red and far-red light was added with either low R:FR ratio (0.098) or high R:FR ratio (2.1) for 3 more days (light spectrum in Fig. 6 A).

As shown in figure 6 B, *abp1* mutants seedlings displayed much longer hypocotyls under low R:FR ratio light, while wild type and *ABP1-OX* seedlings showed relative shorter hypocotyls. *abp1-8* and *abp1-9* mutants were even longer than the constitutive shade-avoidance *phyA-211* mutant seedlings. We also analyzed growth responses to high R:FR ratio in *abp1*mutants. Similarly insensitive responses as in low R:FR ratio data were observed in *abp1* mutants. They produced longer hypocotyls in comparison to wild type and *ABP1-OX* seedlings. Interestingly, *abp1* mutants showed a length similar to hypocotyls as displayed in the *phyB-9* mutant (Fig. 6 C). This indicated that *abp1* mutants might be defective in phyB-mediated responses to shade light, particularly in the hypocotyl length response. Shade responses are regulated redundantly by PHYB, PHYD, and PHYE (Franklin, 2008; Deng et al., 2010; reviewed in Stamm and Kumar, 2010).

Arabidopsis impaired in *phyB* function has a constitutively early flowering phenotype in short days (Halliday et al., 2003). We investigated flowering time in the *abp1* mutants in short days. All *abp1* mutants flowered 6-10 days ($p < 0.001$) earlier in comparison to wild type and two days earlier ($p < 0.05$) than *ABP1-OX* plants (Fig. 7 A and B). All *abp1* mutants produced a smaller rosette leaf number at flowering time compared to wild type and *ABP1-OX* (Fig. 7 C). Our flowering time data in *abp1* mutants support the notion that phytochrome-mediated mechanisms, particularly phyB-mediated signal mechanisms, are defect. We compared also leaf phenotypes of the *abp1* mutants to wild type as well as with *ABP1-OX*, since *phyB* mutation have been reported to have enhanced leaf area (Robson et al., 1993). We observed that all *abp1* mutants have longer and wider leaf blades in comparison to wild type and *ABP1-*

OX (Fig. 7 D and F), suggesting *ABP1* might contribute to the repression of leaf blade expansion in the wild type and in the *abp1* mutants leaf expansion a phyB-regulated property might be also defect.

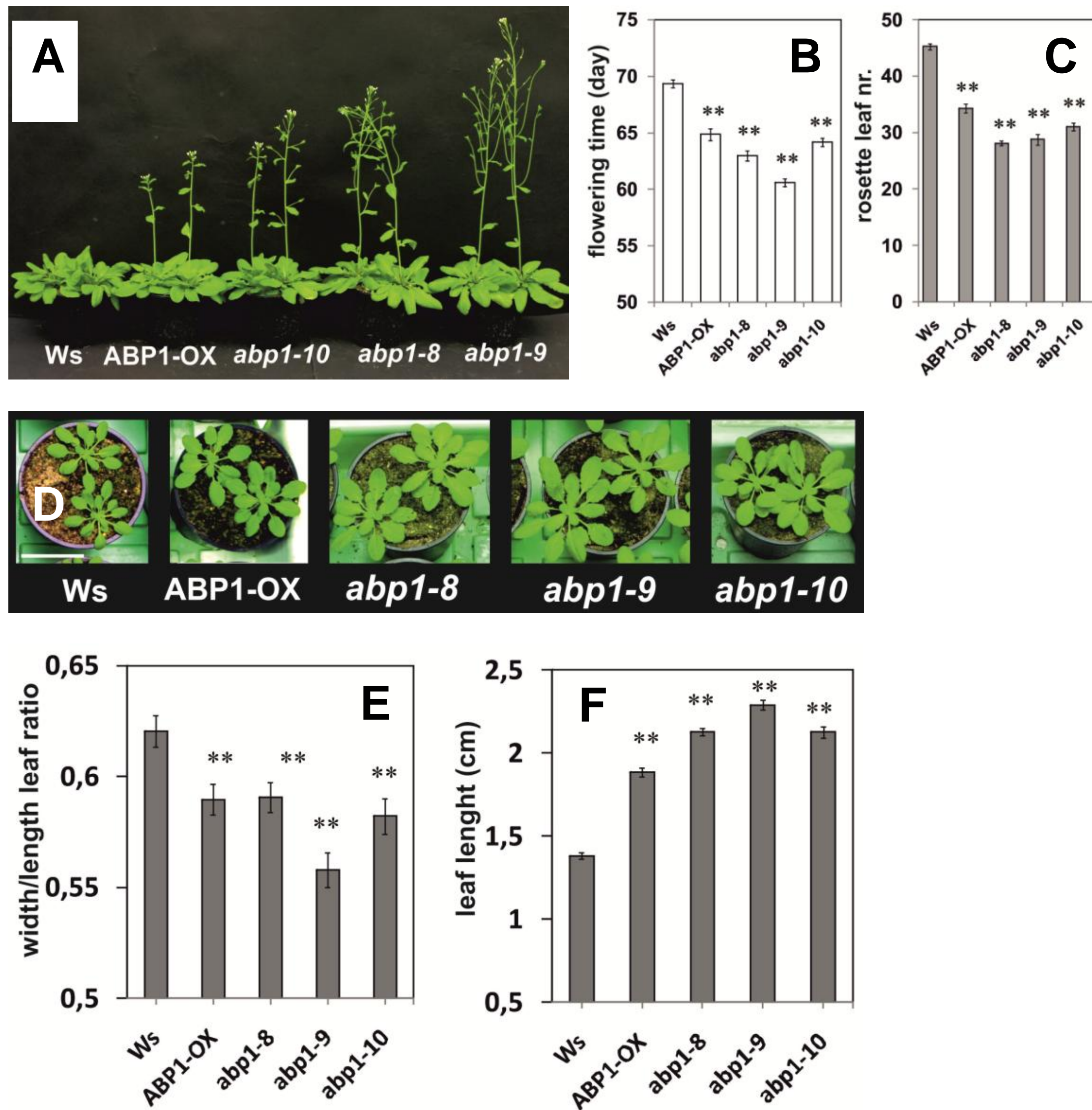

Figure 7. Early flowering phenotypes in in-vitro *abp1* mutants under short-days condition (8h/16h light/dark).
(A) Representative images of 59 days-old plants of Ws, *ABP1-OX*, *abp1* mutants (*abp1-10, abp1-8, abp1-9*). Plants were grown under short-days and flowering time was defined as the time of the first flower emerging which was indicated by opening of the first bud and white petals became visible. (B) Flowering date. (C) Rosette leaves number. Experiments were conducted in two independent replications. From each replication, 30 plants for each genotype were recorded for their flowering date and rosette leaf number. Values are means with S.E. (D). Representative images of leaves appearance of Ws, *ABP1-OX*, *abp1-8, abp1-9,* and *abp1-10.* Ratio of width:length of leaf blades (E) and length of leaf blade (F) were measured from 59 days-old plants. Three biggest leaves from each plant were taken as samples and measured and data were obtained from 60 plants for each genotype. Values are means with S.E. (p <0.001). Bar = 5 cm.

Taken together, the experiments in far red- and red-monochromatic light and shade light indicate defects in phyB-regulated responses but also in monochromatic light, a partial defect in phyA-regulated responses is indicated.

Transcriptional expression of light-induced genes in *abp1* mutants

abp1 mutants exhibited defects that were shown to be stronger in response to shade light. Thus, we reasoned that *ABP1* might also be required for transcriptional regulation of genes involved in shade responses. Several shade-responsive genes have been identified (Devlin et al., 2003; Salter et al., 2003; Sessa et al., 2005; Hortnischek et al., 2009) and some of them were known as primary targets in a shade-regulated transcriptional cascade (Carabelli et al., 1993, 1996; Steindler et al., 1999; Morelli and Ruberti, 2002; Roig-Villanova et al., 2006; review in Stamm and Kumar, 2010; review in Ruberti et al., 2011). To investigate whether mutated *ABP1* resulted in defective responses to shade light, we investigated transcriptional expression of nine shade-induced genes (*ATHB2, HFR1, PIL1, PIF1, PIF5, IAA19, IAA29, PIN3,* and *FIN219*). We used a modified shade light set-up condition as previously described in Wang *et al.* (2011) for our experiment to restrict light influence to a short induction period. Samples were prepared by growing seedlings on agar media under constant white light (24.5 µmol m^{-1} s^{-2}) for 7 days and then transferred to white light supplemented either with far-red (R:FR ratio of 0.098) for 1 hour (Fig. 8 A) or red (R:FR ratio 2.1) for 1 hour (Fig. 8 B).

Expression of tested far red light-induced genes in the *abp1* mutants was found to differ significantly from wild type in each mutant. 50%-90% of the tested genes were expressed statistically significant different. In response to FR-rich light (low R:FR ratio), a basically similar pattern in the expression of three shade-induced genes (*ATHB2, HFR1, PIL1*) was observed in wild type, *ABP1-OX* and in *abp1-8* (Fig.8 A). However, *abp1-9 and abp1-10* mutants expressed two or all three of these genes lower in comparison to wild type and *ABP1-OX*. *abp1-9* showed the lowest induction for both *ATHB2 and HFR1* genes, while in *abp1-10* only *HFR1* induction was low in comparison to wild type. No great differences were observed in the expression of *PIL1* between mutants and wild type, only *abp1-10* showed lower *PIL1* expression than wild type. Interestingly, *ABP1-OX* also showed low induction of *HFR1*, a gene which suppresses elongation to balance *ATHB2* (Sessa et al., 2005; Hortnischek et al., 2009). Higher induction was observed in *PIF1* expression in *abp1-10* in comparison to wild type, but the other *abp1* mutants were not significantly different in comparison to wild type.

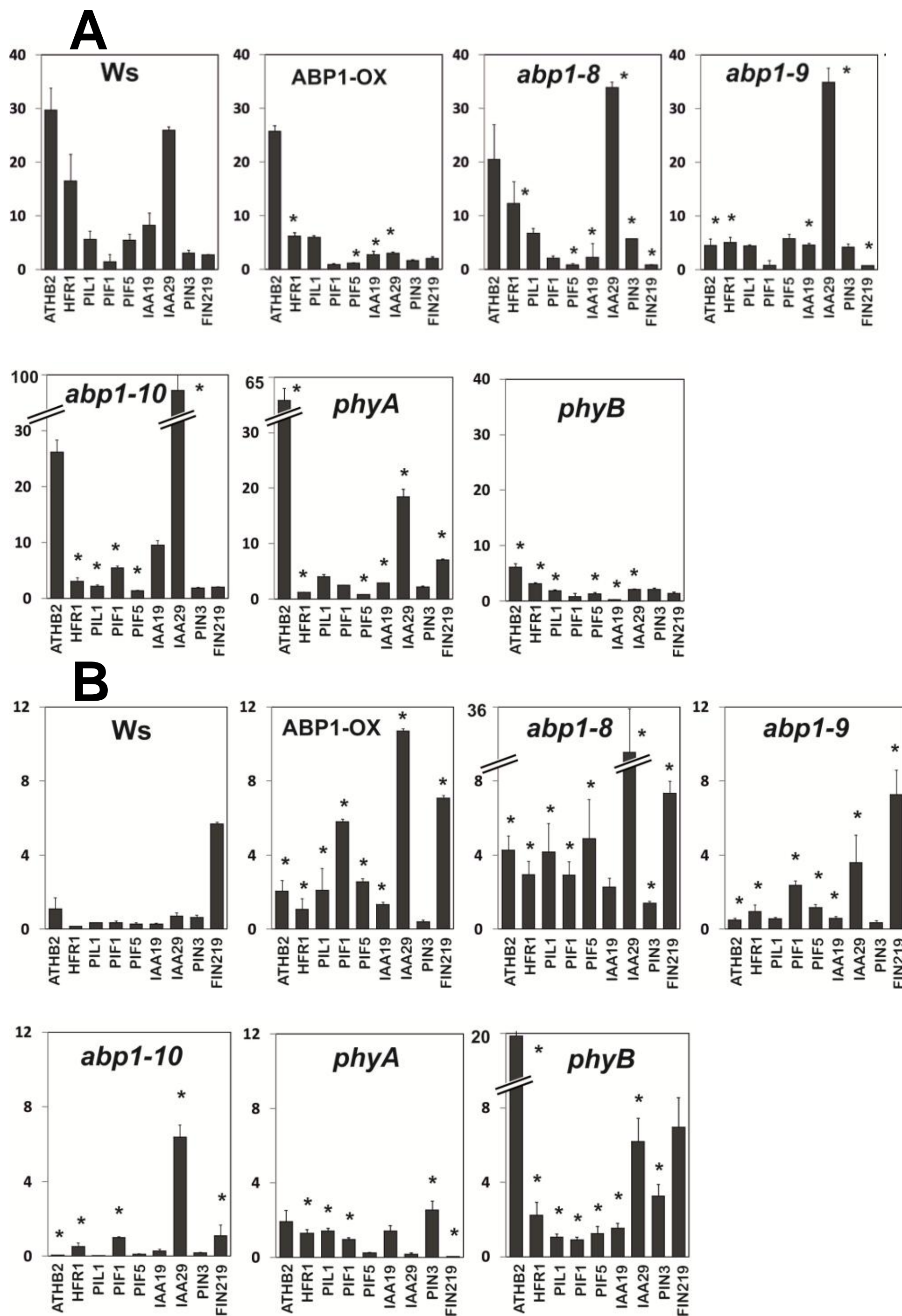

Figure 8. Transcriptional expression of light-induced genes under far-red riched- (A) and red riched-growth (B) condition in Ws, *ABP1-OX, abp1-8, abp1-9, abp1-10, phyA-211*, and *phyB-9* seedlings. Seedlings were grown 7 days under 24.5 m^{-1} s^{-2} white light before treatment with low R:FR ratio (0.098) or high R:FR ratio (2.1) for 1 hour. Seedlings were frozen and used for RNA extraction material. For detail of RNA extraction and cDNA synthesis, see Experimental Procedures. Quantitative Real Time PCR (qRT-PCR) data were obtained from at least three biological replications with three technical replications for each gene target. Statistical analysis was performed as described by Livak and Schmittgen (2001) and verified using method described by Pfaffl *et al*. (2002). Values are means with S.E. ($p < 0.05$). Significant differences (*) were relatively compared to Ws with at least $p < 0.05$.

PIF5 is positively up-regulated by low R:FR ratio light (Lorrain et al., 2008) as was observed in wild type and in *abp1-9* statistically significant, but not in other *abp1* mutants and *ABP1-OX*.

In *phyA-211*, *HFR1* induction was absent and *ATHB2* induction was very high. *PIF5, IAA19* and *IAA29* induction was low but *PIF1* was similar to wild type. Still with the exception of shade repressor *HFR1*, the expression pattern of *phyA-211* after far red induction was overall similar to wild type. In *phyB-9*, induction of all these genes was very low.

Expression of *IAA19* and *IAA29* genes was up-regulated by simulated shade light (Hortnischek et al., 2009). In our experiments, *IAA19* expression was variable in *abp1* mutants. *abp1-8* and *abp1-9* as well as *ABP1-OX* showed lower transcriptional levels of *IAA19* in comparison to wild type (Fig. 8 A), while *abp1-10* showed induction similar to wild type. Moreover, we found that *IAA29* expression was similar in all *abp1* mutants, *phyA-211* and wild type; only in *ABP1-OX* induction was lower just as in *phyB* (Fig. 8 A). We found slight up-regulation in *PIN3* expression in all genotypes, but only *abp1-8* and *abp1-9* showed a slightly higher induction of *PIN3* than wild type (Fig. 8 A). All together, it is obvious that *abp1* mutants and, to some extent *ABP1-OX,* have quantitative reductions in expression of shade-induced genes compared to wild type, suggesting that *ABP1* is involved in the mediating of shade avoidance responses which is controlled mainly by *phyB*.

Under red-rich light (high R:FR ratio), all tested genes, except *FIN219,* were not up-regulated in wild type, while *abp1* mutants displayed various expression patterns under the same light condition (Fig. 8 B). *ABP1-OX* and *abp1-8* showed induction in almost all genes by red light addition (Fig. 8 B), while *abp1-9* and *abp1-10* displayed only slight elevation in transcriptional levels of few genes or were similar to wild type. Similar gene expression patterns were observed in *ABP1-OX* and *abp1-8*, and the generally high induction by added red light of many of the tested genes was also found in *phyB-9*. In high red, lack of suppression of the tested genes is apparent in *phyB-9*. As general pattern in *phyB-9* and *ABP1-OX* and *abp1* mutants, relatively high induction of *IAA29*, low induction of *PIN3*, and high induction of *FIN219* was found. Only *FIN219* was induced in wild type so that induction of *IAA29* and *PIN3* may be a "signature" for *phyB-9* which was also found in *abp1* mutants.

DISCUSSION

Choosing engineered point mutations is convenient to investigate ABP1 functions. With heterozygous *abp1/+* we could perform our experiments only with seed mixtures of 2:1 heterozygous:wild-type (Effendi et al., 2011). Because the loss of function of *ABP1* in homozygous plants is embryo lethal (Chen et al., 2001) viable homozygous *abp1* mutants should provide a better chance in experimental handling and in obtaining new phenotypes. Here we present three viable, engineered *abp1* mutant lines, *abp1-8, abp1-9,* and *abp1-10*, containing mutations in the auxin binding domain (Woo et al., 2002; Napier 2002) and expressing no wild-type *ABP1* allele in *Arabidopsis*. Choosing to mutate the residues Thr54 to Ile54 in *abp1-8* and Leu25 to Tyr25 in *abp1-9* in the binding domain for auxin (Woo et al., 2002) might change binding of auxin to ABP1, and mutation in His106 to Asn106 in *abp1-10* might change binding to the zinc ion in ABP1 and indirectly of auxin to zinc, similarly as in *abp1-5* where another zinc chelator, His59, is mutated (Robert et al., 2010). Those other artificial mutations were tried by us which involved the Trp151 residue proved non-viable plants. This tryptophan was shown to be highly important for function (David et al., 2007). As a receptor, proteins should bind ligands with strict structural and steric specificity. Mutations of critical amino acids in the presumed receptor will affect signal transduction and the downstream functions (Jones and Sussman, 2009) as shown here.

abp1 mutants exhibit altered developmental responses to auxin which resemble a hyposensitive phenotype

Our vector was constructed to code for an inserted strep-flag double tag right before the ER retention signal KDEL so that even expression of the wild type cDNA in the construct in *ABP1-OX* potentially could confer mutant properties. Expression of all mutated *abp1* cDNAs in *ABP1*-null background indeed showed impaired responses in auxin-related functions but not expression of wild type cDNA in *ABP1-OX*. From plants expressing additional point mutations we expected stronger auxin-related phenotypes. All mutants shown here had strong slanting root angles and waving roots (Fig. 1). Plants with strong slanting and waving root phenotypes often have reduced gravitropic responses (Okada and Shimura, 1990; Luschnig et al., 1998) as well as other auxin-related function such as a reduction in auxin sensitivity (Simmons et al., 1995; Sedbrook et al., 1999; Ferrari et al., 2000; Sedbrook et al., 2002; Santner and Watson, 2006; Sedbrook and Kaloriti, 2008; Effendi et al., 2011). Additionally, under our experiment conditions, *abp1* mutants had longer hypocotyls than wild type

indicating defects in auxin functions or in light signaling or both (Liscum and Hangarter, 1991; review in Halliday et al., 2009). Light-related functions of ABP1 will be discussed below.

Hyposensitivity to exogenous auxin was tested in the classical root responses; main root growth inhibition and lateral root formation and in testing rapid induction of auxin-regulated genes. Whereas *ABP1-OX* was like wild type in developmental responses all point mutants were hyposensitive to auxin (Fig. 2). Using gene regulation as a test, delayed regulation was evident in the point mutations but also in a few genes in *ABP1-OX* (Fig. 3). Exogenous auxin did not evoke altered developmental responses in *abp1-5* or *abp1-SS12K* (Braun et al., 2008; Robert et al., 2010) or *abp1/+* (Effendi et al., 2011) but delayed gene regulation was also found in them (Effendi et al., 2011; Effendi et al., 2012-submitted). Defects in early auxin-induced gene regulation had proven to be a sensitive tool to identify functional defects in *abp1* mutants before (Effendi et al., 2011; Effendi and Scherer, 2011; Effendi et al., 2012-submitted). So, the data were presented here described new mutants have stronger auxin-related phenotypes than previous ones manifested in morphological responses and regulatory responses.

***abp1* mutants are defect in phototropism and gravitropism**

Regulation of auxin transport from cell to cell via polar auxin transport mechanism is suggested to start tropic responses (Friml et al., 2002; Esmon et al., 2005; Esmon et al., 2006; Petrásek et al., 2006; Rakusova et al., 2011). PIN2 and PIN3 were identified mainly mediating tropic responses (Müller et al., 1998; Friml et al., 2002). Recent experimental evidence (Wisniewska et al., 2006; Abas et al., 2006; Robert et al., 2010; Xu et al., 2010; Deng et al., 2011; Effendi et al., 2011; Effendi and Scherer, 2011; Effendi et al., 2012-submitted) and our data presented in this study indicate that ABP1 could mediate regulation of auxin transport in tropism by rapid changes in PIN subcellular distribution. We therefore suggest that ABP1 acts through the activity changes of PIN proteins induced by endocytosis and transcytosis (Klein-Vehn and Friml, 2008).

Transcriptional regulation could become important for a more sustained type of response. In line with this are low transcript levels of *PIN2* and *PIN3* in responses to auxin in the *abp1* mutants so that a decreased phototropic and gravitropic response of roots and hypocotyls in the *abp1* mutants can not be sustained (Fig. 3). Among genes that were identified as *Tropic*

Stimulus-Induced (TSI) are auxin-dependent genes activated in *Brassica oleracea* (Esmon et al., 2006). Among these are *GH3.5* and *IAA19* which are expressed lower in *abp1* mutants in response to auxin (Fig. 3). Repressor protein IAA19 has been identified to be involved in the regulatory feedback loop for the control of auxin-dependent tropic responses by making heterodimer with ARF7. Lacking activity of auxin-regulated transcriptional activator NPH4/ARF7 in *Arabidopsis* seedling will promote the disruption of photo- and gravitropic responses (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997; Stowas-evans et al., 1998; Watahiki et al., 1999). Although current knowledge of transcriptional regulation of auxin-responsive genes identify the receptor TIR1 as the key player in gene regulation (Mockaitis and Estelle, 2008), recent studies have demonstrated that ABP1 may contribute in the regulation of early auxin-responsive genes (Braun et al., 2008; Tromas et al., 2009; Effendi et al., 2011; Effendi et al., 2012-submitted). Taken together, we suggest that ABP1 contributes to the control of gravitropic and phototropic responses by modulating PIN action and regulating the expression of some auxin-induced Tropic Stimulated-Induced genes.

abp1 mutants are red-insensitive in response to monochromatic light

The *abp1* mutants were investigated here, having a stronger auxin-related phenotype as those were investigated before (Braun et al., 2008; Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011; Effendi et al., 2012-submitted). Surprisingly, the *abp1* mutants were all insensitive to monochromatic red and far red light (Fig. 6). There seems to be only few mutants *like pft1 (phytochrome and flowering time1)*, *prr7 (pseudo-response regulator7)* and *rf2-1* (*red and far-red insensitive2 to 1*), which is insensitive to both light condition (Cerdán and Chory, 2003; Kaczorowski et al., 2003; Chen and Ni, 2006). As in all other red or far red light experiments, *ABP1-OX* had a mutant phenotype as well, not only the *abp1* mutants, which was usually clearly weaker than in *abp1* mutants. We assume that the tags which were inserted closely to the mobile C-terminus of ABP1 confers conformational change(s) which cause slightly aberrant signal transduction (Bertośa et al., 2008; Scherer, 2011), especially, in pathways leading to interaction with red light signaling. The auxin-induced responses seemed to be less affected or not at all by this presence of a tag.

abp1 mutants show constitutive or hypersensitive response to shade light conditions

Surprisingly, *abp1* mutants showed constitutive shade responses. As "shade" is defined white light enriched with far red light i.e. having a low ratio of R:FR. Using phytochromes, plants are able to detect the presence of neighboring plants by monitoring the change in R:FR ratio

of light (Franklin, 2008). Reduction in the R:FR ratio due to selective absorption of red light by photosynthetic pigments (Ballaré et al., 1990) is happened in shade from neighboring plants. Thus, the plants respond to this condition by promoting a complex growth mechanism for obtaining more light which, in summary, are known as shade avoidance responses, such as hypocotyl and shoot elongation, petiole elongation, leaf hyponasty and early flowering (Franklin, 2008; Lau and Deng, 2010; Stamm and Kumar, 2010).

The long hypocotyls of *abp1* mutants grown under monochromatic red or far red light respectively (Fig. 4) indicate that *PHYB*- and *PHYA*-related functions, respectively, could be impair in the *abp1* mutants. Plants with similar phenotypes are often having defectives in *phyB* or *phyA* respectively (McCormac et al., 1993; Whitelam et al., 1993). In fact, *abp1* mutants show longer hypocotyl than single *phyB-9* or *phyA-211* mutant, suggesting that both *phyA*- and *phyB*-related functions could be defect. In comparison to single mutant *phyA* or *phyB, phyAphyB* double mutants grown under any monochromatic and mixture of R:FR light radiation have taller hypocotyls (Smith and Whitelam, 1997). In line with this notion, the early flowering time and wider and longer leaf blades in the *abp1* mutants (Fig. 5 and 6) provide evidences that particularly *PHYB*-related functions are defect (Halliday et al., 2003; Robson et al., 1993; Keller et al., 2011) since low signaling activity of PHYB is the basis for the shade avoidance responses (Casal, 2012).

abp1 mutants misregulate far red and red light-induced genes

abp1 mutants are red light-signaling mutants and misregulated about two-third of the shade-induced genes we tested here (Fig. 8). How exactly auxin is involved in light signaling, particularly in shade responses, remains as yet unknown (Franklin, 2008; Stamm and Kumar, 2010; Keller et al., 2011; Nozue et al., 2011). One suggested mechanism is induction of auxin biosynthesis by TAA1 in shade (Tao et al., 2008). Other mechanisms are gene regulation (review in Halliday et al., 2009; review in Ruberti et al, 2011) and diversion of polar auxin transport to regulate growth. Obviously, all three mechanisms could or even should be cooperating (Steindler et al., 1999; Hsieh et al., 2000; Devlin et al., 2003; Salter et al, 2003; Sessa et al., 2005; Carabelli et al., 2006; Lorrain et al, 2007; Roig-Villanova et al., 2007; Hortnischek et al., 2009; Keuskamp et al., 2010).

We used shade-induced genes as indicator genes and as a means to identify defects in light-induced gene regulation in the *abp1* mutants. *ATHB2, HFR1, PIL1, PIF1, PIF5, IAA19, IAA29, PIN3,* and *FIN219* are far red- or shade-dependent genes (Steindler et al., 1999; Devlin

et al., 2003; Salter et al, 2003; Sessa et al., 2005; Lorrain et al, 2007; Roig-Villanova et al., 2007; Hortnischek et al., 2009; Keuskamp et al., 2010). *ATHB2, IAA19, IAA29, PIN3* and *FIN219* were also identified as auxin-related genes (Steindler et al., 1999; Devlin et al., 2003; Roig-Villanova et al., 2007; Hortnischek et al., 2009; Keuskamp et al., 2010). In *phyA-211* plants, tall hypocotyls in this light are correlated with high transcript levels of *ATHB2, HFR1,* and *IAA29* (Fig. 7). Of these genes, high level of *ATHB2* transcript could be a cue for hypocotyl elongation under shade light (Schena et al., 1993; Steindler et al., 1999; Carabelli et al., 2006) and it is known as a positive regulator for hypocotyl elongation (Kunihiro et al., 2011). However, as a negative regulator of shade responses, *HFR1* inhibits the action of *ATHB2* (Sessa et al., 2005; Hortnischek et al., 2009), to prevent exaggerated hypocotyl elongation under shade light. The low transcription level of *HFR1* in the *phyA-211* and in the *abp1* mutants might provide the even more importance cue in determining hypocotyl elongation rather than a high level of *ATHB2* alone. Exception is *abp1-8* were both *ATHB2* and *HFR1* genes were at low level induced. ATHB2/HFR1 interaction is part of the gas and brake mechanism of positive and negative regulators of shade avoidance responses (Sessa et al., 2005; Jiao et al., 2007) which in the *abp1* mutants could be defect at least at the level of *ATHB2* and *HFR1* expression.

After short induction by red light added to white light a “signature” of a PHYB-like response could be the high-low-high expression pattern of *IAA29-PIN3-FIN219* (Fig. 8) in *phyB-9* which, to some extent similarly, was found in the *abp1* mutants including the *ABP1-OX*. *phyB-9* and the *abp1* mutants and *ABP1-OX* all exhibited tall hypocotyls in red light while wild type and *phyA-211* did not showed it and had low *IAA29* expression. These features indicate that red light-dependent light genes are regulated similarly in the *abp1* mutants and in *phyB-9*. Moreover, this high-low-high pattern was also found in the far red light condition where *phyA-211* grew tall hypocotyls. Noteworthy, *IAA29* has been shown to be a component of auxin-mediated elongation growth in shade avoidance responses (Tao et al., 2008). The *taa1/wei1/sav3* mutant has a defect in the locus encoding the TAA1 protein involved in IAA biosynthesis. This mutant also shows a reduction in transcript level of *IAA29* and is unable to elongate in simulated shade light (Tao et al., 2008). Thus, high level of *IAA29* transcript in *abp1* mutants as well as in the *phyB-9* response to red light but not in wild type correlated with tall hypocotyl. High expression of *FIN219* correlated with a tall hypocotyl (Wang et al., 2011), here seen correlated in *phyA-211* in far red, and red in *phyB-9* and the *abp1* lines. Contradicting this correlation is the high expression in wild type in red but there the low *IAA29* expression may not allow a long hypocotyl. This complicated regulatory interaction of

red light-activated genes (Jiao et al., 2007) may not allow a simple straightforward explanation of the transcription results but, clearly, the *abp1* mutants had aberrant red- and far red-induced gene expression which provides an explanation of the observed response phenotypes.

Shade avoidance encompasses also leaf expansion, petiole length and early flowering all of which are regulated by low signaling activity of PHYB redundantly together with PHYD and PHYE in comparable manner (Smith and Whitelam, 1997; Devlin et al., 1998; 1999; Franklin et al., 2003; Hornitschek et al., 2009). This corresponds to phenotypes exhibited in leaf expansion and early flowering (Fig. 7 B) in the *abp1* mutants and the weak phenotype of *ABP1-OX* (Fig. 7 D-F).

Recently, a new model of interaction between light and auxin has been put forward (Keuskamp et al., 2010; 2011) based on previous postulates of a diversion of the polar auxin transport by shade from inner tissue to the epidermis (Morelli and Ruberti, 2000, 2002; Ruberti, 2002). Redistribution of PIN3 subcellular distribution from basal to lateral plasma membranes of endodermal cells is hypothesized to induce lateral auxin transport from the inner cells toward the more lateral cell layers which, in turn, is supposed to lead to hypocotyl elongation. *PIN3* expression is enhanced by shade light in the course of several days (Keuskamp et al., 2010). Our short duration far red or red light did not strongly induce *PIN3* transcription but interaction of ABP1 with auxin polar transport has been proposed (Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011) and the regulation of transcriptional expression of *PIN* genes via ABP1 action were also addressed (data in this paper, Effendi and Scherer, 2011; Effendi et al., 2012-submitted).

Our postulated model for the ABP1 main function is that ABP1 is involved in regulating PIN protein activity, likely by protein phosphorylation and other cytosolic reactions, including phospholipase A activation (Effendi et al., 2011; Scherer et al., 2012). Likely, other auxin transport proteins could also regulated by ABP1 but experimental evidence for this is lacking. We assume that regulation of auxin transport regulates auxin concentration so that TIR1 regulates auxin-induced genes correspondingly. Hence, consistent with this hypothesis, ABP1 also controls other functions which need regulation of polar auxin transport, like phototropism and gravitropism (Effendi et al., 2011; data this paper). If diversion and regulation of polar auxin transport proves to be a main component of the shade avoidance responses this would explain why not TIR1 (Effendi et al., 2012-submitted) but ABP1 is the auxin receptor involved in shade avoidance.

EXPERIMENTAL PROCEDURES

Plant material and growth condition

Arabidopsis thaliana Wassilevskija (Ws) and Columbia (Col-0) wild type plants were used for transformation. Light mutants *phyA-211* (Col) was obtained from C. Luschnig (BOKU, Vienna - Sweden). *ABP1* cDNA containing flag-tag and strep-tag II directly prior to the C-terminal KDEL under control of the 35S promoter was kindly provided by T. Reinard – Institute of Plant Genetics, University of Hannover. This construct was then cloned into pENTR D-TOPO (Invitrogen) where site-directed mutation was performed using QuikChange™ Site-Directed Mutagenesis Kit (Stratagen). Entry vectors were cloned into destination vector pB2GW7 (Karimi et al., 2002) and transformed into *Arabidopsis thaliana* heterozygous *abp1/+* (Chen et al., 2001). Progenies of the transformed plants were selected on agar plates containing kanamycin (50µg/ml) and BASTA (30µg/ml). Surviving seedlings were grown further on soil and PCR genotyping to identify homozygous null ABP1 wild type plants. PCR genotyping was done using reverse ABP1 genomic primer (5'-CCT GAG ATC TCA AGT AGG AAG CGT C-3') and right border primer (5'-TCC CAA CAG TTG CGC ACC TGA ATG-3') primer (Chen et al., 2001).

Most experiments were performed on sterile agar or liquid half-strength Murashige and Skoog (MS) media. Seeds were surface sterilized, stratified for 4 days at 4°C, and germinated on 10cm X 10cm square plates containing half-strength basal salt mixture supplemented with 1% sucrose and either 1% agar or 0.5% Gelrite (Duchofa-Biochemie). Seedlings grown on plates then were used for various treatments. Phototropism was performed by placing the plates in darkness for 4 days before applying with 10 µmol m^{-1} s^{-2} lateral blue light for 8 hours (LED chamber, PlantClimatics). For gravitropism experiment, plates were placed for 4 days in the dark, then were tilted by 90° for 24 h. Experiments were repeated three times independently and each replication consisted of more than 90 seedlings for Ws and 75 seedlings for each of the *abp1* mutants. Quantifications were done by scanning the plates with CanonScan 8800F (resolution of 600 dots per inch; Canon, http://www.canon-europe.com) and evaluating lengths or angles with AXIOVISIO LE version 4.6 software (Zeiss, http://www.zeiss.com/).

Auxin sensitivity

Four days old seedlings were transferred to fresh half-strength MS agar media containing increasing 1-NAA concentrations, 0.01 µM, 0.03 µM, 0.05 µM, 0.1 µM, 0.3 µM and 1.0 µM

without 1-NAA. For each concentration, thirty seedlings were used and the experiment was repeated two times. The plates were placed in a vertical position at 22°C under constant white light (50 m^{-1} s^{-2}) and 16/8h (light/dark condition) for 6 days, then scanned and quantified as described in the *plant material and growth condition* section. Data were analyzed using the t-test in Microsoft Excel.

Light condition and shade avoidance experiments

Seeds were prepared as described in plant material and growth condition above. After 4 days stratification, plates were placed in a horizontal position at 22°C under white light for 2 h before transfer for 1 day into darkness. Then they were treated either with 1 µmol m^{-1} s^{-2} red or 1 µmol m^{-1} s^{-2} far red for 3 days. All these light condition were prepared in a LED light chamber (CLF, PlantClimatics). For shade avoidance experiment, the plates were prepared like above and placed in the LED light chamber and exposed to 24.5 µmol m^{-1} s^{-2} constant white LED light for 3 days. Following this treatment either low R/FR ratio (0.098) or high R/FR ratio (2.1) was added for 3 days. Light spectrum was measured using spectrometer USB4000 (Ocean Optic) and analyzed using software Spectrasuite (Ocean Optic). For monochromatic light treatments, hypocotyls lengths and hypocotyls bilateral dropping angles were measured while for shade avoidance experiment hypocotyl length was quantified. Quantification was performed as described in the previous section in *plant material and growth condition* above. Data were obtained from three independent replications and each replication was consisted of more than 40 seedlings. Culture in soil was performed in the greenhouse. For flowering time data collection and *Arabidopsis* was maintained at 22°C constant, 16h/8h (light/dark condition) on peat-based compost soil (Einheitserde, http://www. einheitserde.de/) containing 30% silica sand. Flowering time was defined as the time of the first flower arising which was indicated by opening of the first bud and white petal are shown (Effendi et al., 2011). Ratio of width:length of the leaf was measured from adult plants.

Nucleic acid analysis

For transcriptional expression measurements seedlings were grown in half-strength MS agar medium for 14 days at 22°C under long-day conditions (16h/8h, light/dark condition). Then the seedlings were carefully transferred into fresh half-strength liquid medium for equilibration with gently shaking for 1 h. The medium was replaced by fresh liquid half-strength MS medium with 1µM 1-NAA for 60 min. Seedlings were blotted on filter paper and frozen in liquid nitrogen for further use. For transcriptional expression measurement of red

light responses, after 4 days stratification at 4°C, the seedlings were grown at 22°C in a LED light chamber (CLF, PlantClimatics) 7 days under 24.5 m^{-1} s^{-2} continuous white light. Then R/FR mix light was added either with low R/FR ratio (0.098) or high R/FR ratio (2.1) for 1 hour (light spectra in Fig. 6 A). Samples were blotted and frozen in liquid and used for RNA extraction. For quantitative RT-PCR, 4-5 µg of total RNA was prepared using a TRIzol modified method (Maniatis et al., 1989) and transcribed to first-strand cDNA using a RevertAidTM H Minus first-strand cDNA synthesis kit (Fermentas, http://www.fermentas.com). Primers were designed and selected using PRIMER 3 software (http://frodo.wi.mit.edu/) and checked against primer dimer formation and primer efficiency using NETPRIMER software (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). Transcriptional expression measurements and the primers of qRT-PCR for auxin treatments were described in Effendi *et al.* (2011). The primers for shade avoidance were described in supplementary table 1. PCR efficiency of the primers was > 99%. Data were collected from two to three biological repeats and three technical replicates for each determination. Relative expression was calculated according to the ΔΔCt method (Livak and Schmittgen, 2001) and relative to 18S rRNA expression. The expression level for the control treatment was set as 1-fold. REST 2008 software (Pfaffl et al., 2002) was used for verify the statistical analysis. Means are statistically significantly different when error bars do not overlap ($p < 0.05$ or lower).

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SUPPORTING INFORMATION

Supplemental Table 1.

Primer for light experiment	Sequence (5' – 3')
18S-For	GGC TCG AAG ACG ATC AGA TAC C
18S-Rev	TCG GCA TCG TTT ATG GTT
*ATHB2*_F	GAG GTA GAC TGC GAG TTC TTA CG
*ATHB2*_R	GCA TGT AGA ACT GAG GAG AGA GC
*HFR1*_F	CAC AAG ACG GAC AAG GTT TCG
*HFR1*_R	GTC AGC ATG TGG TTG TGC ATT C
*PIL1*_F	TGG TGC CTT CGT GTG TTT CTC A
*PIL1*_R	GGA CGC AGA CTT TGG GAA TTG
*PIF1*for	CCC GTC AAG AGT CTT TGT ACC
*PIF1*rev	CCC GAG GTT GGA TCA TAC TG
*PIF5*_F	GAT GCA GAC CGT GCA ACA AC
*PIF5*_R	CTT TTA TGC TTG CTT AGG CG
*IAA19*forw	GGT GAC AAC TGC GAA TAC GTT ACC
IAA19 rev	CCC GGT AGC ATC CGA TCT TTT CA
*IAA29*for	TCC TCT GGA ATC CGA GTC TTC
*IAA29*rev	GGT GGC CAT CCA ACA ACT T
PIN3-forw	GAG TTA CCC GAA CCT AAT CA
PIN3-rev	TTA CTG CGT GTC GCT ATA GT
*FIN219*for	TGG TGC CTT CGT GTG TTT CTC A
*FIN219*rev	TCT CTT TCG GTG TTC TTG TCG ATG

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of this thesis was to investigate the potential role of ABP1 as auxin receptor in mediating auxin-related physiological functions, expression of auxin-responsive genes and interaction with light signaling in *Arabidopsis thaliana*. To elucidate this problem we used two available *abp1* mutant lines and designed new engineered in-vitro *abp1* mutants. At the time when we designed our in-vitro *abp1* mutants in 2007, only the heterozygous *abp1/+* mutant was available (Chen et al., 2001b). Later on, two different groups, the Perrot-Rechenmann's group in 2008 and Alan Jones's group in 2010 described two more mutants, *abp1-SS12K* line is a conditional repressor of ABP1 and the other a point mutant *abp1-5*, respectively (Braun et al., 2008; Tromas et al., 2009; Robert et al., 2010; Xu et al., 2010). Using the *abp1/+* and *abp1-5* mutants we were able to demonstrate that impairment of ABP1 induces various defects in auxin physiology-related responses in *Arabidopsis*. Gravitropism in root and hypocotyls as well as phototropism were defect (Chapter 2, 4 and 5). Transcriptional expression of some early-auxin induced genes and PIN genes also were identified to be up-less regulated in these *abp1* mutants (Chapter 2, 3, 4 and 5). We suggest these defects are linked to auxin transport regulation. As an example, it was shown that basipetal auxin transport in root was reduced in heterozygous *abp1/+* (Chapter 2). We proposed also that, in part, these defects might be caused by reducing transcription of PIN genes or auxin-induced genes (Chapter 2, 3, 4 and 5).

We then addressed potential interactions between auxin and light responses. The hypersensitive response in hypocotyl elongation to shade light and the altered transcription of some light-induced genes in *abp1* mutants (Chapter 3, 4 and 5) provide initial evidence for this interaction, which might involve regulation of *PIN3* and perhaps other PIN genes as intermediates between ABP1 and PHYB.

ABP1 is required in the regulation of auxin physiology-related responses

To gain knowledge on the mechanism of phytohormone signal transduction pathways frequently the identification of a receptor initiated a new chapter (Löbler and Klämbt, 1985; Chang et al., 1993; Inoue et al., 2001; Dharmasiri et al., 2005a; Kepinsky and Leyser, 2005). The specific binding of a ligand to its receptor(s) is the first step in the signal transduction pathway. Identification of mutants carrying specific defective phenotype in many cases were

used as an important method to search a receptor, for example for *coi1, ahk4, etr1,* and *gid1* (Bleecker et al., 1988; Feys et al., 1994; Yamada et al., 2001; Ueguchi-Tanaka, 2005).

In case of ABP1, early studies have shown that it binds auxin specifically and with high affinity (Hertel et al., 1972; Dohrmann et al., 1978; Jones et al., 1989; Löbler and Klämbt, 1989; Tian et al., 1995). The assignment of specific functions to ABP1 was hampered by several properties of ABP1, first of all the property of being a small glycoprotein with no transmembrane domain (Hesse et al., 1989; Watanabe and Shimomura, 1997). From discovery (Hertel et al., 1972) until about 2002 only few functions, like channel regulations in the plasma membrane, could be associated with ABP1 but not clearly with other known auxin physiology. Especially, gene regulation was not linked to ABP1 until the report by Braun *et al.* (2008) and our publication (Effendi et al., 2011) but regulation of auxin-induced genes was and is generally accepted to be executed by the other auxin receptor, TIR1. Only recently, experimental reports associated more auxin functions with ABP1 (Badescu and Napier, 2006; Braun et al., 2008; Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011; Scherer, 2011).

Functions of ABP1 in auxin signal transduction and physiology more recently described are more than those described during the decades before. Conditional modification of *ABP1* expression and investigation of *abp1-5* showed that alteration in the *ABP1* gene or its expression can induce mutant phenotypes (Chen et al., 2001a; 2001b; Braun et al., 2008; Tromas et al., 2009; Xu et al., 2010). Using conditionally downregulated *ABP1* activity in the intact plant Braun *et al.* (2008) showed that decreased ABP1 activity leads to a severe retardation of leaf growth which may due to alteration in cell division and decreased cell expansion. Tromas *et al.* (2009) using the same plant system, revealed that the ABP1 is essential for maintenance of the root meristem and implicated in the regulation of gene expression in response to auxin. Moreover, ABP1 had been shown to mediate interdigitated growth and development of leaf epidermal pavement cells. Xu *et al.* (2010) demonstrated that the *abp1-5* mutant, containing a point mutation in the presumed auxin-binding pocket, has a defect in pavement cell interdigitation.

Consistent with available studies, our experimental data suggest that impaired *ABP1* gene in plants could induce alterations in auxin physiology-related responses and confer a mutant phenotype (Chapter 2, 4 and 5). General auxin physiological-related responses such as root slanting and waving, root gravitropic, and hypocotyl gravitropic and phototopic responses as well as apical dominance are defect in the heterozygous *abp1/+, abp1-5* and in-vitro

mutagenized *abp1* mutants with different levels of defects (Chapter 2, 4 and 5). In addition, auxin sensitivity of root developmental responses was also reduced in all these *abp1* mutants (Chapter 2 and 5). The physiological defects in the *abp1- SS12K* mutant induced by ethanol were severe growth defects in shoot, leaves, and root (Braun et al., 2008; Tromas et al., 2009) which we could not found defect in *abp1/+, abp1-5* and our own *abp1* mutants. Besides the light-induced phenotypic properties we found close to normal growth of adult mutant plants investigated here (Chapter 2,3,4, and 5). A probable reason could be that in the mutant *abp1-SS12K* the amount of ABP1 was reduced greatly (80%) whereas *abp1/+* may have some reduction in ABP1 protein but unlikely such a high decrease. *abp1-5* and our own *abp1* mutants likely have normal ABP1 protein amounts or are overexpressions so that a qualitative change rather than quantitative change in signaling output by the mutated protein was the cause of physiological changes observed in the mutants investigated in this thesis. However, the physiological defects described here (tropism defect, lower auxin sensitivity, apical dominance, decreased auxin transport) were not found or not described for *abp1-SS12K.* So, we suggest that decreased binding affinity of auxin to ABP1 in *abp1-5* and in-vitro generated *abp1* mutants as well as modest reduction in number of *ABP1* in the heterozygous *abp1 /+* may have caused all these defects. A critical feature of hormone receptors is that the activated receptor pool size limits the amplitude and/or rate of signal transduction at physiological concentrations of the cognate hormone (Levitzki, 1981; Kenakin, 2004). We speculated that proper stoichiometry of ABP1 and the hypothetical binding protein was rate limiting for signal output and any disturbance of stoichiometry caused a mutant phenotype. Reduction of the dosage effect for ABP1 function by improper binding may also be suggested as the reason underlying all these mutant phenotypes. In addition, the embryo lethal property in the *abp1* null mutant (Chen et al., 2001b) suggests that the presence of an *ABP1* wild type allele determines normal growth in *Arabidopsis* which could be taken as an explanation for the quantitative differences between the *abp1-SS12K* and the *abp1/+* mutants. Thus, it is suggested that the defected auxin physiology-related functions in the *abp1-5* (Chapter 3), *abp1-8, abp1-9*, and *abp1-10* (Chapter 4) may caused by the same reason, the defect in *ABP1* gene which probably caused by mis-binding of auxin to ABP1.

How the impairing of *ABP1* gene regulated these defects in auxin physiology-related responses is still unknown. ABP1 has been reported to be involved in the regulation of subcellular PIN protein localization by enhancing clathrin-dependent endocytosis (Robert et al., 2010). Auxin inhibits this process by interrupting clathrin recruitment to the plasma membrane (Dhonukshe et al., 2007). In *abp1-5* mutant this process disappeared. Thus, ABP1

promotes the clathrin-dependent endocytosis of PIN1 protein and the auxin binding to ABP1 will inhibits this process (Robert et al., 2010). It may be suggested that similar processes can be found for other PIN proteins. Based on this study, we reasoned that defects in tropic responses and hypocotyl elongation in heterozygous *abp1/+, abp1-5,* and in-vitro generated *abp1* mutants can be caused by mis-regulation in auxin transport. Our data in auxin transport in heterozygous *abp1/+* showed that basipetal auxin transport of root in this mutant was decreased (Chapter 2), which to some extent may due to defect in PIN2 function, or not excluding other PIN proteins. The similar pattern of transcriptional expression levels of auxin-related genes in heterozygous *abp1/+* and the *eir1/pin2* mutant (Chapter 2) indicate that ABP1 and PIN2 are in the same signaling chain network. Any defect in one strand of the signaling network will affect the downstream processes and further alter certain physiological functions (Jones and Sussman, 2009). In addition, PIN3 was identified as major factor in gravistimulation responses (Rakusová et al., 2011). Regulation of tropic responses, particularly root gravitropic responses seem to be mediated by the redundant action of several PIN proteins (Kleine-Vehn et al., 2010). Moreover, light induced-polarization of subcellular localization of PIN3 in hypocotyl endodermis cells resulted in changes in auxin distribution and differential growth. Following light activation of PIN3 polarization redirection of auxin flow towards the shaded side will happen and this promotes growth thus hypocotyls to bend towards the light (Ding et al., 2011). PIN3 polarization is facilitated by repression of PINOID transcription and activation of PIN3 by GNOM ARF GTPase GEF (guanine nucleotide exchange factor)-dependent trafficking (Ding et al., 2011).

ABP1 is involved in the regulation of gene expression of auxin-responsive genes

Initially after the discovery of TIR1/AFB, most of efforts in auxin receptor research had been focussed on these complex proteins. The success in elucidating the nuclear auxin pathway and the regulation of gene expression placed TIR1/AFB as the main auxin receptor. However, some rapid auxin-induced processes are unlikely to be initiated by TIR1/AFB action since gene transcription as well as protein synthesis is not involved in these very fast processes (see review Scherer, 2011). Also, more recent experimental data provided evidence that these rapid processes may be regulated by ABP1 action (Steffens et al., 2001; Paciorek et al., 2005; Robert et al., 2010; Xu et al., 2010). However, still the role of ABP1 was proposed to be mainly restricted for non-genomic functions (Tromas et al., 2010).

But the involvement of ABP1 in the regulation of gene expression recently has been demonstrated. Reducing in the steady state of transcript accumulation of 10 genes out of 14 IAA genes following 8 hours of ABP1 inactivation revealed the importance of ABP1 for controlling auxin responses genes (Braun et al., 2008). A similar result was also reported by Tromas *et al.* (2009) who demonstrated reduced accumulation of some IAA genes depended on the conditional repression of ABP1 in the *abp1-SS12K* plant. Consistent with these results, our experimental data showed reduced transcription in all auxin-responsive genes in heterozygous *abp1/+,* in *abp1-5,* as well as in in-vitro generated *abp1* mutants already at 30 min (Chapter 2, 4 and 5). This indicates that auxin responsiveness in the *abp1* mutants used by us is reduced.

How ABP1 mediates auxin gene expression is as yet unknown. However, the different subcellular localization of TIR1 within the nucleus and of ABP1 in the ER and plasma membrane indicates there is no direct physical contact between both proteins. The fact that an effect on TIR-regulated gene expression can be measured already after 30 min in all *abp1* mutant we tested makes clear that a close coupling between ABP1 and TIR1 must exist. We proposed the contribution of ABP1 in gene expression is presumably realized by regulation of auxin efflux and auxin polar transport (Effendi et al., 2011; Effendi and Scherer, 2011; Scherer, 2011). Our data on auxin gene expression in a *pin2* mutant, *eir1*, showed that almost all 12 tested genes were mis-regulated (Chapter 3), suggesting that mis-regulation of polar auxin transport may lead to changes in auxin induction transcription. In fact, the similar reduction in gene expression in the same genes or even stronger reduction was observed in heterozygous *abp/+* and in-vitro generated *abp1* mutants (Chapter 3 and 5). This suggests that PIN2 and ABP1 might operate in the same signaling chain or network. In this case, PIN2 regulation by ABP1 could be one example for our hypothesis, but other auxin transport proteins may be involved in this mechanism. Supporting evidence for this hypothesis is the regulatory role of ABP1 in cycling of PIN proteins as has been proposed by Robert *et al.* (2010). The authors showed that ABP1 functions as a positive regulator in clatrin-dependent endocytosis. The binding of auxin to ABP1 will inhibit endocytosis which in turn maintains PIN protein in the plasma membrane, leading to increased efflux of auxin (Petrásek et al., 2002; Paciorek et al., 2005). In contrast, *abp1-5* showed reduced auxin sensitivity and was resistant to the auxin effect on endocytosis, leading to reduction of PIN internalization even in the presence of auxin (Robert et al., 2010). This mechanism suggested that there is potential link between ABP1 as plasma membrane receptor with TIR1/AFB as cytocolic auxin receptor in gene regulation. ABP1 may contribute to the controlling of the auxin concentration in the

cytosolic compartment via regulation of PIN subcellular localization by perceiving the apoplastic auxin concentration. This dynamic changing PIN localization and, thus, of intracellular auxin might mediate regulation of expression of auxin-induced genes via TIR1/AFB action (Scherer, 2011; Scherer et al., 2012). Placing the emphasis on polar PIN distribution, Kramer (2009) suggested that such a polar distribution which could create an auxin minimum in the cytocol would also need a PIN maximum at this cell pole. TIR1 could be the nuclear cytoplasmic sensor for auxin but details are not clear. Especially, the ER and perinuclear localization of PIN5 is not considered (Mravec et al., 2009). Thus, ABP1 might function as the plasma membrane receptor portion of the auxin gradient sensor (Kramer, 2009).

Interaction between ABP1-mediated signaling with light responses particularly in shade avoidance responses.

During their life, plants monitor the quantity, intensity and quality of the light that serves as their main energy sources. For this reason, plants have evolved photoreceptor systems that specifically recognize different wavelength of light. Four photoreceptor families for the visible part of the spectrum have been identified in plants, the red (R)/far-red (FR) sensing phythochrome family, the blue sensing cryptochrome family, the blue-sensing phototropism, and UV-B photoreceptors (Franklin et al., 2005; Rizzini et al., 2011; Heijde and Ulm, 2012). In competition for light, particularly under close proximity growth condition, plants are able to monitor change in the light quality using phytochromes for the detection of reduction in the ratio of R:FR (Franklin and Whitelam, 2007) due to selective absorption of red light by photosynthetic pigments (Ballaré et al., 1990). Following a reduction of R:FR ratio several morphological changes are triggered such as elongation growth in hypocotyl and petioles (Franklin, 2008).

The importance of auxin in light responses has been demonstrated in many studies. Auxin is synthesized in young aerial parts and transported downward to the root tip through the vascular bundle sheath cells. It has been shown that auxin specifically and actively transported via the efflux carrier PIN proteins and AUX auxin-influx transporters, to generate local asymmetric accumulation of auxin in specific cells and tissues (Bennett et al., 1996; Gälweiler et al., 1998; Luschnig et al., 1998; Chen et al., 1998). This provides a necessary prerequisite for various developmental roles of auxin (Sabatini et al., 1999; Friml et al., 2002; Friml et al., 2003; Benková et al., 2003). Auxin biosynthesis is stimulated by shade light (Tao

et al., 2008) and probably closely linked to this. In addition, auxin distribution has been reported to be affected by light (Keuskamp et al., 2010). Another mode of cooperation of auxin and light is the transcriptional expression of some light-dependent genes. So, some genes were identified responsive to auxin as well as responsive to shade light (Steindler et al., 1999; Morelli and Ruberti, 2002; Tanaka et al., 2002; Devlin et al., 2003; Sessa et al., 2005; Roig-Villanova et al., 2007; Lorrain et al., 2008; Tao et al., 2008; Hortnischek et al., 2009; Sorin et al., 2009; Kozuka et al., 2010; Keuskamp et al., 2010).

The initial observations on a potential light phenotype stem from experiments on flowering time, red light-induced, and shade-induced hypocotyl elongation in this study, which suggested that light signaling in *abp1* mutants presumably is defect. Heterozygous *abp1/+* and in-vitro generated *abp1* mutants flowered earlier when grown in short day conditions (Chapter 2 and 5), while *abp1-5* showed no different flowering time when grown in short day conditions (Chapter 4). The flowering phenotype common to the heterozygous *abp1/+* and in-vitro generated *abp1* mutants is found in plants with a defect in PHYB (Halliday et al., 2003).

This led to systematic investigation of effect of monochromatic red or far red light and of shade light having low or high ratio R:FR on the hypocotyl growth in all *abp1* mutants. In addition, expression of shade-induction genes in red- and far red-enriched white light was investigated in all *abp1* mutants. For reasons of time restriction, further aspects, like petiole length, petiole angle, growth phenotype in blue light and other potential features of light physiology (except phototropism) were not investigated. When grown under monochromatic light (red only and far-red only) long hypocotyl in all in-vitro generated *abp1* mutants were reminiscent of a far-red grown *phyA* mutant and a red grown *phyB* mutant (McCormac et al., 1993; Whitelam et al., 1993). In addition, in-vitro generated *abp1* mutants showed an even longer hypocotyl phenotype than both single *phyA* mutant and *phyB* mutant alone when they were grown under far-red or red light, respectively (Chapter 5). This could mean that both *PHYA*- and *PHYB*-related signaling might be defect in the in-vitro generated *abp1* mutants. A previous study showed similar phenomena in the *phyAphyB* double mutants when were grown under either monochromatic light or a mixture of R:FR light radiation sources in comparison to a single mutant *phyA* or *phyB* (Smith and Whitelam, 1997). Currently, mutants with defects in both red and far red responses are only present in a few number. Chen and Ni (2006) identified a light mutant *rf2-1* (*red and far-red insensitive 2 to 1*) which showed a constitutively elongated hypocotyl under red and far-red light. Interestingly, this mutant has other defects like in the phytochrome-mediated end-of-day-far-red, a response variant of

simulated shade condition. Such aberrant responses are to some extent reminiscent of those in *abp1* mutants so that future investigations of more light responses in *abp1* mutants are warranted.

Consistent with data on growth in colored continuous light, hypersensitive responses to shade simulated light (far-red rich light) in hypocotyl elongation in *abp1-5* and in-vitro generated *abp1* mutants (Chapter 4 and 5) support our notion that *PHYB*- and/or partially *PHYA*-related signaling is defect. We propose that *PHYA*-related signaling only partially is defect based on phenotypic data in in-vitro generated *abp1* mutants. Only few typical deficiency responses of the *phyA* mutant are present in *abp1* mutants (Chapter 5) especially long hypocotyls in continuous far red light and transcription of shade-induced genes similar to those found in *phyA*. In contrast, all *abp1* mutants opened the hypocotyl hook in far red which is not found in *phyA* (Nagatani et al., 1993; Whitelam et al., 1993). *phyB* null mutant showed typical shade avoidance responses such as elongated hypocotyl, petioles and leaves, and acceleration of flowering when grown under low R:FR ratio (far-red rich light) conditions (Franklin and Whitelam, 2005; Morelli and Ruberti, 2002). These typical phenotypic elements were also observed in the in-vitro generated *abp1* mutant and partially in *abp1-5* (Chapter 4 and 5). Other studies proposed that shade avoidance is a redundant function of PHYB, PHYD and PHYE functions (Aukerman et al., 1997; Devlin et al., 1998, 1999). To some extent, this may explain the strong elongated hypocotyl phenotype of in-vitro generated *abp1* mutants in comparison to the lesser hypocotyl elongation in *abp1-5* (Chapter 4 and 4). *abp1-5* is in *Arabidopsis* Columbia ecotype, while the in-vitro generated *abp1* mutants are in the Wassilewskija ecotype which lacks functional PHYD but contain normal levels of PHYA, PHYB, and PHYC (Aukerman et al., 1997). Lack of PHYD might decrease the combined effects of PHYB, PHYC, and PHYD in providing a balance to PHYA. Among the in-vitro generated *abp1* mutants *ABP1-OX* has a special position (Chapter 5). *ABP1-OX* does not have an auxin-regulated phenotype. Gravitropism, phototropism, auxin sensitivity and apical dominance was like in wild type, only few auxin-induced genes were mis-regulated. It does, however, have partial early flowering and shows aberrant regulation of some shade-induced genes (Chapter 5, and see below). We speculate that the tag inserted close to the C-terminus confers this weak light-related phenotype. The tag is also close proximity to the important Trp151 which is part of the auxin binding box (Woo et al., 2002) and causes severe disfunction when mutated (David et al., 2007). Moreover, all in-vitro generated *abp1* mutant constructs containing a Trp151>Ala failed to be selected as viable mutant (data not shown).

So far, there are no molecular mechanism available that describe a regulatory role of auxin in light signaling particularly in shade responses (Franklin, 2008; Stamm and Kumar, 2010; Keller et al., 2011; Nozue et al., 2011). Besides up-regulation of IAA biosynthesis (Tao et al., 2008), which we did not investigate, gene regulation controlled by auxin and/or light could be a mechanism. However, regulation of light genes seems not under control of TIR1/AFB. Application of auxin receptor TIR1 inhibitor a-(phenylethyl-2-one)-IAA (PEO-IAA) for blocking signal perception via TIR1 and its homologues to seedlings showed reduced but not completely absent responses to low-blue light induction, a typical shade simulating condition (Keuskamp et al., 2011). The failure of the *tir1-1* mutants to show a response to shade in this study (Chapter 4) could be a part of a mechanism relying on genes which are co-regulated by auxin and light (Effendi et al., 2012a-submitted). However, at the same time, this reveals an involvement of auxin signaling mediated not by TIR1/AFB.

We have shown that ABP1 regulates auxin-regulated genes somehow in conjunction with TIR1 (Chapter 2-5) but as a mechanism we suggested that ABP1 regulates genes only indirectly by actually regulating PIN protein and, thus, polar auxin transport (Chapter 2; Scherer, 2011; Scherer et al., 2012). This does not exclude transcription regulation of some PIN proteins as was found by us and others (Chapter 3, 4, 5; Keuskamp et al., 2010 and 2011). But effects exerted by regulation of PIN transcription are to slow (discussed in Scherer, 2011) to explain the rapid effects of auxin on auxin efflux transport (Paciorek et al., 2005; Robert et al., 2010) and internal auxin equilibrium concentration (Petrásek et al., 2002). Moreover, changing the direction of auxin polar transport was proposed first as a visionary idea (Morelli and Ruberti, 2002) and, more recently, supported by new experiments (Keuskamp et al., 2010; 2011). The model proposes that auxin synthesized in or led into the shoot by young leaves is transported to the root through the vasculature in non-shade conditions (white light). In the shade, auxin is redistributed laterally to epidermal and cortical cells of the hypocotyl producing the elongation of these two tissues. However, the molecular mechanism underlying this process needs experimentally to be proven. Jensen *et al.* (1998) showed that polar auxin transport is light-dependent and this was implicated in playing a role in the shade avoidance response (Morelli and Ruberti, 2000). Low-fluence red or blue light were identified to induce polar auxin transport, while far red reversed this process and was reduced in *phyA, phyB1*, and *phyB2* mutants in tomato (Liu et al., 2011). These indicated that phytochrome is involved in this response. Light was also shown to determine the cellular localization of PIN3. Thus, polarization of PIN3 is light-dependent (Keuskamp et al., 2010;

Ding et al., 2011). However, neither data on auxin concentration changes in shade (e.g. by DR5-GUS) nor responses in transport measurement were presented.

Several studies demonstrated the possible link between light and auxin signaling and pointed out that light could affect the auxin-response pathway(s) through direct regulation of IAA genes or other early auxin genes (Kim et al., 1998; Cólon-Carmona et al., 2000; Nagpal et al., 2000). Reduced level of all phytochromes was observed in a dominant mutation in *SHY2/IAA3* (Kim et al., 1998) and characterization of the *shy2* mutation suggests functional interactions between PHY and *SHY2/IAA3* gene products (Kim et al., 1996, 1998; Reed et al., 1998; Tian and Reed, 1999). Alteration in subset of phytochrome responses were also shown in *axr2/iaa7* and *axr3/iaa17* mutants (Nagpal et al., 2000). Interestingly, SHY2/IAA3, AXR3/IAA17, and other IAA proteins interact directly with phytochrome and are phosphorylated by recombinant PHYA in vitro (Cólon-Carmona et al., 2000). Following phosphorylation a higher specific activity and/or nuclear concentration of IAA protein is achieved as was shown in phytochrome-mediated phosphorylation of SHY2/IAA3 (Kim et al., 1998; Tian and Reed, 1999; Worley et al., 2000). Therefore, it is suggested nuclear localization of IAA3 protein, its metabolic stability, or its ability to regulate transcription by interacting with other proteins, like IAAs and ARFs, or its DNA-binding properties, could regulate genes by auxin and light (Cólon-Carmona et al., 2000). The decreased transcript level of some IAA genes (*IAA19, IAA29*) in heterozygous *abp/+, abp1-5,* and in-vitro generated *abp1* mutants (Chapter 2, 4, and 5) could be understood as hints for mis-regulation of light responses as indeed was found in these mutants.

Auxin stimulates patatin-related phospholipase A (pPLA) activity very quickly in 3-5 min (Paul et al., 1998). In studies on pPLA knockout plants we found that knockout mutants of the gene coding for *pPLA-I-1* have a phenotype very similar to *abp1* mutants. The *pplaI-1* allele in Ws background has agravitropic and aphototropic hypocotyls, reduced apical dominance, flowers early and is hypersensitive to shade light (Effendi et al., 2012b-in revision).The *ppla-I-3* allele in Col-0 background does not show all of these properties but it is also shade hypersensitive and in both alleles auxin-induced genes are less up-regulated at 30 min. These similarities argue that *ABP1* and *pPLA-I* are operating in the same auxin signaling pathway, especially, considering the rapid activation of phospholipase A activity within minutes. None of the *pPLA* genes was up-regulated by auxin, in contrary, *pPLA-I* is even down-regulated (Effendi et al., 2012b-in revision; Labusch et al., 2012-submitted), but in knockout mutants of all *pPLA* genes 40-70% of auxin-induced genes were not properly up-regulated 30 min after

auxin application (Labusch et al., 2012-submitted). How phopholipase A activation by auxin leads to TIR1 and gene activation remains unknown, presently (Scherer et al., 2012).

SUMMARY

This thesis point out potential role of ABP1 as membrane bound auxin receptor in the regulation of auxin-related functions, regulation of expression of auxin-related genes, and interaction between light and auxin signaling, particularly in response to shade light. The obvious role of ABP1 in phytochrome signaling was a complete surprise. The availability of viable ABP1 mutants provides completely new chance to deal with ABP1 experiments in the future. The presence of functional interaction between TIR1/AFB as a cytosolic auxin receptor and ABP1 as membrane-bound auxin receptor will need to be considered for auxin research in the future as well as in light signaling networks.

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APPENDICES

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LIST OF PUBLICATIONS

Effendi Y., Raddatz K., Rietz S., Labusch C., Wimalasekera R., Zeidler M., Galland P., Scherer G.F.E. 2012. Phospholipase A-I is involved in early auxin gene regulation, gravitropism, phototropism, and red/far-red signaling. Plant J. (in revision)

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Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

Für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, das ich meine Dissertation mit dem Titel

Potential role of ABP1 (AUXIN BINDING PROTEIN1) as a receptor for regulation of auxin-related functions and modulation of light signaling

selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den. 10.07.2012

Yunus Effendi