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Journal

Proceedings of the National Academy of Sciences of the United States of America, 112(7)

ISSN

0027-8424

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Publication Date

2015-02-17

DOI

10.1073/pnas.1500365112

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Auxin binding protein 1 (ABP1) is not required for either auxin signaling or *Arabidopsis* development

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Contributed by Mark Estelle, January 12, 2015 (sent for review December 21, 2014; reviewed by William Gray and Ottoline Leyser)

Auxin binding protein 1 (ABP1) has been studied for decades. It has been suggested that ABP1 functions as an auxin receptor and has an essential role in many developmental processes. Here we present our unexpected findings that ABP1 is neither required for auxin signaling nor necessary for plant development under normal growth conditions. We used our ribozyme-based CRISPR technology to generate an *Arabidopsis abp1* mutant that contains a 5-bp deletion in the first exon of *ABP1*, which resulted in a frameshift and introduction of early stop codons. We also identified a T-DNA insertion *abp1* allele that harbors a T-DNA insertion located 27 bp downstream of the ATG start codon in the first exon. We show that the two new *abp1* mutants are null alleles. Surprisingly, our new *abp1* mutant plants do not display any obvious developmental defects. In fact, the mutant plants are indistinguishable from wild-type plants at every developmental stage analyzed. Furthermore, the *abp1* plants are not resistant to exogenous auxin. At the molecular level, we find that the induction of known auxin-regulated genes is similar in both wild-type and *abp1* plants in response to auxin treatments. We conclude that ABP1 is not a key component in auxin signaling or *Arabidopsis* development.

auxin | ABP1 | plant development | receptor | CRISPR

The auxin binding protein 1 (ABP1) was first isolated from maize plants based on its ability to bind auxin (1). The crystal structure of ABP1 demonstrated clearly that ABP1 has an auxin-binding pocket and, indeed, binds auxin (2). However, the elucidation of the physiological functions of ABP1 has been challenging because the first reported *abp1* T-DNA insertion mutant in *Arabidopsis* was not viable (3). Nevertheless, *ABP1* has been recognized as an essential gene for plant development and as a key component in auxin signaling (4–9). Because viable *abp1* null mutants in *Arabidopsis* were previously unavailable, alternative approaches have been used to disrupt *ABP1* function in *Arabidopsis* to determine the physiological roles of the protein. Cellular immunization approaches were used to generate ABP1 knockdown plants (10, 11). Inducible overexpression of the single chain fragment variable regions (scFv12) of the anti-ABP1 monoclonal antibody mAb12 both in cell lines and in *Arabidopsis* plants presumably neutralizes the endogenous ABP1 activities (10, 11). Two such antibody lines, SS12S and SS12K, have been widely used in many ABP1-related studies (4, 6, 9–11). The results obtained from the characterization of the antibody lines suggest that ABP1 regulates cell division, cell expansion, meristem activities, and root development (4, 6, 10, 12, 13). Transgenic plants that overexpress *ABP1* antisense RNA were also used to elucidate the physiological functions of ABP1 (4, 10). Moreover, missense point mutation alleles of *abp1* have also been generated through the *Arabidopsis* TILLING project. One such TILLING mutant, named *abp1-5*, harbors a mutation (His94 >Tyr) in the auxin-binding pocket and has been widely used in many ABP1-related studies (4, 8, 9). Previous studies based on the antisense lines, antibody lines, and *Arabidopsis* mutant alleles have led to the conclusion that ABP1 is essential for embryogenesis, root development, and many other developmental processes. However, the interpretation of results generated by using the *ABP1* antisense and antibody lines are not straightforward and off-target effects have not been completely ruled out. We believe that characterization of *abp1* null plants is urgently needed to unambiguously define the roles of ABP1 in auxin signaling and in plant development.

In the past several years, studies of the presumed ABP1-mediated auxin signal transduction pathway were carried out in several laboratories. It has been hypothesized that ABP1 is an auxin receptor mediating fast, nongenomic effects of auxin (4–6, 8, 9), whereas the TIR1 family of F-box protein/auxin receptors are responsible for auxin-mediated gene regulation (14, 15). One of the proposed functions of ABP1 is to regulate subcellular distribution of PIN auxin efflux carriers (6, 9, 13). Furthermore, a recent report suggests that a cell surface complex consisting of ABP1 and transmembrane receptor-like kinases functions as an auxin receptor at the plasma membrane by activating the Rho-like guanosine triphosphatases (GTPases) (ROPs) in an auxin-dependent manner (8). ROPs have been reported to play a role in regulating cytoskeleton organization and PIN protein endocytosis (5, 6). However, it is important to unequivocally determine the biological processes that require ABP1 before extensive efforts are directed toward elucidating any ABP1-mediated signaling pathways.

In this paper, we generate and characterize new *abp1* null mutants in *Arabidopsis*. We are interested in elucidating the molecular mechanisms by which auxin regulates flower development because our previously identified auxin biosynthetic mutants display dramatic floral defects (16–18). Because *ABP1* was reported as an essential gene and ABP1 binds auxin (2, 3),

Significance

The plant hormone auxin is a key regulator of plant growth. It has been hypothesized that some auxin responses are mediated by a candidate auxin receptor called auxin binding protein 1 (ABP1). Support for this hypothesis mainly comes from the analyses of *Arabidopsis ABP1* knockdown lines generated by cellular immunization or antisense approaches. However, these approaches are subject to off-target effects. As an alternative, we have recovered two new null alleles of *abp1*. Surprisingly, neither of the mutants exhibits defects in growth and development, or auxin response, indicating that ABP1 does not have a major role in these responses under normal growth conditions. These results require that the role of ABP1 in plant growth and auxin response be reexamined.

Author contributions: Y.G., Y. Zhang, D.Z., X.D., M.E., and Y. Zhao designed research; Y.G., Y. Zhang, D.Z., X.D., and Y. Zhao performed research; Y.G., Y. Zhang, D.Z., X.D., M.E., and Y. Zhao analyzed data; and M.E. and Y. Zhao wrote the paper.

Reviewers: W.G., University of Minnesota; and O.L., University of Cambridge.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1500365112/-/DCSupplemental.

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we decided to determine whether ABP1 plays a role in flower development. We used our recently developed ribozyme-based CRISPR gene editing technology (19) to specifically inactivate *ABP1* during flower development. Unexpectedly, we recovered a viable *abp1* mutant (*abp1-c1*, *c* stands for alleles generated by using CRISPR) that contains a 5-bp deletion in the first exon of *ABP1*. We also isolated a T-DNA *abp1* allele (*abp1-TD1*) that harbors a T-DNA insertion in the first exon of ABP1. We show that both *abp1-c1* and *abp1-TD1* are null mutants. Surprisingly, the mutants were indistinguishable from wild-type (WT) plants at all of the developmental stages we analyzed. Our data clearly demonstrate that *ABP1* is not an essential gene and that ABP1 does not play a major role in auxin signaling and *Arabidopsis* development under normal growth conditions.

Results and Discussion

Generation of Loss-of-Function *abp1* Mutants in *Arabidopsis* Using CRISPR Technology. In an attempt to determine the roles of *ABP1* in *Arabidopsis* flower development, we used the latest CRISPR technology (19) to specifically knockout the *ABP1* gene during *Arabidopsis* flower development. We designed a ribozyme–guide RNA–ribozyme (RGR) unit that specifically targets a stretch of DNA in the first exon of *ABP1* gene (Fig. 1*A*). The RGR unit was placed under the control of the strong constitutive *CaMV 35S* promoter. Primary transcripts of *RGR* undergo self-processing to release the mature functional guide RNA (gRNA) as we demonstrated (19). We controlled the expression of the *Cas9* nuclease by using the *APETALA 1* (*AP1*) promoter (Fig. 1*A*). We expected that the gRNA would bring the Cas9 protein to the *ABP1* target site where it will generate double-stranded breaks. Deletions and insertions will be produced during non-homologous end joining repair of the double-stranded break. We hypothesized that the gene editing will take place only during flower development as the expression of the Cas9 nuclease is under the control of a floral meristematic promoter.

We were disappointed that no obvious floral defects were observed in the T1 transgenic plants that contained the expression cassettes for *Cas9* and the *RGR*. We then grew T2 plants to identify homozygous *Cas9*/*RGR* insertion plants, which may have higher efficiency of editing *ABP1* because of potentially higher expression of *RGR* and *Cas9* in the homozygous lines. Unexpectedly, we recovered T2 plants that are homozygous *abp1* deletion mutant plants (named *abp1-c1*). The *abp1-c1* contains a 5-bp deletion in the first exon (Fig. 1*B*). The deletion presumably leads to a frameshift and would generate premature stop codons. Therefore, *abp1-c1* is likely a null mutant. Because our *abp1-c1* results appear to contradict a previous report that a T-DNA insertion *abp1* mutant was embryo lethal (3), we hypothesized that perhaps the Cas9 protein or the CRISPR construct or an off-target site mutation partially rescued the presumed embryo lethal phenotypes of *abp1-c1*. We then backcrossed the *abp1-c1* to WT plants to segregate out the CRISPR construct and potential off-target background mutations. We genotyped the F_2 population generated from the backcross and identified *Cas9* free, *abp1-c1* homozygous plants. It was clear that *abp1-c1* plants were not embryo lethal. The mutation in *abp1-c1* was stable and transmitted to next generations in a Mendelian fashion (Fig. S1).

The *abp1-c1* Mutant Is a Null Allele. The 5-bp deletion in the first exon is predicted to cause a frameshift and to introduce several early stop codons. Because our results were not consistent with what was previously reported regarding an *abp1* null mutant, we investigated whether the 5-bp deletion in *ABP1* might generate cryptic splicing junctions that might still lead to the production of functional *ABP1* mRNA and ABP1 protein. We extracted mRNA from *abp1-c1* and WT plants, and amplified *ABP1* cDNAs by RT-PCR. The *ABP1* cDNA from WT plants was the

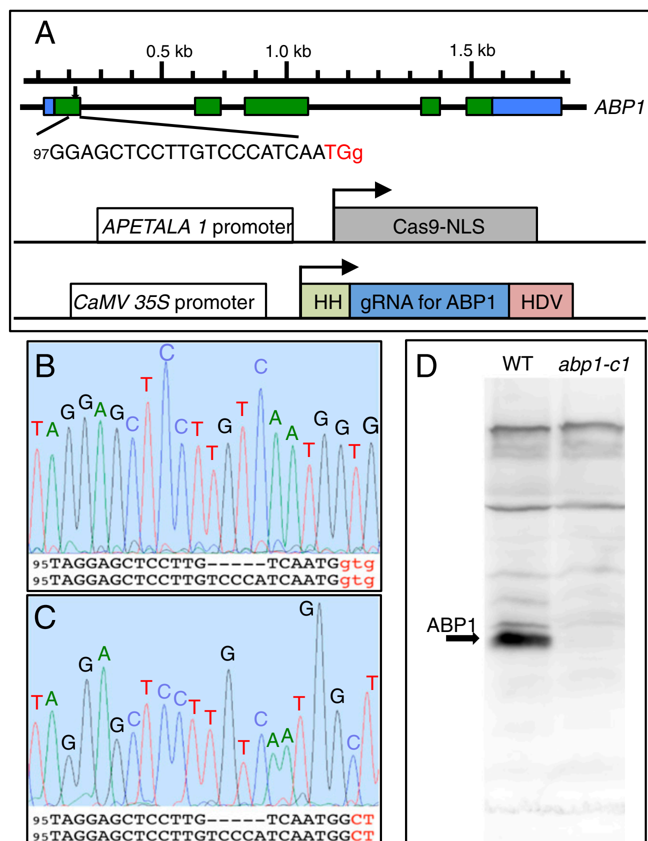

Fig. 1. Generation of a null allele of *abp1* mutant using the ribozyme-based CRISPR gene editing technology. (*A*) A schematic description of the CRISPR construct that contains a Cas9 expression cassette and a CaMV 35S promoter controlled gRNA production unit. (*B*) A 5-bp deletion was detected in genomic DNA of *abp1-c1* mutants. The intron sequences are in lowercase and in red. (*C*) The *abp1-c1* cDNA also contained the same 5-bp deletion. (*D*) There was no detectable ABP1 protein in *abp1-c1* as shown in this Western blot image.

same as reported (3). The *ABP1* cDNAs from *abp1-c1* all contained the 5-bp deletion (Fig. 1*C*). The mutant *abp1-c1* cDNA contained several premature stop codons and was unlikely to produce a functional ABP1 protein. To further demonstrate that our *abp1-c1* is a null allele, we performed a Western blot by using anti-ABP1 polyclonal antibody (8). The results in Fig. 1*D* show that the antibody detected ABP1 and several nonspecific bands. Although both the WT and *abp1-c1* lanes had the same nonspecific bands, the ABP1 band in *abp1-c1* sample was clearly missing, demonstrating that the *abp1-c1* is a null mutant.

The *abp1-c1* Plants Are Indistinguishable from WT Plants. In previous studies, ABP1 knockdown was associated with a number of developmental defects including changes in root and hypocotyl elongation, leaf expansion, and maintenance of the root meristem (4, 10, 11, 20–23). To determine whether *abp1-c1* plants exhibited any of these defects, we compared them to WT plants grown under the same growth conditions. As shown in Fig. 2*A*, light grown *abp1-c1* seedlings looked similar to WT seedlings. Both WT and *abp1-c1* plants had similar hypocotyl lengths (Fig. 2*B*). Hypocotyl elongation is sensitive to changes in auxin concentration or auxin response (24, 25). The length of primary roots of *abp1-c1* seedlings was also like that of WT plants (Fig. 2 *A* and *C*), and the cellular organization of primary roots of the mutant, including the meristem, appeared similar to that of WT plants (Fig. 2 *D* and *E*). We did not observe any alterations of

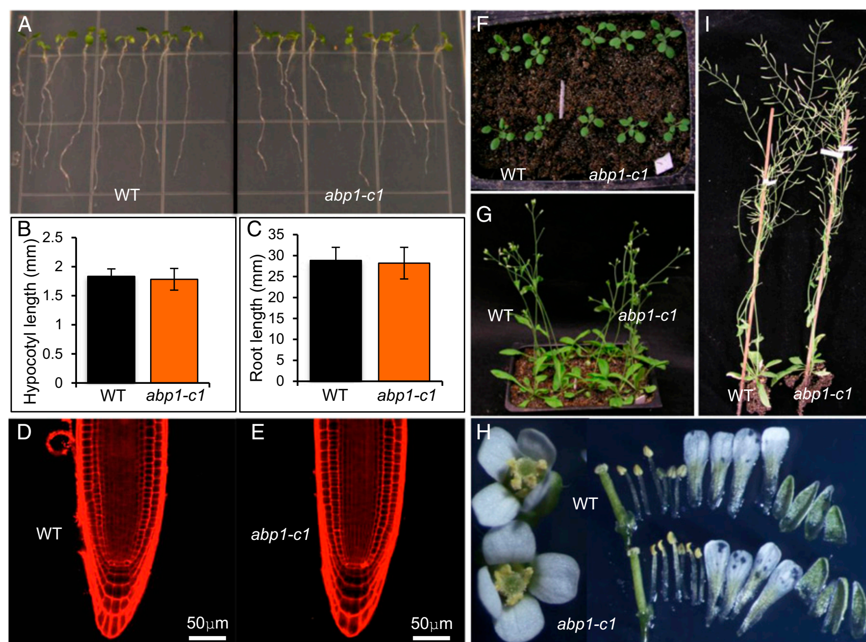

Fig. 2. The *abp1-c1* and WT plants display no significant differences at various developmental stages. (*A*) Seven-day-old seedlings on regular MS plates. (*B* and *C*) Hypocotyl length and root length of 7-d-old WT and *abp1-c1* seedlings. Shown are average ± SD ($n = 50$). (*D* and *E*) Root cell shape of 7-d-old WT and *abp1-c1*. (*F*, *G*, and *I*) Phenotype of WT and *abp1-c1* at juvenile stage (*F*), floral transition stage (*G*), and mature plant stage (*I*). (*H*) Flowers and floral organs of WT and *abp1-c1*.

cell size or changes in spatial arrangement of the different cell types (Fig. 2 *D* and *E*). The microscopic structure of *abp1-c1* roots is not different from that of WT plants. At young adult stages, *abp1-c1* plants developed normally and appeared as healthy as WT plants (Fig. 2*F*). WT plants and *abp1-c1* plants had similar flowering time (Fig. 2*G*). Flowers of *abp1-c1* had the same numbers of floral organs as WT flowers (Fig. 2*H*). Lastly, mature *abp1-c1* plants and WT plants had similar architecture and *abp1-c1* plants were as fertile as WT plants (Fig. 2*I*).

Dark-grown seedlings of the ABP1 antibody lines were partially de-etiolated with short hypocotyls and lacked an apical hook (11). However, the *abp1-5* weak allele was indistinguishable from WT when grown in total darkness (26). Because dark-grown conditions vary little from laboratory to laboratory, we tested whether *abp1-c1* displayed any phenotypes in the dark. Dark-grown *abp1-c1* appeared similar to WT seedlings in terms of hypocotyl length and the formation of an apical hook (Fig. S2).

One of the key phenotypic readouts of *abp1* knockdown or weak alleles in previous studies is a reduction of pavement cell interdigitation (8, 9). The reduction of interdigitation in *abp1* knockdown lines or *abp1-5* cannot be rescued by exogenous auxin (8, 9). We analyzed pavement cell interdigitation in both WT and *abp1-c1* with and without auxin treatments (Fig. 3). In the absence of exogenous auxin, *abp1-c1* and WT showed the same levels of pavement cell interdigitation (Fig. 3). Auxin treatments slightly increased interdigitation of pavement cells in both WT and *abp1-c1* (Fig. 3). We did not observe any differences between *abp1-c1* and WT plants in terms of pavement cell interdigitation.

Overall, the *abp1-c1* plants were indistinguishable from WT plants at the various developmental stages we analyzed, demonstrating that ABP1 probably does not play a major role in *Arabidopsis* development under normal growth conditions.

The *abp1-c1* Plants Are Not Auxin Resistant. Several studies have reported changes in auxin response in ABP1 knockdown lines (20, 21). We used a classic root elongation assay (27) to determine whether *abp1-c1* had altered sensitivity to exogenous auxin. We tested both the natural auxin indole-3-acetic acid (IAA) and the synthetic auxin 1-naphthaleneacetic acid (NAA), because ABP1 has been reported to have a higher affinity for NAA than IAA (28). In the presence of increasing concentrations of auxin in the growth media, primary roots of WT plants became progressively shorter (Fig. 4). Both auxins also inhibited the elongation of primary roots of *abp1-c1* (Fig. 4). The dose–response curves to IAA treatments for WT and *abp1-c1* were almost superimposable, indicating that there was not a significant difference between WT and *abp1-c1* plants in response to auxin treatments (Fig. 4*A*). Similar results were also observed when NAA was used in the treatments (Fig. 4*B*).

The *abp1-c1* and WT Plants Respond to Auxin Similarly at the Molecular Level. Although ABP1 was suggested to mainly function in nongenomic pathways, several studies have reported that reduction in ABP1 function affects auxin-regulated gene expression (10, 20, 21). Furthermore, it was recently reported that ABP1 regulates the degradation of AUX/IAA proteins (7). Therefore, we analyzed the expression levels of a set of well-characterized auxin inducible genes in both *abp1-c1* and WT plants with and without auxin treatments to determine whether disruption of *ABP1* affects auxin signaling. The tested auxin responsive genes were induced by auxin in WT plants (Fig. 5). The same set of auxin-inducible genes was also induced in *abp1-c1* plants (Fig. 5). The overall expression levels of the genes in *abp1-c1* and WT were similar, indicating that disruption of *ABP1* did not affect auxin-mediated gene expression.

A New T-DNA *abp1* Null Mutant Was Not Embryo Lethal and Displayed No Obvious Developmental Defects. We have provided clear evidence that *abp1-c1* is a null mutant and that *abp1-c1* plants do not display any obvious defects at the various developmental stages we analyzed. Further, the *abp1-c1* plants did not show altered auxin responses. Because of the lack of any visible and molecular phenotypes in *abp1-c1*, it is difficult to completely rule out the possibility that a tight-linked unknown *abp1* suppressor

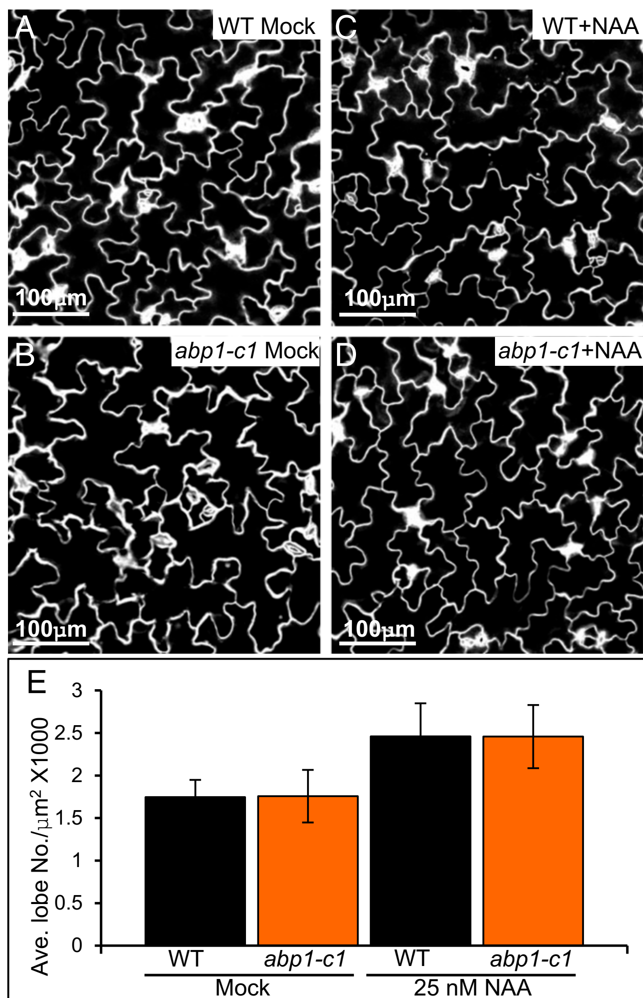

Fig. 3. Pavement cell development in *abp1-c1* and WT. Confocal images of cotyledon pavement cells of WT (*A* and *C*) and *abp1-c1* (*B* and *D*) with auxin (*C* and *D*) and without auxin (*A* and *B*) treatments. Five-day-old light-grown seedlings were transferred to MS plates with or without 25 nM NAA for 2 d. Samples were treated with 5 μg/mL FM1-43 (Life Technologies; F-35355) for 30 min before confocal imaging. (*E*) Quantification of pavement cell lobes. One hundred fifty cells for each treatment and each genotype were quantified. Images were gridded to 25 of 20,000 μm^2 squares by using ImageJ before counting. Error bars are SD.

may have completely masked the effects of *abp1* mutation. We believe that analysis of additional alleles of *abp1* that were generated by using non-CRISPR methods will help us to further confirm our findings. We obtained a T-DNA insertion mutant from the *Arabidopsis* stock center (Fig. 6). The mutant (*abp1-TD1*) had a T-DNA insertion at 27 bp downstream of the ATG start

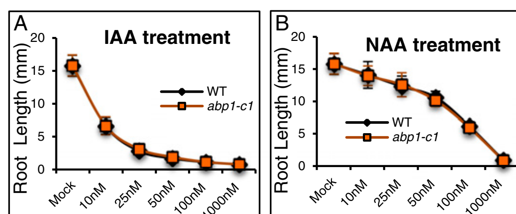

Fig. 4. Effects of auxin treatments on *abp1-c1* root elongation. Quantification of root elongation of WT and *abp1-c1* with various concentrations of IAA (*A*) or NAA (*B*) for 2 d. Shown are average ± SD ($n = 50$).

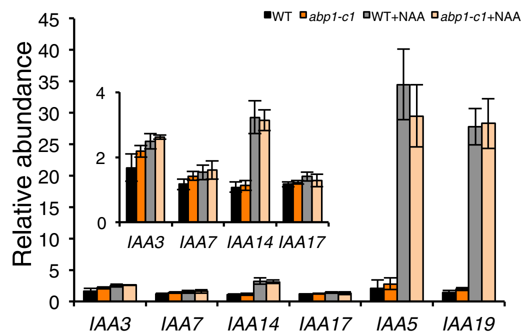

Fig. 5. *AUX/IAA* transcripts abundance in *abp1-c1* with NAA treatments. Light grown, 7-d-old seedlings were treated with or without 1 μM NAA for 2 h and were collected for RNA extraction. For each genotype and treatment, five biological replicates were performed. Expression of *IAA3*, *IAA7*, *IAA14*, and *IAA17* with reduced *y* axis are shown as inset. Error bars are SD.

codon in the first exon (Fig. 6*A*). Interestingly, the T-DNA insertion site was close to the previously reported embryonic lethal T-DNA insertion mutant, which had an insertion at 51 bp from the ATG (3). The *abp1-TD1* plants were viable and displayed no obvious differences from WT plants (Fig. 6*B*). At the mature stage, *abp1-TD1* and WT were similar in size and both were fertile (Fig. 6*C*). We investigated whether *abp1-TD1* still produced *ABP1* mRNA by RT-PCR analysis. We first used a pair of primers (A5P + A3P, please see Table S1 for primers used in this study) (Fig. 6*A*) that can amplify the entire ORF from ATG start codon to the TAA stop codon. It was clear that the primers efficiently amplified the *ABP1* cDNA from WT samples, whereas no *ABP1* cDNA was amplified in the *abp1-TD1* sample (Fig. 6*D*). We then used another pair of primers (A2E and A3P) (Fig. 6*A*) to determine whether *abp1-TD1* can produce partial *ABP1* mRNA, which might still produce functional ABP1 protein. As shown in Fig. 6*D*, *abp1-TD1* did not produce such partial mRNA. Moreover, our Western blot analysis (Fig. 6*E*) indicated that *abp1-TD1* is a null allele. The finding that *abp1-TD1* was viable, normal, and fertile further supports the conclusions that ABP1 is not essential.

In summary, the new *abp1* mutants presented in this paper offer the genetic materials needed to unambiguously define the physiological roles of ABP1. The mutants are viable, stable, and more importantly, they are nulls. Moreover, the mutants are generated by using different methods and the *abp1-c1* and *abp1-TD* harbor different types of mutations. Our results clearly demonstrate that plants do not need ABP1 for auxin signaling and for their growth and development under normal growth conditions. At this point, the reasons for the differences between the phenotype of our mutants and previously described *ABP1* knockdown lines are not clear. However, both cellular immunization and antisense approaches can be susceptible to off-target effects. For example, a recent study in zebrafish showed that 80% knockdown mutants induced by Morpholinos (antisense) were not recapitulated by true null mutants (29).

Materials and Methods

Plant Materials. The *abp1-TD1* (SK21825) was obtained from the *Arabidopsis* stock center. All plants were grown under long-day conditions (16-h light and 8-h darkness) at 22 °C if not otherwise specified. For hypocotyl and root length measurements, seedlings were grown on Murashige and Skoog (MS) media containing 1% sucrose under long-day conditions on vertical plates for 7 d. The plates were scanned, and NIH Image J software was used to quantify hypocotyl and root lengths.

Generation of *abp1-c1* using CRISPR technology. Our ribozyme-based CRISPR technology was described (19). WT *Arabidopsis* plants, *Columbia-0* ecotype, were transformed with the CRISPR construct by floral dipping. The *abp1-c1* plants were identified at the T2 stage.

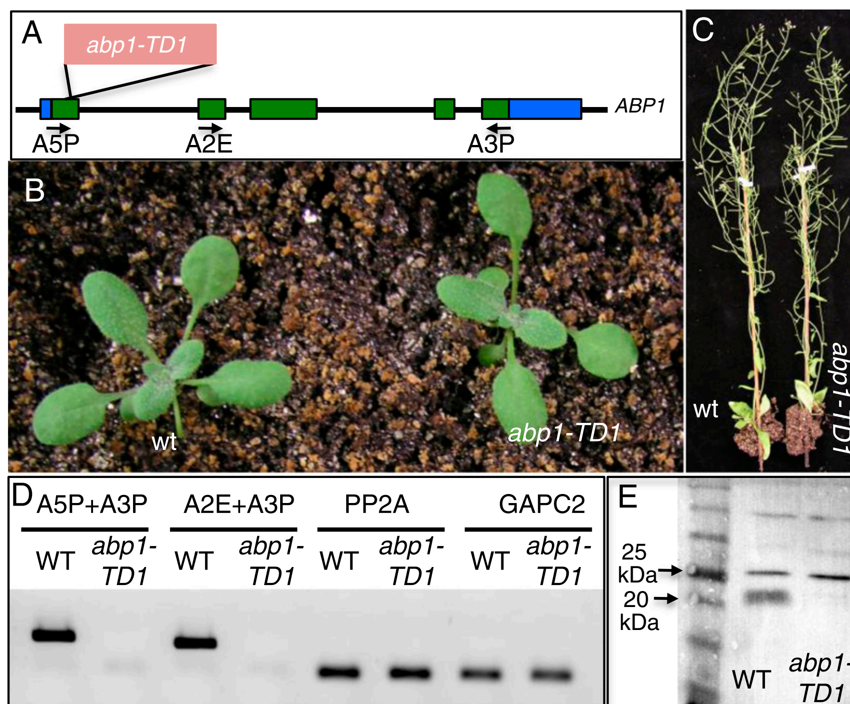

Fig. 6. Identification of a T-DNA insertion null allele of *abp1*. (*A*) Schematic presentation of the T-DNA insertion site in *abp1-TD1*. The T-DNA insertion is 27 bp downstream of ATG start codon of the first exon. (*B*) *abp1-TD1* is viable and does not have obvious developmental defects. (*C*) *abp1-TD1* is fertile and similar to WT in size. (*D*) RT-PCR results indicate that *abp1-TD1* plants do not produce *ABP1* mRNA. The A5P and A3P pair amplifies the full length *ABP1* cDNA from the start codon to the stop codon. The A2E and A3P primers amplify the *ABP1* cDNA that does not contain the sequences of the first exon. The positions of the PCR primers are schematically indicated in the panel A. The RT-PCR products were amplified with 45 saturated cycles and loaded onto 1.2% agarose gel. (*E*) A Western blot image indicates that *abp1-TD1* lacked ABP1 protein. The band between 25 and 20 kDa in WT lane is ABP1, which has a predicted size of 22 kDa.

Genotyping *abp1* Mutants. The T-DNA insertion mutant was genotyped by using a PCR-based method described (30, 31). Genotyping primers for *abp1-TD1* were as follows: ABP1-U409F, ABP1-586R, and the T-DNA specific primer pSKTAIL-L3 (please see Table S1). For genotyping *abp1-c1*, we amplified an *ABP1* fragment by PCR using the following two primers: ABP1-U409F and ABP1-586R. The resulting PCR product was digested with the restriction enzyme BslI, which cuts WT PCR product once and does not cut the mutant band (Fig. S1).

Western Blot. Plant extracts were loaded onto SDS/PAGE gels. The gel was run until bromophenol blue was approximately 1 cm above the bottom of the gel, and the proteins were transferred to a PVDF membrane. The membrane was blocked in 5% (wt/vol) nonfat milk overnight at 4 °C and with anti-ABP1 antibody at room temperature for 3 h. The membranes were washed in TBST (20 mM Tris, 150 mM NaCl, pH = 8.0 plus 0.05% Tween 20) three times, incubated with goat anti-rabbit secondary antibody for 3 h, and washed in TBST three times. Results were visualized by ECL Plus Western Blotting Detection System (Amersham; RPN2232).

Analysis of Auxin Responses. Five-day-old seedlings grown on MS plates were transferred to MS plates containing various concentrations of IAA or NAA, or mock. The root tips of seedlings were marked. After grown vertically for 2 d, plates were scanned. The root elongation that occurred during the 2-d period, and hypocotyl length was measured by using NIH ImageJ.

To analyze auxin-induced gene expression, 7-d-old seedlings were treated with or without 1 μM NAA for 2 h. Five biological replicates were prepared for both WT and *abp1-c1* mutant, with or without the treatment. Total RNAs were extracted by using the RNeasy Plant Mini Kit (Qiagen; 74904) according to the instructions from the manufacturer. RNA samples were treated with DNase and purified before performing quantitative RT-PCR. PCR primers are listed in Table S1.

ACKNOWLEDGMENTS. We thank Dr. Alan Jones for kindly providing us the ABP1 antibody. This work was supported by NIH Grants R01GM068631 (to Y. Zhao) and R01GM43644 (to M.E.), National Science Foundation (Plant Genome Grant DBI-0820729 (to Y. Zhao), the Gordon and Betty Moore Foundation (M.E.), and the Howard Hughes Medical Institute (M.E.).

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