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COMMENTARY

Genome Sequencing of Arabidopsis *abp1-5* Reveals Second-Site Mutations That May Affect Phenotypes

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Auxin regulates numerous aspects of plant growth and development. For many years, investigating roles for AUXIN BINDING PROTEIN1 (ABP1) in auxin response was impeded by the reported embryo lethality of mutants defective in *ABP1*. However, identification of a viable *Arabidopsis thaliana* TILLING mutant defective in the ABP1 auxin binding pocket (*abp1-5*) allowed inroads into understanding ABP1 function. During our own studies with *abp1-5*, we observed growth phenotypes segregating independently of the *ABP1* lesion, leading us to sequence the genome of the *abp1-5* line described previously. We found that the *abp1-5* line we sequenced contains over 8000 single nucleotide polymorphisms in addition to the *ABP1* mutation and that at least some of these mutations may originate from the Arabidopsis Wassilewskija accession. Furthermore, a *phyB* null allele in the *abp1-5* background is likely causative for the long hypocotyl phenotype previously attributed to disrupted ABP1 function. Our findings complicate the interpretation of *abp1-5* phenotypes for which no complementation test was conducted. Our findings on *abp1-5* also provide a cautionary tale illustrating the need to use multiple alleles or complementation lines when attributing roles to a gene product.

INTRODUCTION

The plant hormone auxin regulates cell division and cell expansion to affect all aspects of plant growth (reviewed in Perrot-Rechenmann, 2010). Auxin regulates a wide range of developmental processes, and tight control of auxin response is maintained by multiple modes of regulation. These include regulating auxin biosynthesis and metabolism, transport, and signaling (reviewed in Enders and Strader, 2015). To date, nuclear auxin signaling components have been well characterized (reviewed in Chapman and Estelle, 2009; Salehin et al., 2015). In addition, a nontranscriptional auxin response pathway has been proposed, with AUXIN BINDING PROTEIN1 (ABP1) acting in the apoplast as an auxin receptor and transmitting a cytoplasmic signal to regulate auxin responses such as auxin transport and cytoskeletal rearrangements (reviewed in Shi and Yang, 2011; Sauer et al., 2013).

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Study of ABP1 has a long, complicated history. Experiments demonstrating auxin binding activity for ABP1 were published as early as the 1980s (reviewed in Jones, 1994). Reverse genetics proved to be a complicated approach to study ABP1 function in *Arabidopsis thaliana*, as two independently generated *abp1* mutants, presumed to be null alleles, appeared to be embryo lethal (Chen et al., 2001; Tzafrir et al., 2004; Meinke et al., 2008; Sassi et al., 2014). Knockdown lines of *ABP1* provided some ability to study ABP1 function by reverse genetics. Arabidopsis lines expressing an inducible ABP1 antisense transcript or an inducible single-chain fragment variable scFv12 (David et al., 2007) from a monoclonal antibody raised to ABP1 (Leblanc et al., 1999), targeted to either the apoplast (SS12S) or the endoplasmic reticulum (SS12K), led to phenotypes consistent with decreased auxin activity (Braun et al., 2008; Tromas et al., 2009). Furthermore, the *abp1-5* mutation, first described 5 years ago, provided a much needed tool to study ABP1 function. This ethyl methanesulfonate (EMS)-generated TILLING (Henikoff et al., 2004) allele carries a histidine-to-tyrosine point mutation at position 94 in the auxin binding pocket of ABP1, which presumably alters auxin binding affinity (Robert et al., 2010; Xu et al., 2010). Studies using the ABP1 antisense lines, the inducible monoclonal antibody lines, and *abp1-5* have uncovered many auxin-related roles for ABP1.

Decreased ABP1 activity in the antisense lines, antibody-expressing lines, and *abp1-5* has been reported to result in both morphological and molecular phenotypes. Reported morphological changes in these lines include small epinastic cotyledons and leaves caused by decreased cell size and infrequent cell divisions (Braun et al., 2008), reduced root growth (Tromas et al., 2009), decreased epidermal pavement cell lobing (Xu et al., 2010), reduced hypocotyl elongation in dark-grown seedlings (Paque et al., 2014), and long hypocotyls when grown under low red:far-red light conditions (Effendi et al., 2013). On a molecular level, affecting ABP1 function using antisense, antibodies, or the *abp1-5* allele has been reported to result in decreased auxin transcriptional responses (Tromas et al., 2009), reduced activation of ROP small GTPases (Xu et al., 2010), enhanced auxin efflux protein internalization in epidermal pavement cells (Xu et al., 2010), reduced Brefeldin A-induced

internalization of auxin efflux proteins in root cells (Robert et al., 2010; Chen et al., 2012), altered auxin-responsive rearrangement of microtubules (Chen et al., 2014), and altered xyloglucan structure (Paque et al., 2014). These phenotypes suggest roles for ABP1 in many processes.

Inconsistent with research suggesting multiple roles for ABP1 throughout plant development, Gao et al. (2015) recently reported that two independent CRISPR and T-DNA insertion alleles of *abp1*, which fail to accumulate ABP1 protein, display no obvious phenotypes. These findings cannot be reconciled easily with the embryo lethal phenotypes of the two original lethal T-DNA insertion lines (Chen et al., 2001; Tzafrir et al., 2004; Meinke et al., 2008; Sassi et al., 2014), the phenotypes of the ABP1 antisense line (Braun et al., 2008; Chen et al., 2014), the phenotypes of the lines expressing anti-ABP1 monoclonal antibody fragments (Braun et al., 2008; Tromas et al., 2009; Paque et al., 2014), or the phenotypes of the *abp1-5* TILLING allele (Robert et al., 2010; Xu et al., 2010; Effendi et al., 2013; Chen et al., 2014) and thus require explanation. The situation is far from clear at present and might be explained, for example, by off-target effects in the antibody and antisense expression lines in conjunction with background mutations in the knockout lines as well as in the *abp1-5* allele. Or, for the opposite interpretation, these differences may be explained by background suppressor mutations or compensatory systems in the CRISPR knockout line. The differences observed between the various T-DNA lines remain confusing (Chen et al., 2001; Tzafrir et al., 2004; Meinke et al., 2008; Sassi et al., 2014; Gao et al., 2015). Resolving these differences in reported *abp1* phenotypes will be an important task for the community in the coming years.

In our studies using *abp1-5*, we found that the long hypocotyl phenotypes ascribed to *abp1-5* segregated independently of the *abp1-5* lesion. We therefore performed whole-genome sequencing of the *abp1-5* line (originally described in Xu et al., 2010) and found numerous additional mutations, some of which may account for the phenotype differences between the two recently reported *abp1* null alleles (Gao et al., 2015) and *abp1-5*.

abp1-5 LIGHT SIGNALING DEFECTS ARE LIKELY CAUSED BY A SECOND-SITE MUTATION IN *PHYB*

abp1-5 displays red light phenotypes consistent with ABP1 roles in light signaling (Effendi et al., 2013, 2015). In particular, *abp1-5* displays longer hypocotyls under red light (Effendi et al., 2013; Figure 1). Although less dramatic, *abp1-5* also displays shorter hypocotyls than the wild type when grown in darkness and longer hypocotyls than the wild type under white light and blue light (Figure 1A). Because these *abp1-5* phenotypes were strikingly similar to phenotypes seen in *phytochrome B* (*phyB*) mutants (Reed et al., 1993), we sequenced the *PHYB* gene in *abp1-5* to rule out the possibility that these phenotypes were caused by a second-site mutation in *PHYB*. Rather than ruling out *PHYB* mutations as a contributor to the *abp1-5* phenotypes, we found that the *PHYB* gene in *abp1-5* contains a C-to-T base change at position 1267 (where the A of the ATG is at position 1) that causes a Glu-423-to-stop mutation (Figure 2A). Because this premature

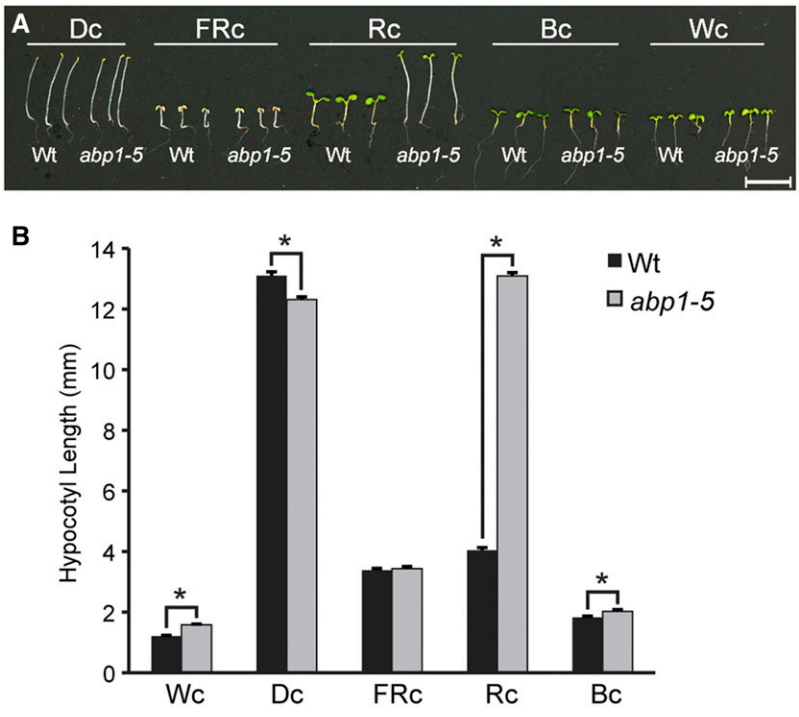

Figure 1. *abp1-5* Displays Altered Light Responses.

(A) Photographs of 7-d-old wild-type (Col-0) and *abp1-5* seedlings grown at 22°C on Phytoblend medium supplemented with 1% sucrose under darkness (Dc), FRc (5 μmol m^{-2} s^{-1}), Rc (50 μmol m^{-2} s^{-1}), Bc (25 μmol m^{-2} s^{-1}), or Wc (100 μmol m^{-2} s^{-1}) light conditions. Bar = 1 mm.

(B) Mean hypocotyl lengths (±SE; $n = 75$) of 7-d-old wild-type (Col-0) and *abp1-5* seedlings grown at 22°C on Phytoblend medium supplemented with 1% sucrose under Dc (darkness), FRc (5 μmol m^{-2} s^{-1}), Rc (50 μmol m^{-2} s^{-1}), Bc (25 μmol m^{-2} s^{-1}), or Wc (100 μmol m^{-2} s^{-1}) light conditions. *abp1-5* hypocotyls were significantly longer than wild-type hypocotyls under Wc, Rc, and Bc conditions ($P \leq 0.005$) in two-tailed *t* tests assuming unequal variance. *abp1-5* hypocotyls were significantly shorter than wild-type hypocotyls under Dc conditions ($P \leq 1 \times 10^{-6}$) in two-tailed *t* tests assuming unequal variance.

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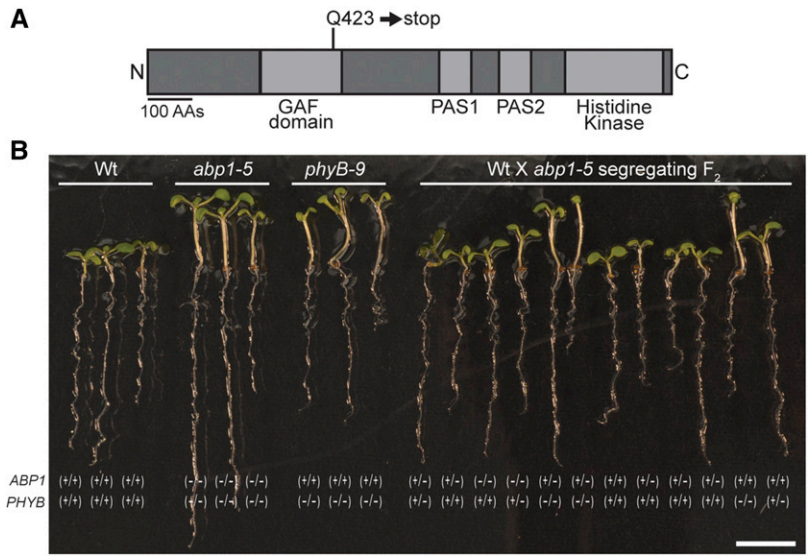

Figure 2. The Long Hypocotyl Phenotype of *abp1-5* Segregates Independently of the *abp1-5* Mutation.

(A) Examination of the *PHYB* gene in *abp1-5* revealed a C-to-T base change at position 1267 (where the A of the ATG is at position 1) that caused a Glu-423-to-stop mutation in the GAF domain.
(B) Images of 7-d-old wild-type (Col-0), *abp1-5*, *phyB-9*, and F2 progeny from the *abp1-5* backcross to Col-0 seedlings grown under yellow light conditions. Genotyping results for *ABP1* and *PHYB* mutations are indicated below each imaged seedling. Bar = 7 mm.

stop is in the GAF domain, the *PHYB* gene in *abp1-5* is unlikely to encode a functional protein (Reed et al., 1993; Bradley et al., 1996; Chen et al., 2003).

Because the *ABP1* and *PHYB* genes are located on different chromosomes, their segregation is unlinked in segregating populations. To determine whether the *PHYB* mutation in *abp1-5* is associated with the long hypocotyl phenotype observed in *abp1-5*, we examined the phenotypes and genotypes of segregating F2 seedlings from *abp1-5* crossed to the wild type (Columbia-0 [Col-0]). The 3:1 segregation of short hypocotyls to long hypocotyls within this segregating backcross suggested that this phenotype was caused by a single, recessive mutation (Figure 2B). We then genotyped these segregating individuals and found that the long-hypocotyl phenotype was associated with the mutation in *PHYB*, but not the mutation in *ABP1* (Figure 2B).

abp1-5 CONTAINS NUMEROUS BACKGROUND MUTATIONS

After discovering a *PHYB* mutation in the *abp1-5* background, we decided to sequence the genome of the original *abp1-5* line (Xu et al., 2010) to discover whether it carried additional background mutations. Whole-genome sequencing of this *abp1-5* line revealed over 8500 single nucleotide polymorphisms (SNPs) (2627 of which are consistent with EMS mutagenesis) in the exome (Table 1), with a concentration of mutations on Chromosome 3, even though *ABP1* is located on the north arm of Chromosome 4 (Figure 3). Of these SNPs, 4034 result in nonsynonymous amino acid changes in the encoded proteins and 66 of the mutations result in the creation of premature stop codons (Supplemental Data Set 1). Although most of the identified SNPs on Chromosomes 1, 2, 4, and 5 are consistent with EMS mutagenesis (G-to-A or C-to-T), a majority of the SNPs found on Chromosome 3 are not EMS related, suggesting that the origin of these polymorphisms may be more complicated, such as a cross to a non-Columbia ecotype during the reported six cycles of backcrossing (Xu et al., 2010). Indeed, the *abp1-5* polymorphisms identified on the south arm of Chromosome 3 are also present in the Wassilewskija (Ws-2) accession (Figure 4A), consistent with the possibility that a cross to

Table 1. SNPs in the *abp1-5* Exome

Chromosome	Synonymous	Nonsynonymous	Stop Gained	ncRNA[a]	Start Lost	Total
1	0	4 (2)	0	0	0	4 (2)
2	23 (20)	48 (46)	2 (2)	0	0	73 (68)
3	4388 (1469)	3936 (984)	61 (22)	51 (19)	8 (1)	8444 (2495)
4	12 (10)	22 (21)	2 (2)	0	0	36 (33)
5	8 (8)	20 (20)	1 (1)	0	0	29 (29)
Mitochondria	4 (0)	4 (0)	0	0	0	8 (0)
Chloroplast	0	0	0	0	0	0
Total	4435 (1507)	4034 (1073)	66 (27)	51 (19)	8 (1)	8594 (2627)

Numbers in parentheses indicate the number of SNPs consistent with EMS mutagenesis (C to T or G to A).
[a]ncRNA, noncoding RNA.

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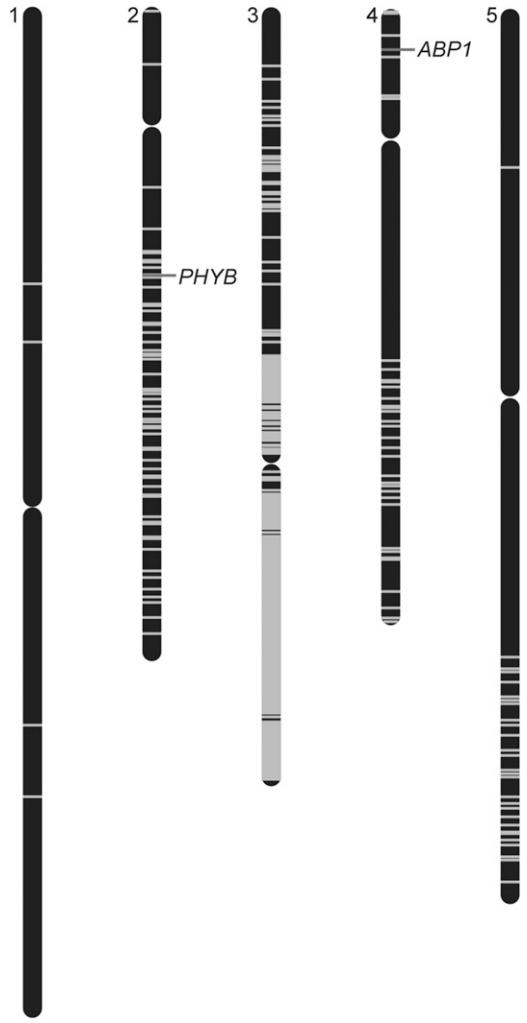

Figure 3. Whole-Genome Sequencing of *abp1-5* Revealed Numerous SNPs.

Map positions of homozygous SNPs (see Supplemental Data Set 1 for a list of mutations identified) are identified by a gray line on each of the five Arabidopsis chromosomes.

the Ws accession at some point in the history of *abp1-5* was the origin of these polymorphisms. Conversely, examined *abp1-5* SNPs on Chromosomes 1, 2, 4, and 5 do not appear to be present in either the Ws-2 or Landsberg *erecta* (L*er*-0) ecotypes (Figure 4B). The *abp1-5* lines characterized in other publications (Robert et al., 2010; Baster et al., 2013; Effendi et al., 2013; Chen et al., 2014; Paque et al., 2014; Xu et al., 2014) likely carry a distinct subset of background mutations, depending on mutation segregation as a result of crosses made to create *abp1-5* carrying molecular reporters.

The extent to which these *abp1-5* background mutations contribute to *abp1-5* phenotypes is unknown; in addition, these mutations are likely to have segregated independently and variably as labs have made additional crosses with *abp1-5*, increasing the complexity of understanding *abp1-5* phenotypes for which no complementation line was used.

REASSESSING *abp1-5* PHENOTYPES

For decades, identification of ABP1 roles in signaling and development was elusive to researchers because of the lack of genetic resources. Identification of the *abp1-5* TILLING allele provided a useful tool to examine ABP1 function and allowed identification of downstream ABP1 signaling components. However, our data revealing numerous background mutations in an *abp1-5* line (Figure 3), combined with recent characterization of two independent new *abp1* null alleles with no discernible phenotypes (Gao et al., 2015), suggest that our community will need to reassess *abp1-5* phenotypes in cases where no complementation line was included in the analysis. Additionally, using the recently identified *ABP1* null alleles (Gao et al., 2015) to reexamine phenotypes may aid in understanding phenotype differences among *abp1* alleles.

Phenotypic inconsistencies among *abp1* alleles have led to confusion regarding ABP1 function. Unlike the recently identified *ABP1* null alleles that display no discernible phenotype (Gao et al., 2015), the inducible repression alleles and *abp1-5* display a wide variety of cell expansion and cell division defects throughout plant development (Braun et al., 2008; Tromas et al., 2009; Robert et al., 2010; Xu et al., 2010; Chen et al., 2012, 2014; Paque et al., 2014). In addition, a recent report demonstrating that expression of wild-type *ABP1* in the *abp1-1* insertion mutant fails to rescue its embryo-lethal phenotype (Grones et al., 2015) suggests the possibility that the *abp1-1* embryo lethality is caused by a defect in a gene other than *ABP1*. Off-target effects in inducible repression lines, background mutations in *abp1-5* or the T-DNA lines, or compensatory systems in the CRISPR and T-DNA insertional knockout lines could contribute to the distinct phenotypes (or lack thereof) observed in different *abp1* alleles. Alternatively, these phenotypic differences might arise from differing plant growth responses under chronic and acute lack of ABP1 activity.

This cautionary tale of background mutations in *abp1-5* is not the first of its kind. Other examples include a *pen2* mutation in

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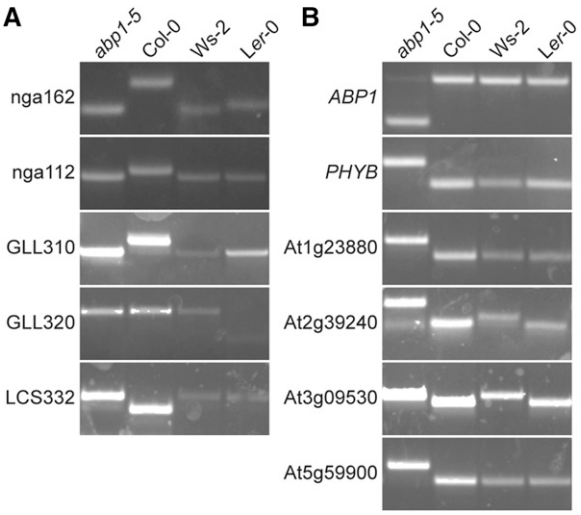

Figure 4. Genotyping Reveals Outcrossing in the History of *abp1-5*.

(A) PCR-based genotyping of *abp1-5*, Col-0, Ws-2, and L*er*-0 using mapping markers nga162 (Bell and Ecker, 1994), nga112 (Bell and Ecker, 1994), GLL310 (Strader et al., 2010), GLL320, and LCS332. See Table 2 for genotyping information.
(B) PCR-based genotyping of *abp1-5*, and the Col-0, Ws-2, and L*er*-0 ecotypes for polymorphisms in the *ABP1*, *PHYB*, At1g23880, At2g39240, At3g09530, and At5g5990 genes identified by *abp1-5* whole-genome sequencing (see Supplemental Data Set for a list of mutations identified). See Table 2 for genotyping information.

the *coi1-16* allele (Westphal et al., 2008) and an *are1* mutation in *ctr1-1* (Shin et al., 2013). Recently, whole-genome sequencing has been used to reveal an unexpected pedigree for a classic "trisomic" line (Salomé and Weigel, 2015). These findings underscore the importance of examining multiple alleles whenever possible and keeping careful records of crossing history. Additionally, as in the case for *abp1-5*, complementation tests are required to ensure that any new phenotypes attributed to a mutation are rescued in a complementation line, preferably using the native promoter to drive expression.

Our findings described here are meant to inform other researchers using the *abp1-5* allele. We encourage the use of a rescue line alongside any future uses of this allele, so that identified phenotypes can be justifiably attributed to the *abp1-5* mutation. Moreover, the newly described *abp1* null alleles (Gao et al., 2015) may provide excellent resources for ABP1 functional studies using reverse genetics, provided they are free of off-target effects, insertion position effects, or background mutations. The numerous background mutations in the *abp1-5* line described here may help explain some of the phenotypic differences between the *abp1* CRISPR and T-DNA insertion null alleles (Gao et al., 2015) and *abp1-5* (Robert et al., 2010; Xu et al., 2010, 2014; Baster et al., 2013; Effendi et al., 2013; Chen et al., 2014; Paque et al., 2014).

METHODS

Phenotypic Assays

Arabidopsis thaliana mutants were in the Col-0 background, which was used as the reference sequence and the wild type in all experiments. For phenotypic assays, seeds were surface sterilized (Last and Fink, 1988), stratified for 2 d at 4°C, and plated on plant nutrient media (Haughn and Somerville, 1986) supplemented with 0.5% sucrose (w/v) and solidified with 0.6% (w/v) agar. To examine hypocotyl elongation, seedlings were grown at 22°C under continuous illumination. Seedlings were imaged after 7 d of growth.

For measurement of hypocotyls length under monochromatic illumination, surface-sterilized seeds were cold-stratified at 4°C for 4 d in darkness and germinated on Murashige and Skoog media containing 1% (w/v) sucrose adjusted to pH 5.7 with KOH and 0.7% (w/v) Phytoblend agar (Caisson Labs). Seedlings were grown at 22°C for 7 d under continuous far-red (FRc; λ_{max}~735 nm), red (Rc; λ_{max}~670 nm), blue (Bc; λ_{max}~470 nm), or white (Wc) light using light sources previously described (Warnasooriya and Montgomery, 2009). Fluence rate of FR was measured using a StellarNet EPP2000 spectroradiometer (Apogee Instruments), and fluence rates of Rc, Bc, and Wc were measured using a LI-250A light meter (Li-Cor). Hypocotyl lengths were measured using Image J software (NIH).

Genetic Analyses

The *abp1-5* mutant used in this study was provided by the lab of Zhenbiao Yang (University of California-Riverside) and was initially described by Xu et al. (2010). The *abp1-5* mutant, originally in the Col-0 background (Robert et al., 2010; Xu et al., 2010), was backcrossed to Col-0 and resultant F2 examined for segregation of the long hypocotyl phenotype. Individual F2 were genotyped using PCR analysis (Table 2).

Whole-Genome Sequencing

Arabidopsis *abp1-5* seeds were surface sterilized and plated on media on top of sterile filter paper. After 7 d of growth, *abp1-5* tissue was collected and genomic DNA extracted (Thole et al., 2014). A library was prepared from the genomic DNA using an Illumina Genomic DNA kit. Libraries were then sequenced at the Washington University Genome Technology Assistance Center (https://gtac.wustl.edu) on an Illumina HiSequation 2000 using multiplexing in a 100-bp paired end run. Using Novoalign (Novo-craft), reads were aligned to the Arabidopsis Col-0 reference genome with Arabidopsis Information Resource 10 gene annotations (Arabidopsis Genome Initiative, 2000). SNPs were identified using SAMtools (Li et al., 2009) and annotated using snpEFF (Cingolani et al., 2012).

Accession Numbers

Accession numbers for each gene are listed in Table 2 and Supplemental Data Set 1.

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Table 2. Genotyping Primers and Markers

Marker	Gene	Enzyme	Size of Products (bp)				Oligonucleotides[a]
			abp1-5	Col-0	Ws-2	L*er*-0	
ABP1	At4g02980	*Rsa*I	107+43	150	150	150	CATTGGTATCCGCTCGGCTCTTATCTGTG GTACCACTGCCCTTTAGGACAAC
PHYB	At2g18790	*Pst*I	155	130+25	130+25	130+25	AAGGTATGCTTGTGAGTTTTTGCTG GTGTAACAATTCCAGCAGGCGAGTC
At1g23880	At1g23880	*Acc*I	190	169+21	169+21	169+21	GTATTCAGTGGAAACGGTGGTA TGAAATCCACCAAGTCTGACGCAAAAT
At2g39240	At2g39240	*Xho*I	150	130+20	130+20	130+20	GAGAGACTCCTGAAGATGCCCTCGA TGCATACAAAATCAAGTCCAATAAT
At3g09530	At3g09530	*Hinf*I	199	178+21	199	178+21	CCAACAATCTCCAACACGTTGACT GCTTCTTCGGGCGACATCTCCAC
At5g59900	At5g59900	*Acc*I	200	176+24	176+24	176+24	GTGAGCTAGTCTCGAGAAGATTGTAGA CAACAGTGATGAAGCAGGCCAGAAGAGATTA
nga162	At3g13960	–	85	107	85	89	CATGCAATTTGCATCTGAGG CTCTGTCACTCTTTTCCTCTGG
nga112	At3g62650	–	189	197	189	189	CGTGTATGCAGCTGCATAGACAGTGG GGCGTTATCTCCATCACTCCCTATAGC
GLL310	At3g51380	–	204	246	204	204	AGAAGAGACAGTGACAGAATCGGGTAATAAG GTCTATCTCCCCCACTTGTTCATC
GLL320	At3g50550	*Apo*I	191	191	191	114+77	GAATGGCTAGCCCCAAAGAC TATTGCGTAAAGAAGCGAAAAC
LCS332	At3g17850	*Dpn*II	148	118+30	148	148	CTGATGGCGGCAAAGTAGGGCTGAG CGAAGATGGGTGCTGATGGTGGTGAC

[a]Underlined nucleotide is the introduced mutation for the derived cleaved amplified polymorphic sequence marker (Neff et al., 2002).

Supplemental Data

Supplemental Data Set 1. *abp1-5* whole-genome sequencing data.

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AUTHOR CONTRIBUTIONS

T.A.E., Z.Y., B.L.M., and L.C.S. planned the experiments. T.A.E. and S.O. conducted experiments. T.A.E., S.O., Z.Y., B.L.M., and L.C.S. wrote the article.

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