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

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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. 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 cytokinetic 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.

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The authors declare no conflict of interest.

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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. 1A). 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 (API) promoter (Fig. 1A). 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 homoyzgeneous 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. 1B). 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 F2 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 same as reported (3). The ABP1 cDNAs from abp1-c1 all contained the 5-bp deletion (Fig. 1C). 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. 1D 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. 2A, light grown abp1-c1 seedlings looked similar to WT seedlings. Both WT and abp1-c1 plants had similar hypocotyl lengths (Fig. 2B). 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. 2A and C), and the cellular organization of primary roots of the mutant, including the meristem, appeared similar to that of WT plants (Fig. 2D and E). We did not observe any alterations of
We used a classic root elongation assay (27) to determine whether the reduced auxin response in ABP1 knockdown lines (20) had similar flowering time (Fig. 2A). Dark-grown seedlings of the ABP1 antibody lines were partially de-etiolated with short hypocotyls and lacked an apical hook (11). However, the abp1-3 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. 4B). Similar results were also observed when NAA was used in the treatments (Fig. 4B).

The abp1-c1 and WT Plants Respond to Auxin Similarly at the Molecular Level. Although ABP1 was suggested to mainly function in non-genomic 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 (29) 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
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 codon in the first exon (Fig. 6A). 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. 6B). At the mature stage, abp1-TD1 and WT were similar in size and both were fertile (Fig. 6C). 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. 6D) 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. 6D). We then used another pair of primers (A2E and A3P) (Fig. 6D) to determine whether abp1-TD1 can produce partial ABP1 mRNA, which might still produce functional ABP1 protein. As shown in Fig. 6D, abp1-TD1 did not produce partial mRNA. Moreover, our Western blot analysis (Fig. 6E) 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. 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 μm² squares by using ImageJ before counting. Error bars are SD.

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.
Genotyping abp1 Mutants. The T-DNA insertion mutant was genotyped by using a PCR-based method described (30, 31). Genotyping primers for the T-DNA insertion site in abp1-TD1 were as follows: ABP1-U409F, ABP1-586R, and the T-DNA specific primer pSKTAIL-L3 (please see Table S1). For genotyping, 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.

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. This work was supported by NIH Grants R01GM068631 (to Y. Zhao), and R01GM36444 (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.).

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.

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Supporting Information

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Fig. S1. The CRISPR allele of abp1 is stably transmitted to next generations according to Mendel genetics. Ninety-six progenies from a single abp1-c1/− plant were genotyped by using methods described in the text. The gel picture shows the patterns of WT, heterozygous, and homozygous abp1-c1 samples. The actual number of plants for each genotype is shown in parentheses.

Fig. S2. The abp1-c1 seedlings grown in the dark were similar to WT grown under the same conditions. (A) Four-day-old seedlings grown in total darkness: WT (Left) and abp1-c1 (Right). Note that both WT and the mutant had an apical hook. (B) Quantification of hypocotyl length of dark-grown seedlings.
Table S1. Primers used in this study

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