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Summary

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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

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). Suppression 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 exclusively to TIR1 and its homologs (Mockaitis and Estelle, 2008). We previously showed that auxin activates phospholipase 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 phenotypic 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 downwards, 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 (Figure 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 seedlings 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

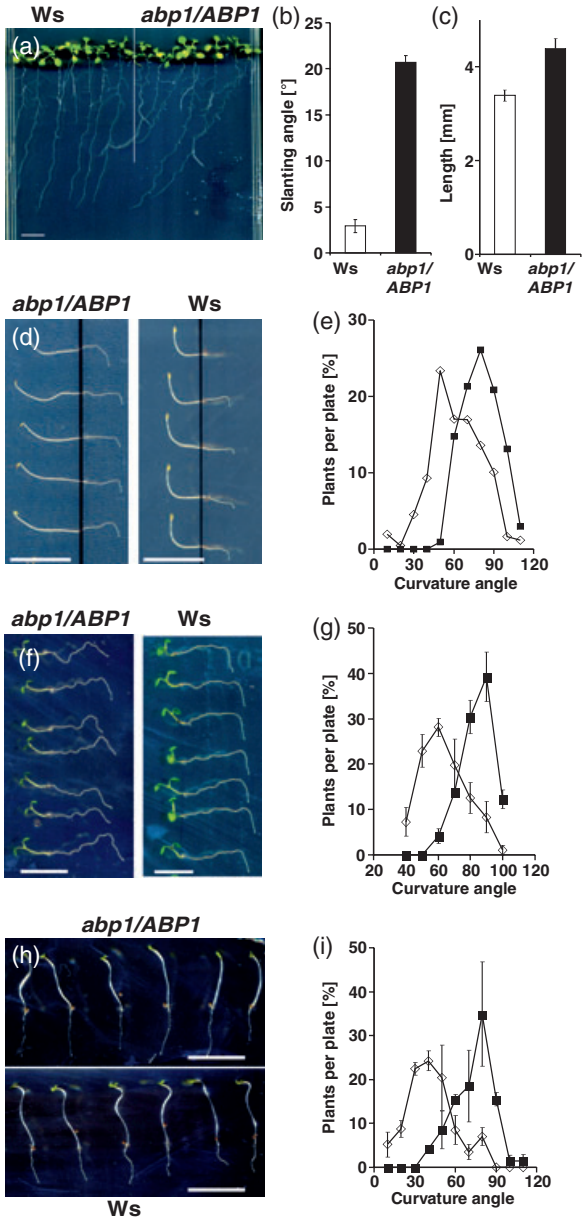


Figure 1. Phenotype and responses to gravity and light in wild-type Ws and heterozygous *abp1/ABP1* seedlings.
(a) Ws seedlings (left) and *abp1/ABP1* seedlings (right). Scale bar = 1 cm.
(b) Slanting angles of seedlings grown for 7 days in the light. White bar, Ws; black bar, *abp1/ABP1*. Values are 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 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 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

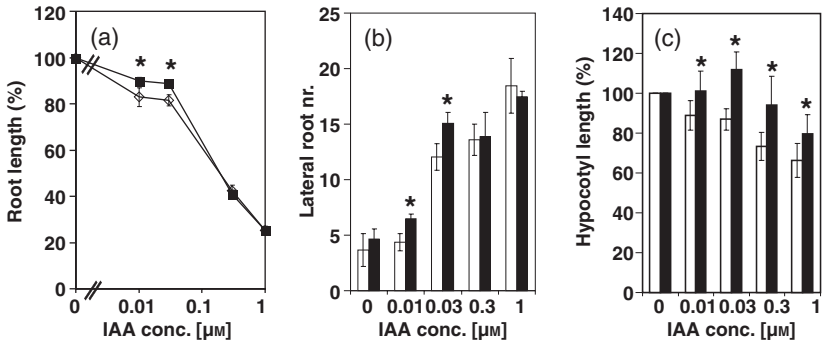
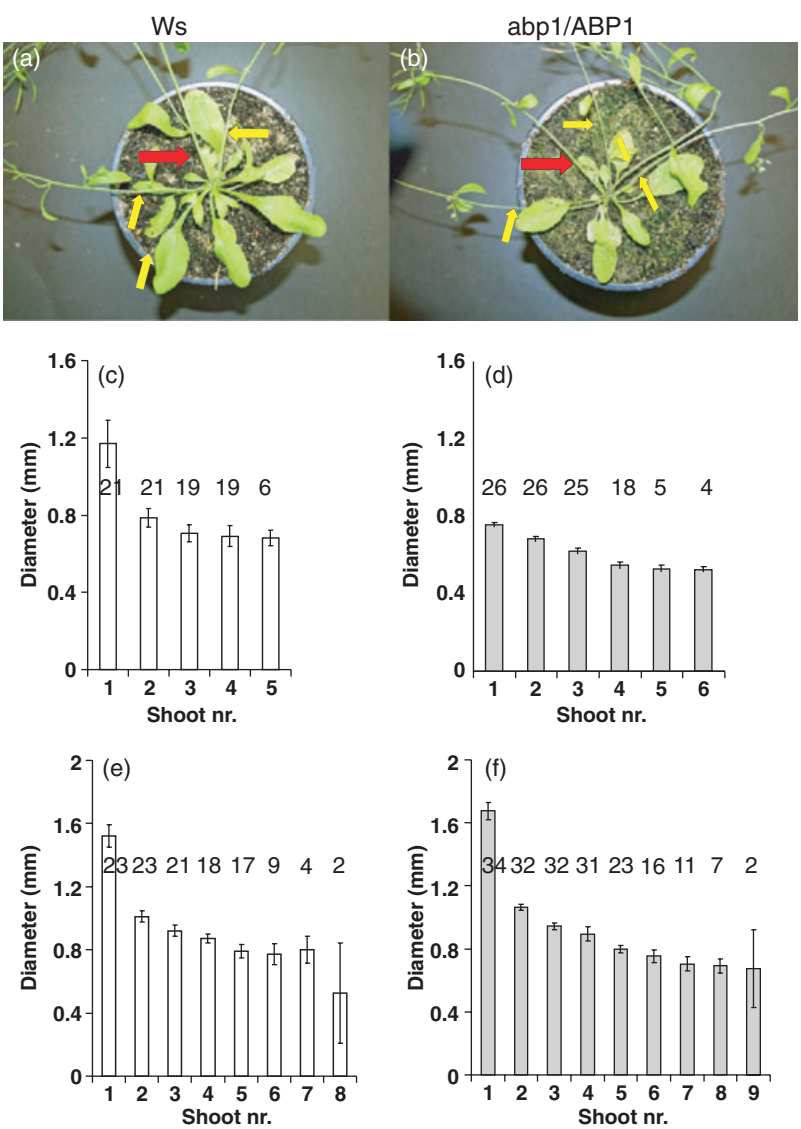


Figure 2. Auxin sensitivity of wild-type Ws and *abp1/ABP1* seedlings.
All seedlings were grown on vertical agar plates without auxin for 4 days, and then transferred to plates containing increasing concentrations of IAA. The *abp1/ABP1* seedlings were selected from the segregating population after 3 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 conditions (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.



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

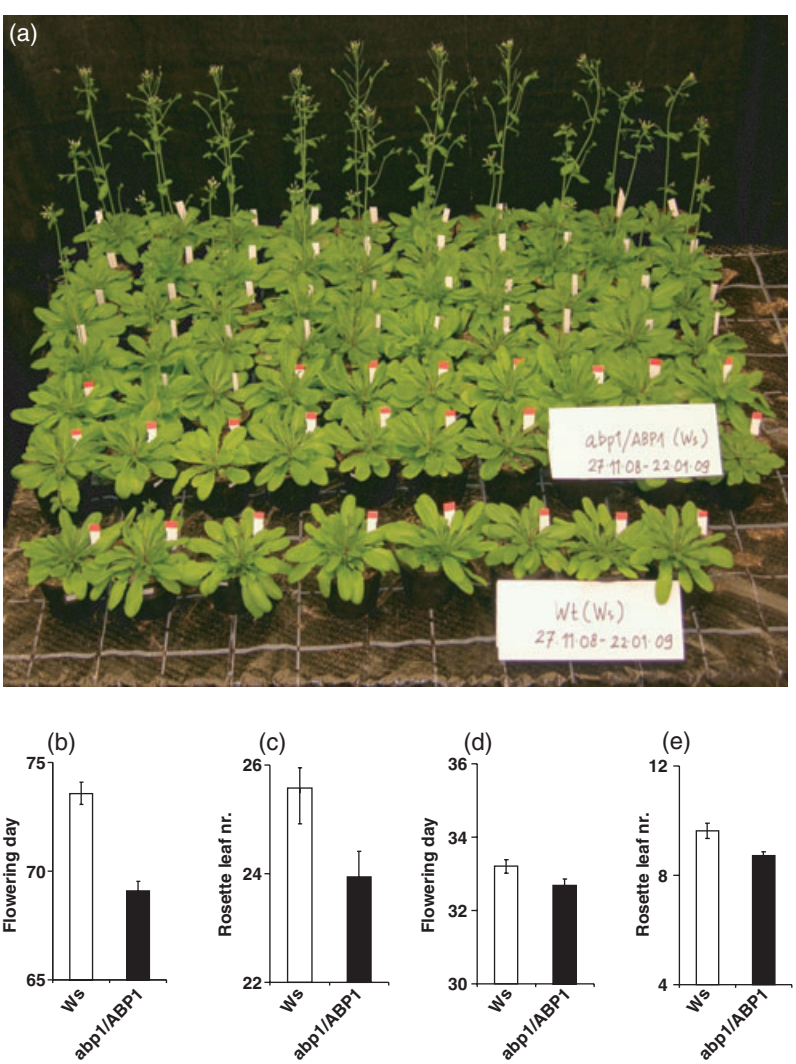


Figure 4. Early-flowering phenotype of wild-type Ws and heterozygous *abp1*/*ABP1* plants grown under short-day conditions (8 h/16 h light/dark) (a–c) or long-day conditions (16 h/8 h light/dark) (d, e).
(a) Plants from seeds of a kanamycin-resistant heterozygous *abp1*/*ABP1* plant and Ws wild-type 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* plants (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, *IAA19* and *IAA20* were more than tenfold up-regulated and *IAA11*, *IAA13* and *IAA14* 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

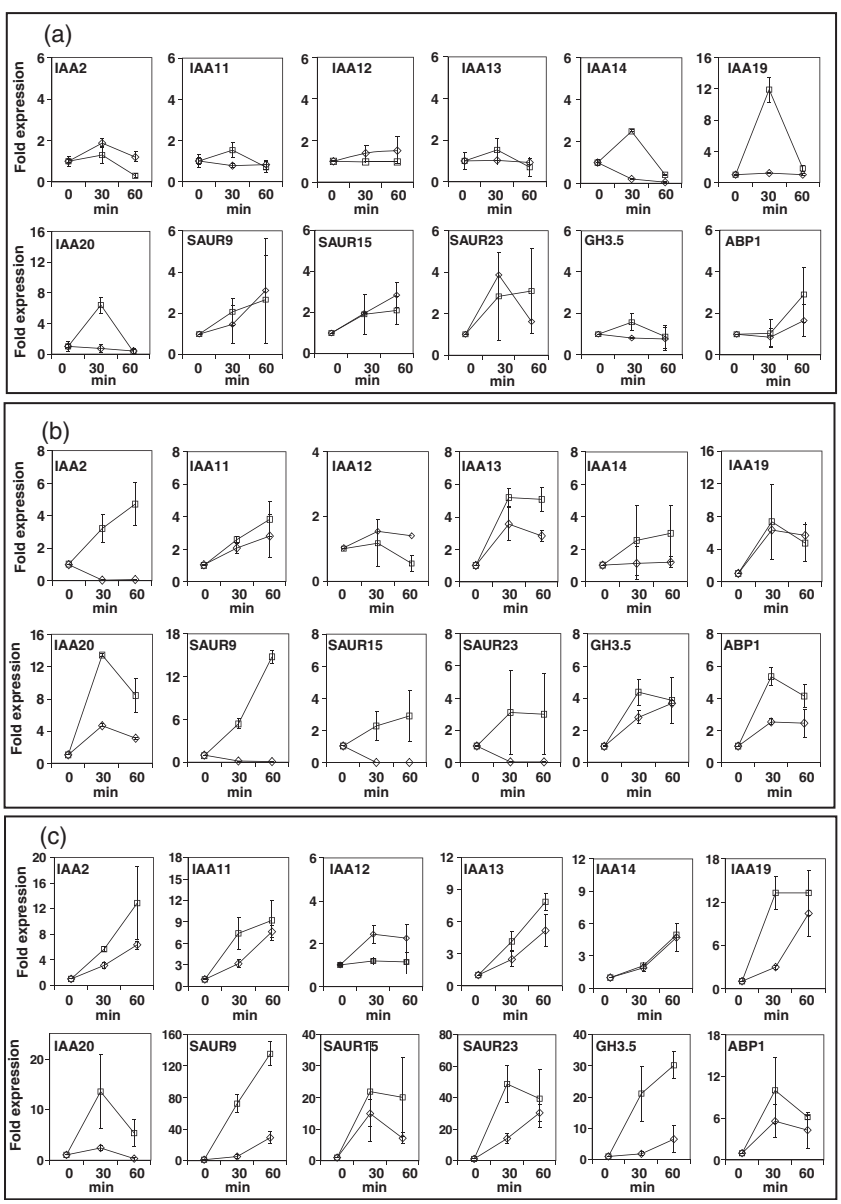


Figure 5. Regulation of early auxin-regulated genes and *ABP1* in light-grown wild-type (Ws) and *abp1*/*ABP1* seedlings.
Wild-type seedlings were grown on half-strength MS agar, and seeds from a kanamycin-resistant *abp1*/*ABP1* plant were grown in the same medium containing 50 μg ml^{-1} kanamycin. 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 measurement. 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.

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 (application 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

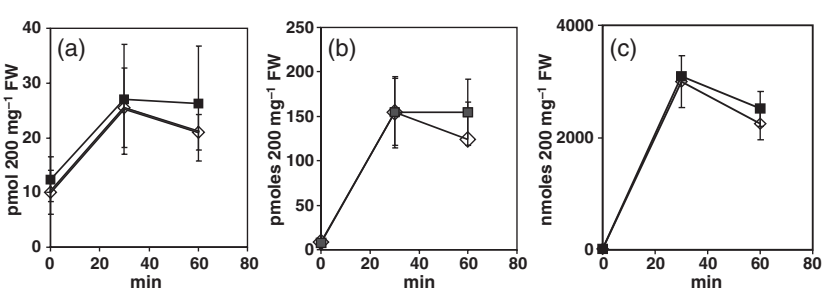


Figure 6. Auxin uptake into wild-type and *abp1*/*ABP1* seedlings.
Plants were grown, selected, and treated with auxin as described in the legend to Figure 5 and in Experimental Procedures. To stop auxin uptake, plants were quickly rinsed five times with sterile water, dried, and quickly frozen in liquid 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.

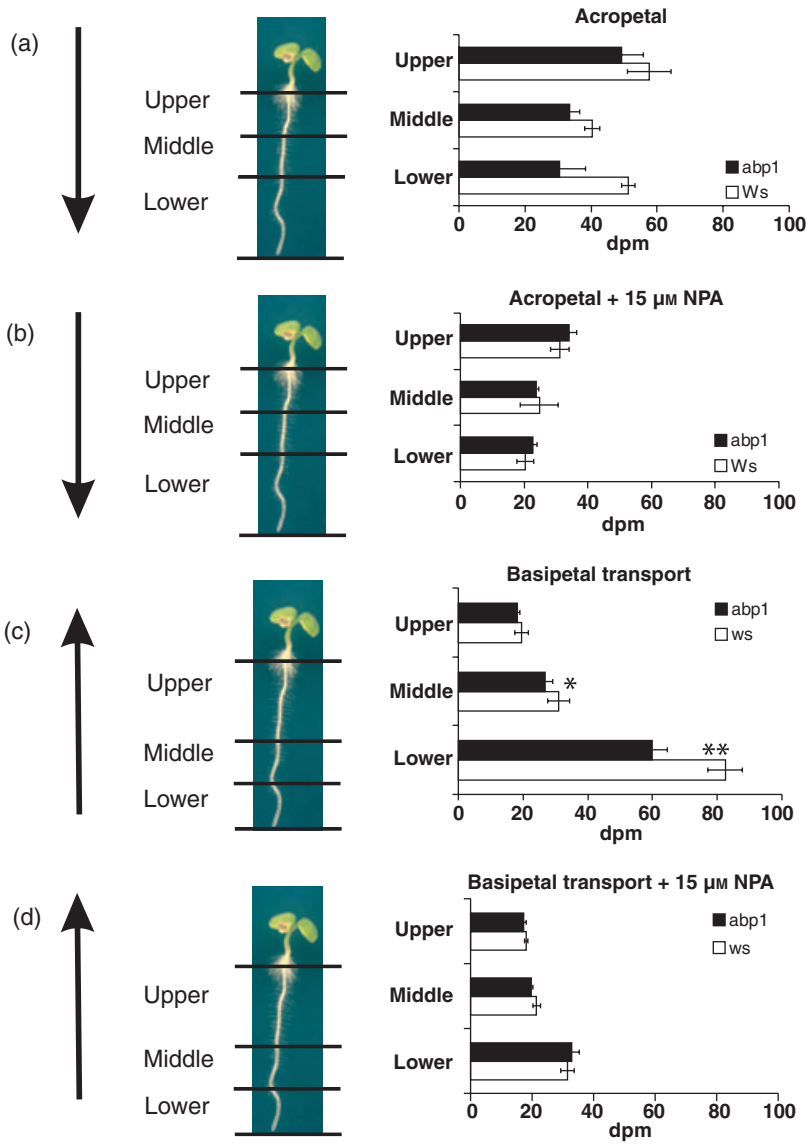


Figure 7. Polar auxin transport in wild-type and *abp1*/*ABP1* roots.
Two consecutive 5 mm pieces 1 mm away from the source and the residual third root piece were used as indicated on the left. Black bars, *abp1*/*ABP1* mutant [n = 18 for (a, d), n = 19 for (b, c)]; white bars, wild-type [n = 12 for (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 statistically significant differences.
(c, d) Basipetal transport in the presence (c) or absence (d) of 15 μM NPA. There were no statistically 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.

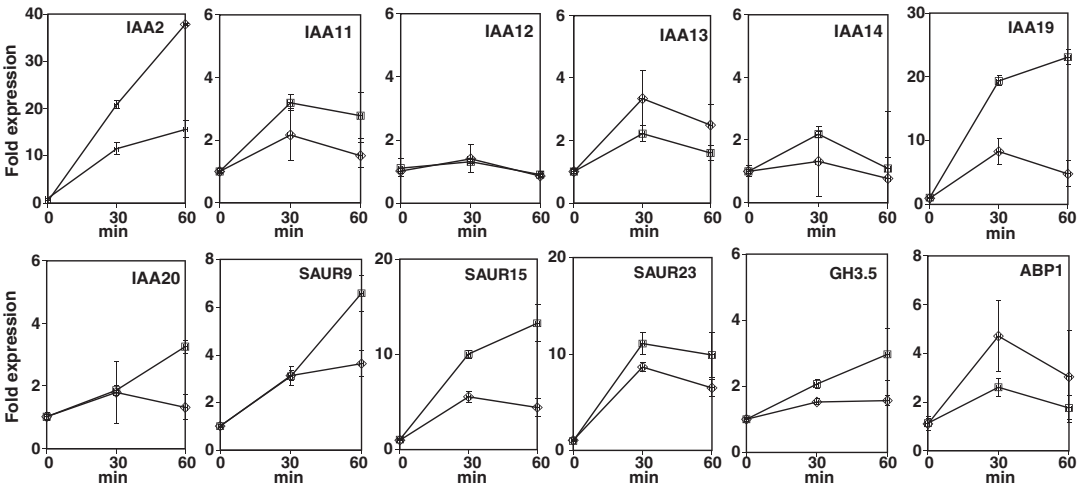


Figure 8. Regulation of early auxin-regulated genes and *ABP1* in light-grown wild-type (Col) and *eir1* seedlings.
Wild-type and *eir1* seedlings were grown on half-strength MS agar. After 14 days, seedlings were washed three times in the medium, and transferred to fresh liquid half-strength MS medium 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 transcriptional co-factor, respectively, and the genes regulated by them, or a subset of these genes, must have a critical function in growth in tropisms. All other gravitropic or phototropic 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

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 André, 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 independently 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.

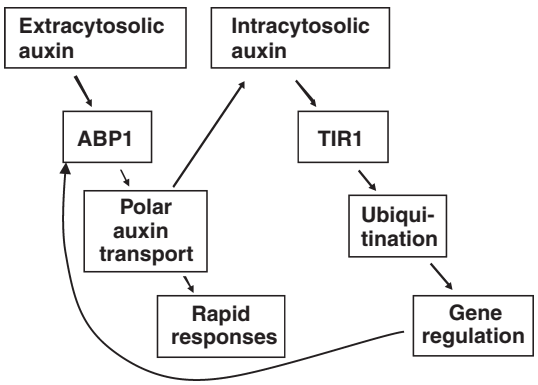


Figure 9 Model of ABP1 action and ABP1–TIR1 interaction.

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 concentrator. Samples were derivatized using 80 μl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (pyridine salt) with 1% trimethylchlorosilane (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-genotyped 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′-GGCTCGAAGACGATCAGATACC-3′0, 18S rRNA reverse (5′-TCGGCATCGTTTATGGTT-3′), *ABP1* forward (5′-ACGAGAAAAT CATACCAATTCGGACTAACC-3′), *ABP1* reverse (5′-GTATCTACGTA GTGTCACAAAACCTCAAC-3′), *IAA2* forward (5′-GGTTGGCCACCA GTGAGATC-3′), *IAA2* reverse (5′-AGCTCCGTCCATACTCACTTTCA-3′), *IAA11* forward (5′-CCTCCCTTCCCTCACAATCA-3′), *IAA11* reverse (5′-AACCGCCTTCCATTTTCGA-3′), *IAA12* forward (5′-CGT TGGGTCTAAACGCTCTG-3′), *IAA12* reverse (5′-TTCCGCTCTTGCTG CCTTCA-3′), *IAA13* forward (5′-CACGAAATCAAGAACCAAACGA-3′),

IAA13 reverse (5′-CACCGTAACGTCGAAAAGAGATC-3′), *IAA14* forward (5′-CCTTCTAAGCCTCCTGCTAAAGCAC-3′), *IAA14* reverse (5′-CCATCCATGGAAACCTTCAC-3′), *IAA19* forward (5′-GGTGACAACTGCGAATACGTTACC-3′), *IAA19* reverse (5′-CCCGGTAGCATCCGATCTTTTCA-3′), *IAA20* forward (5′-CAATATTTCAACGGTGGCTATGG-3′), *IAA20* reverse (5′-GCCACATATTCCGCATCCTCTA-3′), *GH3.5* forward (5′-AGCCCTAACGAGACCATCCT-3), *GH3.5* reverse (5′-AAGCCATGGATGGTATGAGC-3′), *SAUR9* forward (5′-GACGTGCCAAAAGGTCACTT-3′), *SAUR9* reverse (5′-AGTGAGACCCATCTCGTGCT-3′), *SAUR15* forward (5′-ATGGCTTTTTTGAGGAGTTTCTTGGG-3), *SAUR15* reverse (5′-TCATTGTATCTGAGATGTGACTGTG-3′), *SAUR23* forward (5′-ATGGCTTTGGTGAGAAGTCTATTGGT-3), and *SAUR23* reverse (5′-TCAATGGAGCCGAGAAGTCACATTGA-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.appliedbiosystems.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 $\Delta\Delta C_t$ 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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