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Overexpression of a putative *Arabidopsis* BAHD acyltransferase causes dwarfism that can be rescued by brassinosteroid

Mengjiao Wang[1,2,†], Xiayan Liu[1,2,†], Rui Wang[1,2], Wanchun Li[1,2], Steve Rodermel[3] and Fei Yu[1,2,*]

[1] College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, People's Republic of China
[2] State Key Laboratory of Crop Stress Biology in Arid Areas (Northwest A&F University), Yangling, Shaanxi 712100, People's Republic of China
[3] Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA

†These authors contributed equally to this article.
*To whom correspondence should be addressed. E-mail: flyfeiyu@gmail.com

Abstract

Plant growth and development are ensured through networks of complex regulatory schemes. Genetic approaches have been invaluable in dissecting these regulatory pathways. This study reports the isolation of a semi-dominant dwarf mutant designated *abnormal shoot1-1 dominant* (*abs1-1D*) through an *Arabidopsis* T-DNA activation tagging mutant screen. It was shown that the overexpression of a novel BAHD family acyltransferase gene, *ABS1*/At4g15400, was the cause of the dwarf phenotype in *abs1-1D*. Overexpression of *ABS1* led to many phenotypic features reminiscent of brassinosteroid (BR) deficient or signalling mutants, and it was shown that exogenously applied BR could effectively rescue the dwarf phenotype of *abs1-1D*. Furthermore, genetic analyses indicated that *abs1-1D* interacted, in different ways, with the BR-deficient mutant *det2-1*, the constitutive BR response mutant *bes1-D* and the photomorphogenic mutant *phyB-1*. Moreover, *ABS1* expression was activated by BR treatment or in a *bes1-D* mutant background. Genome-wide transcriptome profiling of *abs1-1D* revealed clear reprogramming of metabolic pathways, and it was demonstrated that BR biosynthesis genes were activated in *abs1-1D* and that the flavonoid biosynthesis pathway was repressed in *abs1-1D,* as well as in *det2-1*. This work provides new insights into the possible involvement of BAHD acyltransferase in the regulation of plant growth and development, and indicates a possible role of *ABS1* in maintaining BR homeostasis.

Key words: *Arabidopsis* shoot development, BAHD acyltransferase, brassinosteroid, de-etiolation, dwarfism, genetic interaction.

Introduction

The growth and development of higher plants follow stereotypical developmental programmes that are intricately regulated (Steeves and Sussex, 1989). For instance, in the light, plants undergo photomorphogenesis, while in the dark skotomorphogenesis takes place. The failure to properly elaborate these regulatory programmes often leads to phenotypes that have enabled us to probe many facets of these regulations, including the actions of plant photoreceptors and hormones (Koornneef *et al.*, 1980; Chory *et al.*, 1989; Li *et al.*, 1996; Li and Chory, 1997). The need for coordinated hormone actions necessitates perspectives from two opposing fronts. On one hand, plant hormone biosynthesis and signalling are essential for plant development and have long intrigued researchers (Kim and Wang, 2010; Zhao, 2010). However, on the other hand, an equally important strategy is

applied by plants to ensure the coordination is through hormone inactivation mechanisms, such as conjugation and/or modification, which have been demonstrated to be important in maintaining the homeostasis of hormone actions (Yamaguchi, 2008).

Genetic screens for mutants with phenotypes indicative of plant hormone deficiency or signalling defects have been one major avenue for elucidating plant hormone biosynthesis and regulatory pathways (Clouse, 1996). One class of mutants that was instrumental in the advancement of plant hormone biology is dwarf mutants (Feldmann *et al.*, 1989). The conspicuous miniature light-grown phenotype of this class of mutants immediately suggests an intimate involvement of the mutated genes in the regulation of plant growth and development. Although many processes have been linked with dwarf phenotypes, the dominant examples are mutants defective in plant hormone biosynthesis and signal transduction, particularly brassinosteroid (BR) and gibberellins (GAs). For example, both BR-deficient and BR-insensitive mutants have been identified, leading to the establishment of DET2 as an important BR biosynthesis gene and BRI1 as the receptor for BR signalling (Li *et al.*, 1996; Li and Chory, 1997). Genetic lesions that can lead to a dwarf phenotype have also been identified in mutants deficient in GAs or defective in GA signalling, and the GA-related dwarf trait has been applied successfully in modern agriculture (Peng *et al.*, 1999).

The majority of dwarf mutants identified so far are genetically recessive, suggesting a positive regulatory role of the underlying genes. In contrast, an increasing number of *Arabidopsis* mutants are being reported that showed genetically dominant dwarfism. Based on the dominant nature of these mutants, it can be inferred that their gene products typically act as negative regulators of hormone biosynthesis or signalling. Consistent with this genetic nature, factors that are capable of inactivating distinct plant hormones, and thus negatively regulating plant growth, have been documented in *Arabidopsis* (Neff *et al.*, 1999; Nakamura *et al.*, 2005; Poppenberger *et al.*, 2005; Takahashi *et al.*, 2005; Turk *et al.*, 2005; Varbanova *et al.*, 2007). For instance, the *Arabidopsis BAS1* gene was first identified in a genetic screen looking for suppressors of the phytochrome B-defective mutant *phyB-4* (Neff *et al.*, 1999). *BAS1* encodes a cytochrome P450 (CYP734A1) that probably inactivates BRs through 26-hydroxylation (Neff *et al.*, 1999). Increased expression of a second cytochrome P450 (CYP72C1), which is a close BAS1 homologue known as SHK1/SOB7/CHI2, also leads to a dominant dwarf phenotype (Nakamura *et al.*, 2005; Takahashi *et al.*, 2005; Turk *et al.*, 2005). Another example of an enzyme that can inactivate BR was demonstrated with the overexpression of an *Arabidopsis* UDP-glycosyltransferase (UGT73C5) (Poppenberger *et al.*, 2005). The overexpression of this enzyme, which probably catalyses the 23-*O*-glucosylation of brassinolide and castasterone, leads to transgenic plants that are reminiscent of BR-deficient mutants such as *det2* (Poppenberger *et al.*, 2005). A similar mode of plant hormone inactivation through modifications has also been observed with GA (Varbanova *et al.*, 2007). The overexpression of *Arabidopsis* methyltransferase GMAT1 or GMAT2 inactivates active GAs through methylation and leads to conspicuous dwarfism, conferring phenotypes similar to those of recessive GA biosynthesis mutants (Varbanova *et al.*, 2007).

In our previous work, we isolated a series of *Arabidopsis* mutants that have altered shoot development programmes and we named these mutants *abnormal shoot* (*abs*) mutants. One semi-dominant mutant, *abs1-1D*, that showed dramatic dwarf phenotype in the light was characterized further. We determined that *abs1-1D* phenotypes were the consequence of the overexpression of *ABS1*/At4g15400. *ABS1* encodes a putative BAHD family of acyltransferases that is expressed in many tissues but with prominent expression in roots. We found that *abs1-1D* displayed many features of BR-deficient mutants, including dwarfism in the light, a darker green leaf colour, de-etiolation in the dark, and compromised leaf elongation. Moreover, exogenous applied BR could effectively rescue *abs1-1D* BR-deficient-related phenotypes. Genome-wide *abs1-1D* transcriptome analysis revealed both expected and previously unknown reprogramming of metabolic pathways at the transcriptional level. These results suggest that *ABS1* is capable of regulating plant growth and development, possibly through involvement in BR homeostasis.

Materials and methods

Plant materials and growth conditions

Wild-type, *abs1-1D*, and the T-DNA insertional line WiscDsLox474E11 (The Arabidopsis Biological Resource Center, OH, USA) are in Columbia ecotype background. The *det2-1* (Li *et al.*, 1996), *bes1-D* (Yin *et al.*, 2002), and *phyB-1* (Bo64) (Reed *et al.*, 1993) mutants have been described and are in Columbia, Enkheim-2, and Landsberg *erecta* backgrounds, respectively. *Arabidopsis* plants were grown at 22 °C under continuous illumination (~100 µmol m^{-2} s^{-1}) on commercial soil mix (Pindstrup, Denmark). For plate and liquid culture experiments, seeds were surface sterilized and grown on 0.5× strength Murashige and Skoog (MS) medium supplemented with 1% sucrose and 1% agar (for plates) and grown at 22 °C under continuous illumination (~100 µmol m^{-2} s^{-1} for light experiments).

DNA and RNA manipulations

DNA isolation, Southern blot analysis and plasmid rescue were performed as described by Yu *et al.* (2008). Total cellular RNAs were extracted using Trizol RNA reagent (Life Technologies, CA, USA). Northern blot analyses were performed as described by Yu *et al.* (2008). For semi-quantitative RT-PCR analysis, first-strand cDNA was synthesized from 1 µg DNase-treated total RNA using a PrimeScript reverse transcription kit (Takara, Japan). The gene-specific primers and RT-PCR conditions used in this study are listed in Supplementary Table S1 (at *JXB* online). Where possible, we designed RT-PCR primers flanking introns to distinguish genomic DNA and cDNA.

Generation of transgenic lines

To generate an *ABS1* overexpression construct, a genomic DNA fragment encompassing the full-length *ABS1*/At4g15400 sequence was amplified with *Pfu* Turbo DNA polymerase (Stratagene, CA, USA) using primers 15400F and 15400R. The amplified fragment was cloned into pBluescript KS+ vector and sequenced before subcloning into the binary vector pBI111L (Yu *et al.*, 2004). The resulting construct was transformed into *Agrobacterium tumefaciens* by electroporation, and the floral dip method was used to transform wild-type or *phyB-1* mutant plants (Clough and Bent, 1998). T1 transgenic lines were screened on solid 0.5× MS medium containing 50 mg l^{-1} of kanamycin. The phenotypes of the transgenic lines were examined in both T1 and T2 generations.

To generate an *ABS1* promoter–β-glucuronidase (GUS) fusion construct, a genomic DNA fragment of 1149 bp upstream of the start codon of the *ABS1* gene was amplified using primers 15400PF and 15400PR

and cloned into the *Xba*I and *Bam*HI sites of pCB308 (Xiang *et al.*, 1999). The resulting construct was designated $P_{ABS1}::uidA$ and introduced to wild-type plants. T1 transgenic plants were screened for Basta resistance. GUS activity was examined in T2 plants from independent lines. Histochemical GUS staining was performed following the procedure of Jefferson (1987).

Protoplast transient expression assay and confocal microscopy

The control green fluorescent protein (GFP) vector pTF486 containing the open reading frame of enhanced GFP (eGFP) driven by the cauliflower mosaic virus (CaMV) 35S promoter was designated *P35S::GFP* (Yu *et al.*, 2008). To generate a C-terminal GFP-tagged ABS1 fusion protein, the open reading frame of *ABS1* was amplified using primers 15400F and 15400GFPR and subcloned into pTF486, and the resulting construct was designated *P35S::ABS1–GFP*. *Arabidopsis* leaf protoplast preparation and transient expression of GFP constructs were performed as described by Yoo *et al.* (2007). For nuclei visualization, protoplasts were resuspended in 0.16 M CaCl$_2$ (pH 7.4). Hoechst 33342 (Sigma, St Louis, MO, USA) was added to a final concentration of 20 μg ml^{-1} to stain the nuclei for 15 min in the dark (Meadows and Potrykus, 1981). GFP, Hoechst 33342, and chlorophyll fluorescence signals were monitored using a Nikon A1 confocal microscope.

BR treatment

Synthetic BR epi-brassinolide (epiBL; Sigma) was used in BR treatment experiments. For testing the response of *Arabidopsis* seedlings to exogenous BR, plants were germinated and grown on 0.5× MS plates without epiBL for 6 days and then transferred to 0.5× MS plates with various concentrations of epiBL for 6 days prior to photographing. To analyse the effect of BR on hypocotyl elongation, plants were germinated and grown on vertically placed 0.5× MS plates with various concentrations of epiBL in the dark or in the light for 1 week in the growth room before photographing and hypocotyl length measurements. A one-sided *t*-test was used to compare the mean hypocotyl length of *abs1-1D* or *det2-1* with that of the wild type under the same treatment conditions. For BR treatment in liquid medium, seedlings were grown on solid 0.5× MS medium for 6 days and then transferred into 0.5× MS liquid medium. After 5 days of liquid culture, seedlings were treated with 0.2 μM or 0.5 μM epiBL for 4 h and then collected and flash frozen in liquid N$_2$ for RNA extraction.

Light microscopy

The middle section of leaf petioles from the first pair of true leaves were hand cut and fixed in 4% (v/v) glutaraldehyde in 0.1 mM sodium phosphate buffer (pH 6.8) for 12 h at 4 °C. After fixation, samples were dehydrated in a dilution series of acetone and embedded in SPI-PON 812 resin (Structure Probe, West Chester, PA, USA). Semi-thin sections were stained with 1% (v/v) toluidine blue O and observed under an Olympus BX51 light microscope equipped with a DP70 digital camera.

Chlorophyll concentration measurement

Chlorophyll concentration was determined on a fresh-weight basis. In brief, 2-week-old seedlings were harvested, weighed, and finely ground in liquid N$_2$. Total chlorophyll was extracted with 95% ethanol, and chlorophyll concentrations were calculated according to the method of Lichtenthaler (1987). Differences in chlorophyll concentration between the wild-type and mutants were evaluated using a *P* value generated by a one-sided *t*-test.

Microarray analysis

Total RNAs were prepared from the aerial parts of 3-week-old wild-type and *abs1-1D* plants. Two biological replicates were used for microarray experiments with Affymetrix ATH1 GeneChip. RNA purification, probe labelling, hybridization, gene chip scanning, and microarray data normalization using Affymetrix MicroArray Suite (MAS) 5.0 software were performed at Shanghai Biotechnology Co., Shanghai, China. A single technical replicate was performed for each biological sample. Genes with a minimum twofold change in expression were considered to be differentially regulated.

Results

Isolation of abs1-1D

In our efforts to identify activation tagged gain-of-function *yellow variegated* (*var2*) suppressors, we recovered numerous mutants with altered shoot development (Yu *et al.*, 2008). We named these mutants *abnormal shoot* (*abs*) mutants, and the *abs1-1D* (*D* denotes *dominant*) mutant was investigated further. Because all of our mutants were in a *var2-5* background, we first crossed out the *var2-5* background and obtained the *abs1-1D* mutation in a wild-type background. Genetic analysis indicated that *abs1-1D* behaved in a semi-dominant manner (Supplementary Fig. S1 at *JXB* online). The mutant phenotype was stable during our experiments. Compared with the wild type, the most striking feature of the *abs1-1D* homozygous mutant was its conspicuous dwarf phenotype at the 3-week-old light-grown stage (Fig. 1A). This became even more dramatic at late developmental stages (Fig. 1B). Closer examination of individual leaves from *abs1-1D* plants revealed that, unlike wild-type leaves, the leaves of *abs1-1D* plants showed abnormal curvature and could not be flattened without overlapping between two solid surfaces (Fig. 1C). At 3 weeks old, *abs1-1D* plants developed fewer leaves with significantly shortened petioles compared with those of the wild type (Fig. 1D). We also observed that *abs1-1D* leaves appeared to be darker green under our growth conditions (Fig. 1A).

Cloning of ABS1

We next tested whether the *abs1-1D* dwarf phenotype was linked with activation tagging T-DNA insertions. Southern blotting revealed the presence of a single T-DNA insertion in *abs1-1D* that co-segregated with the mutant phenotype, suggesting that *abs1-1D* was tagged with a T-DNA (Fig. 2A). Applying a standard plasmid rescue procedure, we recovered a plasmid that harboured both plant and T-DNA vector sequences. Subsequent sequencing revealed that the T-DNA was inserted in *Arabidopsis* chromosome 4 between At4g15400 and At4g15410 (Fig. 2B). As At4g15400 is the gene immediately adjacent to the right border region, where the 35S enhancer sequences are located, we examined the expression of At4g15400, and northern blotting indicated that the expression level of At4g15400 was greatly enhanced in *abs1-1D* (Fig. 2C).

To evaluate whether overexpression of At4g15400 was the cause of the *abs1-1D* dwarf phenotype, we recapitulated the *abs1-1D* phenotype with independent CaMV 35S promoter-driven At4g15400 overexpressing transgenic lines (Fig. 2D). Multiple transgenic lines showed the characteristic dwarf phenotype of *abs1-1D* (Fig. 2D) and the phenotypes were associated with elevated At4g15400 expression revealed by semi-quantitative RT-PCR (Fig. 2E). RT-PCR also revealed that At4g15400 was expressed in shoot tissues (Fig. 2E). These data indicated that overexpression of At4g15400 was the cause of the *abs1-1D* dwarf phenotype and that *ABS1* is At4g15400.
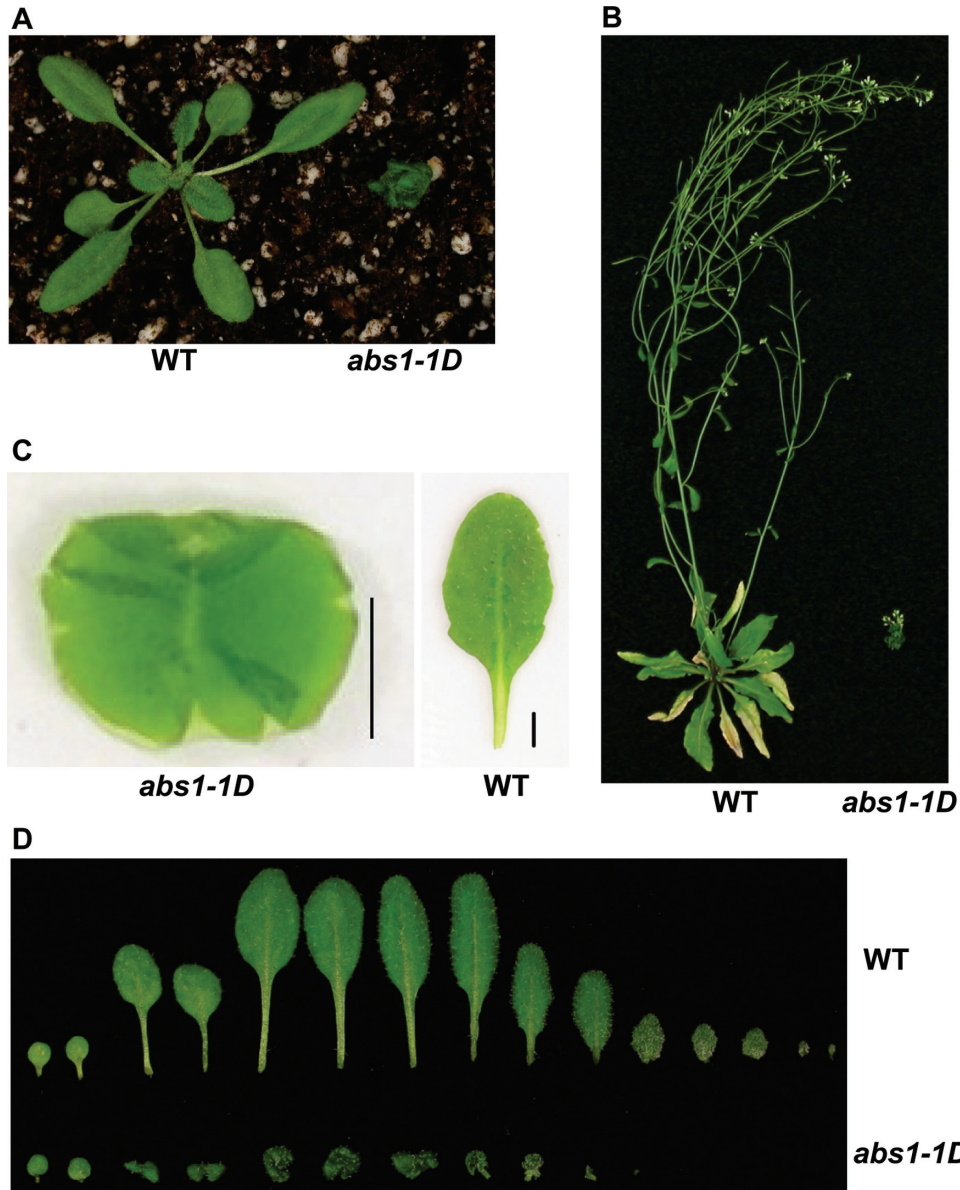
Fig. 1. Phenotype of the *Arabidopsis abs1-1D* mutant. (A, B) Phenotypes of representative 3-week-old (A) and 7-week-old (B) wild-type (WT) and *abs1-1D* plants. (C) An individual rosette leaf of *abs1-1D* and wild-type plants. Leaves were flattened between glass slides before photographing; overlapping leaf areas are darker green. Bar, 0.25 cm. (D) Comparison of rosette leaves from 3-week-old wild-type and *abs1-1D* plants. Leaves were arranged from left to right in the order of their initiation.

To investigate the impact caused by a loss-of-function mutation of *ABS1*, we sought a T-DNA insertional line of *ABS1* from The Arabidopsis Biological Resource Center and obtained one such mutant, WiscDsLox474E11 (Supplementary Fig. S2 at *JXB* online). The T-DNA insertion site in WiscDsLox474E11 is near the 3' region of the *ABS1* coding sequence, 49 bp upstream of the stop codon (Supplementary Fig. S2A). Full-length *ABS1* transcripts were not detectable in plants that were homozygous for the T-DNA insertion (Supplementary Fig. S2B, C). WiscDsLox474E11 homozygous mutants were indistinguishable from the wild type when grown under our conditions (Supplementary Fig. S2D). However, dark-grown WiscDsLox474E11 seedlings showed modest yet significant enhanced elongation of the hypocotyl compared with that of the

wild type (Supplementary Fig. S2E, F). WiscDsLox474E11 was recently identified as *brassinosteroid inactivator1-1* (*bia1-1*) in another study (Roh *et al.*, 2012), and our findings are in line with their results.

ABS1 *encodes a putative BAHD family of acyltransferases*

BlastP analysis using ABS1/At4g15400 amino acid sequences revealed that it is a putative member of the *Arabidopsis* BAHD family of acyltransferases (D'Auria, 2006; Yu *et al.*, 2009). BAHD was named based on the first letters of four canonical enzymes in this family: benzylalcohol *O*-acetyltransferase, *B*EAT; anthocyanin *O*-hydroxycinnamoyltransferase, *A*HCT;
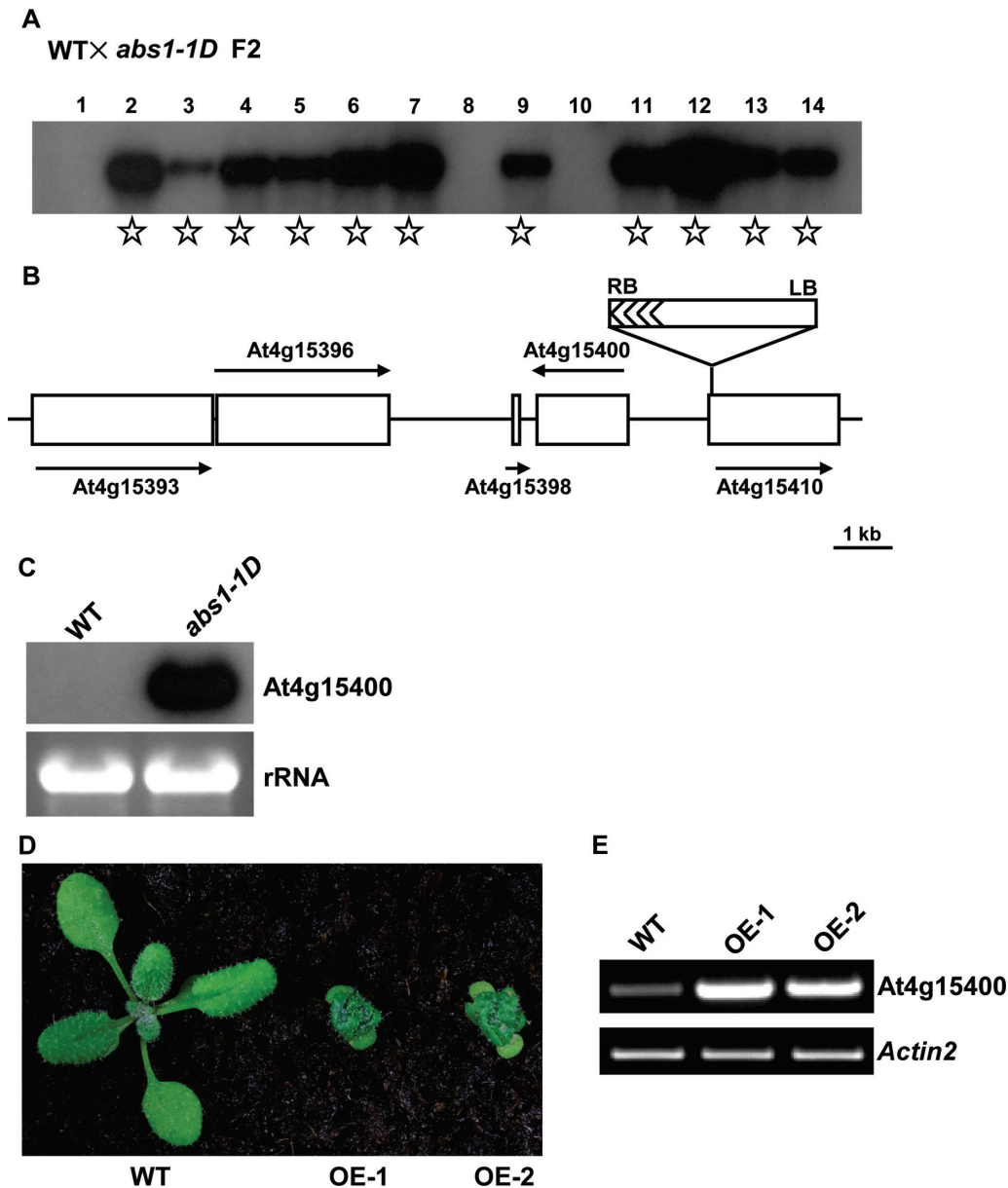
Fig. 2. Cloning of the *ABS1* gene. (A) Co-segregation analysis of *abs1-1D*. Fourteen F2 plants derived from a cross between wild type and *abs1-1D* were randomly selected to score phenotype. Genomic DNAs from these plants were digested with *Bam*HI. A Southern blot was probed with a ^{32}P-labelled *BAR* gene sequence (Yu *et al.*, 2008). Samples from plants showing the *abs1-1D* phenotype are indicated with stars. (B) Schematic representation of the T-DNA insertion site in *abs1-1D*. The positions of genes in the vicinity of the T-DNA are shown as open boxes. Arrows represent the orientation of the open reading frames of these genes. (C) Accumulation of At4g15400 transcripts in wild-type and *abs1-1D* plants. Equal amounts of total RNA (5 μg) extracted from 2-week-old seedlings were separated on a formaldehyde gel and transferred to a nylon membrane. The RNA blot was hybridized with ^{32}P-labelled full-length At4g15400 cDNA. The ethidium bromide-stained RNA gel shown below served as a loading control. (D) Phenotypes of representative 2-week-old wild-type and two independent transgenic lines overexpressing At4g15400 (OE-1, and OE-2). (E) Semi-quantitative RT-PCR analysis of At4g15400 transcript accumulation in the plants shown in (D). All primers and RT-PCR conditions used in this study are listed in Supplementary Table S1. *Actin2* expression was used as a control.

anthranilate *N*-hydroxycinnamoyl/benzoyltransferase, *H*CBT; and deacetylvindoline 4-*O*-acetyltransferase, *D*AT (D'Auria, 2006; Yu *et al.*, 2009). In *Arabidopsis*, there are ~60 putative BAHD acyltransferases that are grouped phylogenetically into five distinctive clades (D'Auria, 2006; Yu *et al.*, 2009). ABS1/At4g15400 was assigned to clade IV, which was previously designated clade III in a different naming system (D'Auria, 2006;

Yu *et al.*, 2009). The closest homologues of ABS1/At4g15400 in *Arabidopsis* are At5g47980 and At5g23970, which share 51.7 and 42.1% amino acid identity with ABS1, respectively (Supplementary Fig. S3 at *JXB* online).

BAHD family acyltransferases share two major features. First is the HXXXD motif, where the histidine residue is probably the catalytic centre (D'Auria, 2006; Yu *et al.*, 2009). In

ABS1/At4g15400, the HXXXD motif corresponds to HKICD and the universally conserved histidine residue is His[151] in ABS1/At4g15400 (Supplementary Fig. S3). At the second conserved motif of BAHD, ABS1 has a DFGSG arrangement (Supplementary Fig. S3), rather than the more prevalent DFGWG, and this seems to agree with data suggesting that the DFGWG domain can tolerate a certain degree of variability in BAHD family acyltransferases (Yu *et al.*, 2009).

We next examined the tissue expression pattern of *ABS1*/At4g15400 and its two closest homologues: At5g47980 and At5g23970 (Yu *et al.*, 2009). Semi-quantitative RT-PCR showed distinct expression patterns for these three genes. *ABS1*/At4g15400 was predominantly expressed in *Arabidopsis* root tissues, with relatively low expressions also detected in seedling, rosette leaves, silique, and flower tissues (Fig. 3A). For At5g47980, we detected major expression in the root tissue but not in other tissues examined (Fig. 3A). Overexpression of At5g47980 also led to transgenic plants with reduced sizes; however, the phenotypes of transgenic lines we recovered were not as dramatic as *ABS1*/At4g15400 overexpression lines (Supplementary Fig. S4). Interestingly, At5g23970 showed an expression pattern associated with floral tissues, with expression in cauline leaves, silique, and the highest level in the flower (Fig. 3A).

We next examined the tissue expression profile of *ABS1* through promoter–GUS reporter analysis. Transgenic lines harbouring GUS driven transcriptionally by a ~1.1 kb *ABS1* endogenous promoter showed strong expression in root tissues (Fig. 3B). In leaves, *ABS1* expression was distributed as distinct foci at the edge of cotyledons and rosette leaves, reminiscent of the distribution of hydathodes along the leaf edge (Fig. 3B; Figueroa-Balderas *et al.*, 2006). *ABS1* expression was also observed in developing seeds in the silique (Fig. 3B). Promoter–GUS tissue expression results were in line with the RT-PCR results (Fig. 3A, 3B).

Arabidopsis BAHD family members have been predicted to be targeted into various subcellular compartments including the cytosol, nucleus, chloroplast, and mitochondria (Yu *et al.*, 2009). To test the subcellular localization of ABS1, we carried out transient expression analysis of ABS1–GFP fusion protein in *Arabidopsis* leaf protoplasts. A control vector containing only the GFP coding sequence under the control of the CaMV 35S promoter (*P35S::GFP*) expressed GFP in the cytosol and nucleus, consistent with previous findings (Fig. 3C; Vert *et al.*, 2002). Transient expression of *P35S::ABS1–GFP* showed green fluorescence distribution mostly in the cytosol, suggesting that ABS1 is probably a cytosolic protein (Fig. 3C).

The abs1-1D *mutant shows a typical BR-deficient phenotype*

The dwarf phenotype of *abs1-1D* closely resembles the canonical BR-deficient or -signalling mutants, such as *det2* (Li *et al.*, 1996). BR-deficient or -signalling mutants have a number of stereotypical phenotypes including dwarfism, a darker green leaf colour, and de-etiolation in the dark (Fujioka and Yokota, 2003). During our routine growing of *abs1-1D*, we observed casually that *abs1-1D* had a darker green appearance, and quantitative analysis of

the chlorophyll concentration confirmed that *abs1-1D* had a significantly higher accumulation of chlorophyll when compared with wild-type plants (Fig. 4A). Chlorophyll accumulation in *ABS1* overexpressing plants was also higher than that of wild-type plants (Supplementary Fig. S5A). We next examined the dark-growth features of *abs1-1D*. After 1 week of dark growth, *abs1-1D* seedlings had significantly shortened hypocotyls when compared with those of wild-type plants (Fig. 4B, 4E). We also examined the hypocotyl lengths of seedlings grown in the light (Fig. 4C, 4D). Light-grown *abs1-1D* plants displayed shortened hypocotyls (Fig. 4C, 4D). A shorter hypocotyl phenotype was also observed in *ABS1* overexpressing plants (Supplementary Fig. S5B, C). Abnormal hypocotyl elongation of *abs1-1D* in both the light and dark prompted us to examine the state of cell elongation in *abs1-1D*. *det2-1*, a known BR deficient mutant defective in a steroid 5-α-reductase, was included as a control (Li *et al.*, 1996). For both *abs1-1D* and *det2-1*, the longitudinal sections of the leaf petioles showed clear abnormal elongation along the leaf axis, with the lengths of cells greatly reduced when compared with those of wild-type plants (Fig. 4F). However, cross-sections of *abs1-1D* and *det2-1* showed that the sizes of cells along the axis perpendicular to the leaf petiole were comparable in the two mutants when compared with those of the wild type (Fig. 4F). Taken together, these data indicated that the phenotypes of the *abs1-1D* mutant, including dwarfism, a darker green leaf colour, de-etiolation, and cell elongation defects, mimics BR-deficient or -signalling mutants.

Exogenous BR rescues abs1-1D

This overall resemblance of *abs1-1D* to BR-deficient or -signalling mutants prompted us to test whether exogenously applied BR could rescue the *abs1-1D* phenotypes, a common procedure for distinguishing BR-deficient from BR-signalling mutants (Clouse, 1996). We tested the responses of *abs1-1D* and *det2-1* to the applications of various concentrations of synthetic BR, epiBL. Fig. 5A shows that, when transferred to control medium, both *abs1-1D* and *det2-1* maintained the characteristic dwarf phenotype. However, when seedlings were transferred to 0.2 μM epiBL medium, both *abs1-1D* and *det2* showed elongation of leaf petioles, a clear sign of the rescue of the dwarf phenotype (Fig. 5A). The rescue effect could also be observed with 0.5 μM epiBL treatment (Fig. 5A).

We next tested the responses of other aspects of *abs1-1D* phenotypes to epiBL. The hypocotyl lengths of light-grown *abs1-1D* and *det2-1* seedlings were significantly shorter than those of the wild type (Fig. 5B; statistical analysis in Supplementary Fig. S6A). However, when germinated on MS medium containing various concentrations of epiBL, both *abs1-1D* and *det2-1* showed enhanced growth of hypocotyls, and at 0.2 μM epiBL, the hypocotyl lengths of *abs1-1D* and *det2-1* were comparable with those of the wild type, which were also enhanced to a lesser degree (Fig. 5B; statistical analysis in Supplementary Fig. S6A). The de-etiolation phenotypes of *abs1-1D* and *det2-1* in the dark were also rescued by exogenously applied epiBL (Fig. 5C; statistical analysis in Supplementary Fig. S6B). Overall, these data indicated that exogenously applied BR can rescue the dwarf and hypocotyl defects of *abs1-1D*, as well as those of *det2-1*, suggesting that *abs1-1D* phenotypes may be associated with BR deficiency.
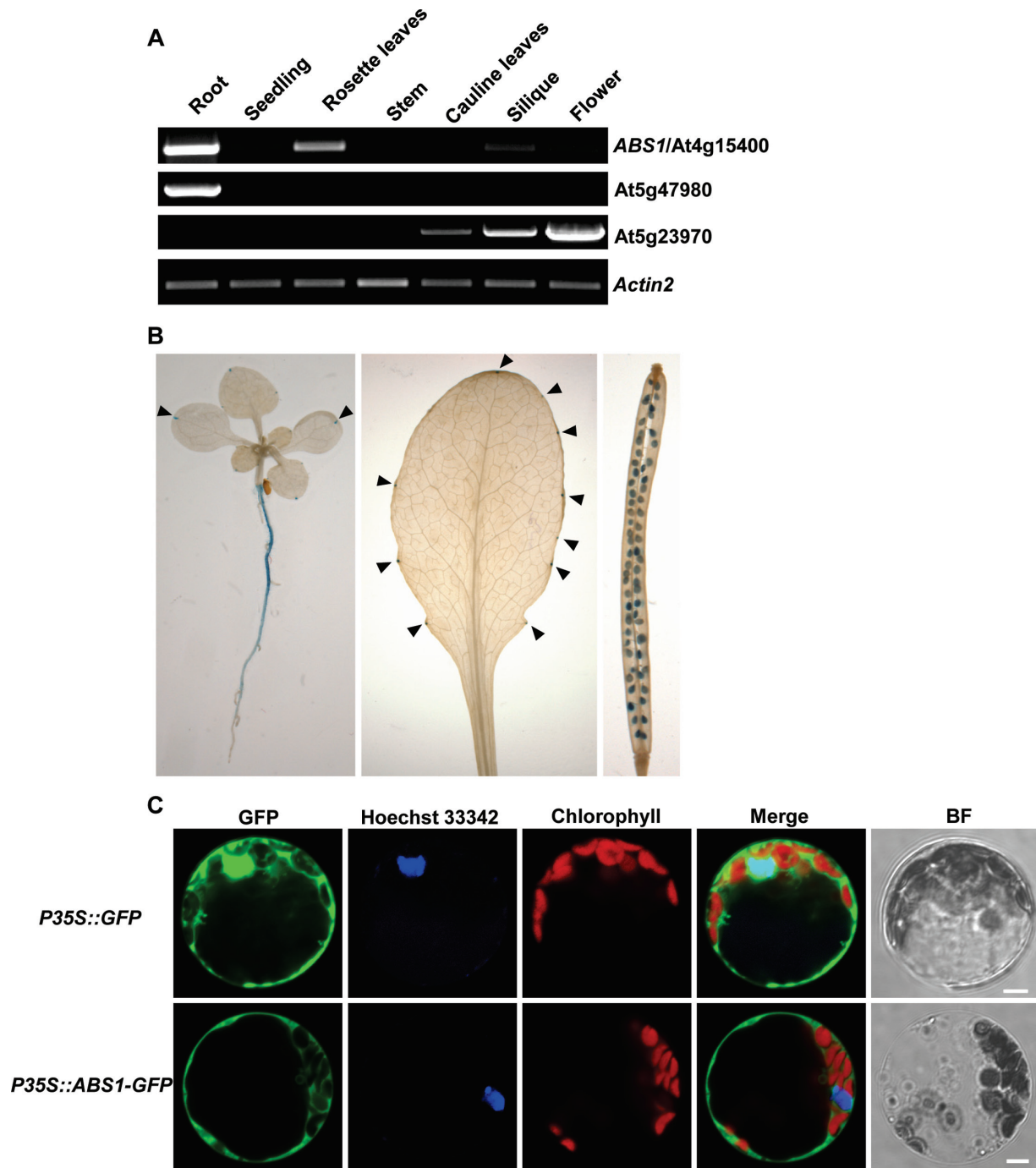
Fig. 3. Expression analysis of ABS1. (A) Semi-quantitative RT-PCR analysis of the expression of *ABS1* and its two close homologues in the indicated tissues from wild-type plants. (B) Histochemical GUS staining of wild-type plants expressing the *ABS1* promoter–GUS fusion construct. Shown are a 10-day-old seedling, fully expanded rosette leaf and silique tissues. Arrowheads indicate GUS staining in the cotyledons and rosette leaf. (C) Cellular localization of the ABS1 protein. Protoplasts prepared from 4-week-old wild-type *Arabidopsis* leaves were transformed with *P35S::GFP* or *P35S::ABS1-GFP*. Hoechst 33342 was used to stain the nucleus. The GFP, Hoechst 33342, and chlorophyll autofluorescence signals were monitored by confocal microscopy. Bright field (BF) images served as controls for protoplast integrity. Representative images of a single protoplast are shown. Bar, 5 μm).

One hallmark feature of BR-deficient mutants is the feedback upregulation of BR biosynthesis genes (Choe *et al.*, 2001; Tanaka *et al.*, 2005; Song *et al.*, 2009). We thus tested the expression of four classic BR biosynthesis genes (*DWF4*, *CPD*, *BR6ox1*, and *DET2*) in *abs1-1D*, two *ABS1* overexpressing transgenic lines (OE-1 and OE-3), and *det2-1* with semi-quantitative RT-PCR (Fig. 5D). Three of the four genes examined (*DWF4*, *CPD*, and *BR6ox1*) showed clearly elevated expression in *abs1-1D*, OE-1, OE-3, and *det2-1* plants when compared with the wild type (Fig. 5D), while expression of *DET2* appeared to be only
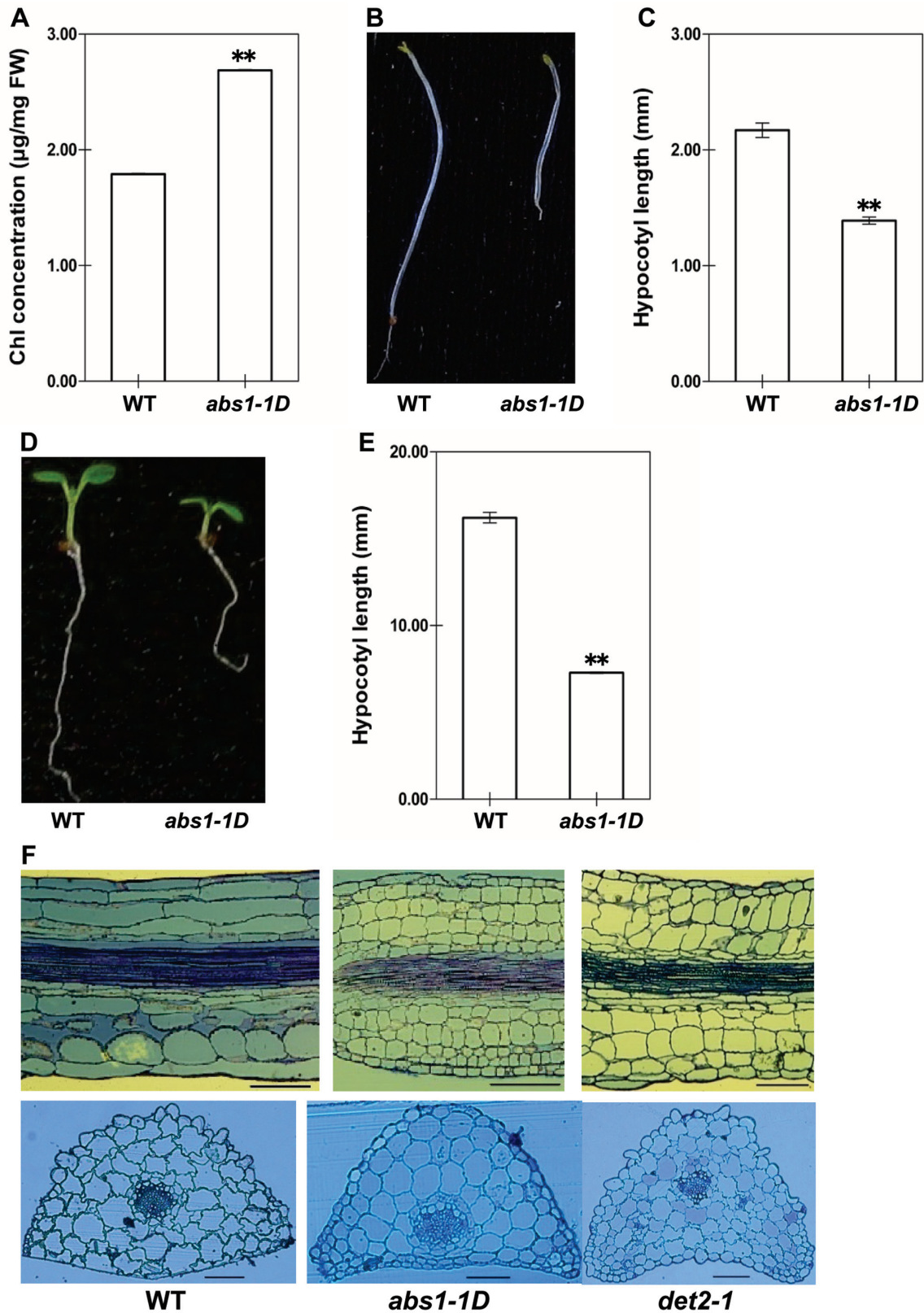
Fig. 4. BR-deficient related morphological phenotypes in *abs1-1D*. (A) Chlorophyll content of 2-week-old wild-type and *abs1-1D* plants. Measurements were calibrated on a fresh weight (FW) basis. Error bars represent standard error of three independent pooled whole seedling samples (**, *P* <0.01). (B, D) Comparison of hypocotyl length of 1-week-old wild-type and *abs1-1D* plants grown in the dark (B) and in the light (D). (C, E) Statistical analysis of hypocotyl length measurements of plants grown on the same plates as shown in (D) and (B), respectively. Each bar represents the mean hypocotyl length of 12 plants. Error bars represent ±standard error (**, *P*<0.01). (F) Longitudinal sections (upper panels) and cross-sections (lower panels) of petioles of the first two true leaves from wild-type, *abs1-1D* and *det2-1* plants. Bars, 50 μm.
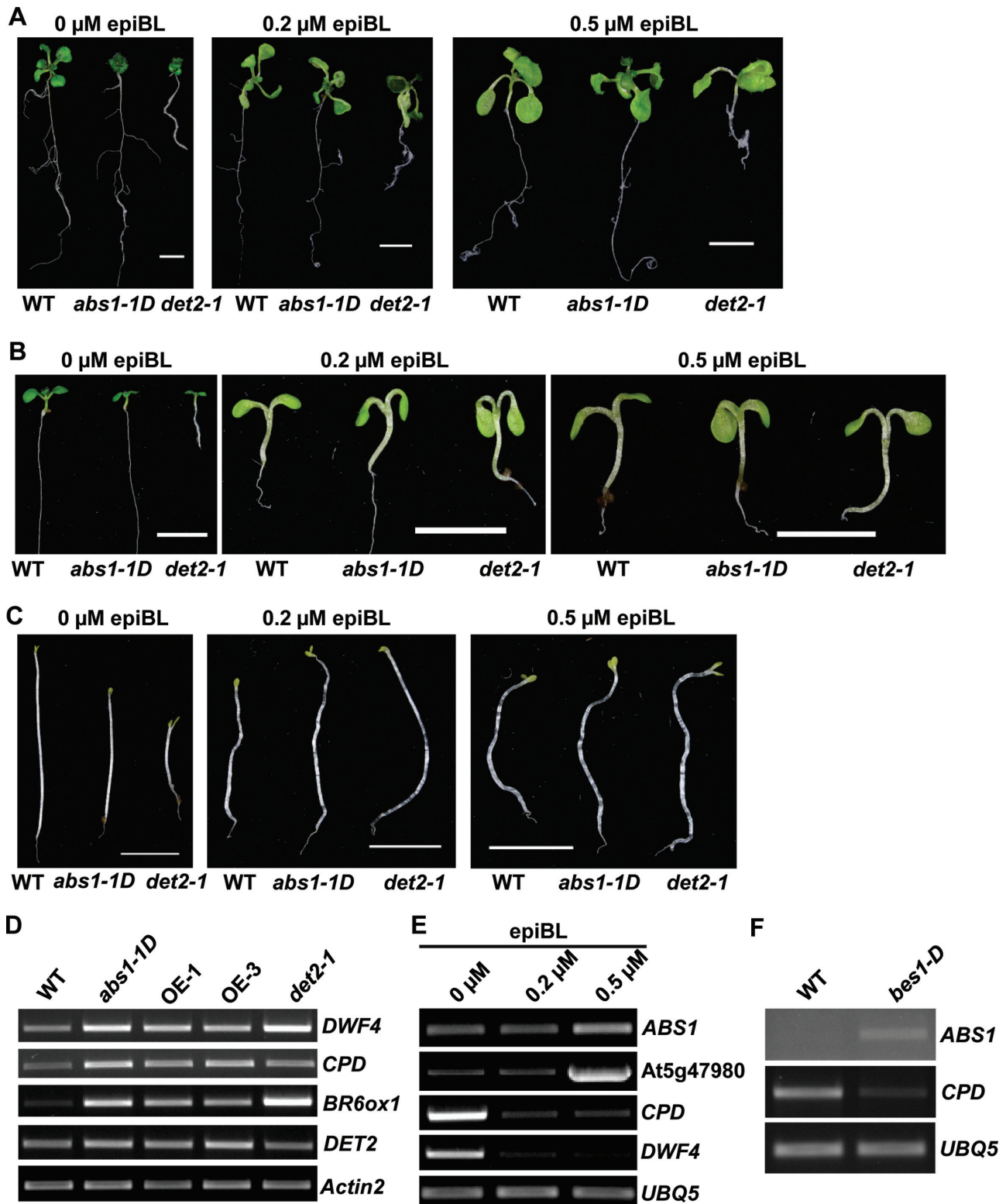
Fig. 5. BR-deficient-related molecular characteristics in *abs1-1D*. (A) Representative phenotypes of wild-type, *abs1-1D*, and *det2-1* plants after transplanted to epiBL-supplemented plates. Plants were germinated on 0.5× MS plates and grown for 6 days prior to transplanting to plates supplemented with 0, 0.2, or 0.5 μM epiBL. Photographs were taken 6 days after transplantation. Bars, 0.5 cm. (B, C) Hypocotyl elongation of 1-week-old wild-type, *abs1-1D*, and *det2-1* plants germinated on 0.5× MS plates containing 0, 0.2, or 0.5 μM epiBL in the light (B) and in the dark (C). Bars, 0.5 cm. (D) Semi-quantitative RT-PCR analysis of expression of the indicated BR biosynthesis genes in wild-type, *abs1-1D*, two *ABS1* overexpression lines (OE-1 and OE-3) and *det2-1* plants. (E) Semi-quantitative RT-PCR analysis of expression of the indicated genes in response to exogenously applied epiBL in wild-type plants. (F) Semi-quantitative RT-PCR analysis of expression of the indicated genes in wild-type and *bes1-D* plants. *Actin2* or *UBQ5* expression was used as a control.

slightly higher in the mutants (Fig. 5D). These data indicated that transcripts of some BR biosynthesis genes accumulate at higher levels in *abs1-1D*.

We next looked at the expression of *ABS1* and its homologue At5g47980 in response to exogenously applied epiBL. When treated with epiBL, expression of the BR biosynthesis genes *CPD* and *DWF4* was repressed as a result of the negative-feedback regulation (Fig. 5E). On the other hand, we observed that, when treated with 0.5 μM epiBL, both *ABS1* and At5g47980 were induced, while the expression of both genes at 0.2 μM epiBL treatment was not significantly altered (Fig. 5E). Furthermore, the expression of *ABS1* in the *bes1-D* mutant, in which BR signalling is constitutively active, was also elevated, while the BR biosynthesis gene *CPD* was repressed (Fig. 5F; Yin *et al.*, 2002). The induction of *ABS1* in *bes1-D* is in agreement with a recent report of upregulated genes in a *bes1-D* background through microarray analysis (Yu *et al.*, 2011). Our results indicated that *ABS1* is induced by activated BR signalling, either by applying BR or by activating BR-signalling components.

Genetic interactions between abs1-1D, det2-1, bes1-D, *and* phyB-1

If *abs1-1D* is defective in BR accumulation, we should be able to test its genetic interactions with known mutants that are related to BR biosynthesis or signalling. Using this concept, we surveyed the genetic interactions between *abs1-1D* and mutants including *det2-1*, *bes1-D*, and *phyB-1*.

We first tested the genetic interaction between *abs1-1D* and *det2-1*. Under our growth conditions, *det2-1* displayed a weaker dwarf phenotype when compared with *abs1-1D* (Fig. 6A; Li *et al.*, 1996). We obtained *abs1-1D det2-1* double mutants (Supplementary Fig. S6C), which showed extreme dwarfism and were sterile (Fig. 6A). Genetic interaction between *abs1-1D* and *det2-1* could also be observed with dark-grown seedlings (Fig. 6B). Compared with the de-etiolation phenotypes of either parent, *abs1-1D* or *det2-1*, the double mutant showed a more dramatic de-etiolation phenotype (Fig. 6B). The synergistic interaction between *abs1-1D* and *det2-1* suggested that *ABS1* and *DET2* may be functionally related.

The semi-dominant BR constitutive response mutant *bes1-D* was originally isolated in a genetic screen for suppressors of *bri1-119*, which is a weak mutant allele of the BR receptor gene *BRI1* (Li and Chory, 1997; Yin *et al.*, 2002). *BES1* encodes a transcription factor that is a positive BR-signalling component downstream of BRI1 (Yin *et al.*, 2002). *bes1-D* can suppress many features of *bri1-119*, including the dwarfism and de-etiolation phenotypes (Yin *et al.*, 2002). When compared with a heterozygous *abs1-1D/+* plant, the double heterozygote *bes1-D/+ abs1-1D/+* plant was much enlarged in size, suggesting that *bes1-D* is a robust *abs1-1D* genetic suppressor (Fig. 6C).

Compromised BR biosynthesis can also rescue the elongated hypocotyl phenotype of *phyB-1* mutant, as demonstrated by the *phyB-1 det2-1* double mutant (Chory, 1992). To test the effect of *ABS1* overexpression on *phyB-1* (Landsberg *erecta* ecotype background), we generated transgenic lines overexpressing *ABS1* in a *phyB-1* mutant background. Multiple transgenic plants showed phenotypes that resembled those of *abs1-1D* (Fig. 6D).

The hypocotyls of transgenic plants were significantly shortened when compared with *phyB-1*, suggesting that the overproduction of *ABS1* can suppress *phyB-1*, reminiscent of what has been observed with the interaction between *det2* and *phyB-1* and of *phyB-1* genetic suppressors such as *bas1-D* (Chory, 1992; Neff *et al.*, 1999).

Global gene expression profile of abs1-1D

To examine the global impact of the overexpression of *ABS1* on plant development, we obtained Affymetrix ATH1 gene chip data of both wild-type and *abs1-1D* mutant plants. Despite the dramatic developmental phenotypes of *abs1-1D*, we only detected a relatively small impact on the transcriptome, with 383 genes upregulated and 238 genes downregulated, using a two-fold cut-off level (Fig. 7A; Supplementary Table S2). The gene ontology of the genes with up- or downregulated expression appeared to cover many different cellular processes, as with gene chip data from other mutants with developmental phenotypes (Fig. 7B). The expression levels of the major up- and downregulated genes in the microarray were examined using semi-quantitative RT-PCR (Fig. 7C). Similar expression pattern changes of these genes were observed in *abs1-1D* plants and two *ABS1* overexpression lines compared with wild-type plants, confirming the reliability of the microarray data (Fig. 7C).

Upon closer examination of the microarray data, two major features of the *abs1-1D* transcriptome stood out. Firstly, many genes that are involved in the BR biosynthesis pathway were upregulated in *abs1-1D*, including *DWF4*, *CPD*, and *BR6ox1*, and this is consistent with our earlier data showing that several BR biosynthesis genes were upregulated in *abs1-1D* (Figs 5D, 7B; Supplementary Table S2). Secondly, many genes related to the flavonoid biosynthesis pathway were downregulated in *abs1-1D* (Fig. 7B; Supplementary Table S2). Semi-quantitative RT-PCR analysis verified the downregulation of a representative set of such genes, including many *TRANSPARENT TESTA* (*TT*) genes (Fig. 7D). A similar expression level change in all genes tested was also observed in *det2-1* plants, suggesting that the phenotypic similarity of *abs1-1D* and *det2-1* is associated with some similar alterations in their transcriptomes (Fig. 7C, 7D).

Discussion

In this work, we have reported the isolation of a semi-dominant *Arabidopsis* mutant, *abs1-1D*, which displayed a dramatic dwarf stature when grown in the light. We subsequently established that overexpression of a BAHD family of acyltransferases, ABS1/At4g15400, was the cause of the dwarf phenotype. The BAHD family of acyltransferases typically catalyses the acylation of a diverse array of substrates, with acyl-CoA thioesters serving as the acyl group donor and the majority of substrates being plant secondary metabolites including flavonoid pathway intermediates (D'Auria, 2006). Currently, we do not know the exact substrate or the nature of the acyl group that ABS1 might be able to exert enzymatic activity towards. However, amino acid sequence alignment with known BAHD family members shows the presence of conserved motifs of BAHD acyltransferases in ABS1, particularly the universally conserved histidine residue that has
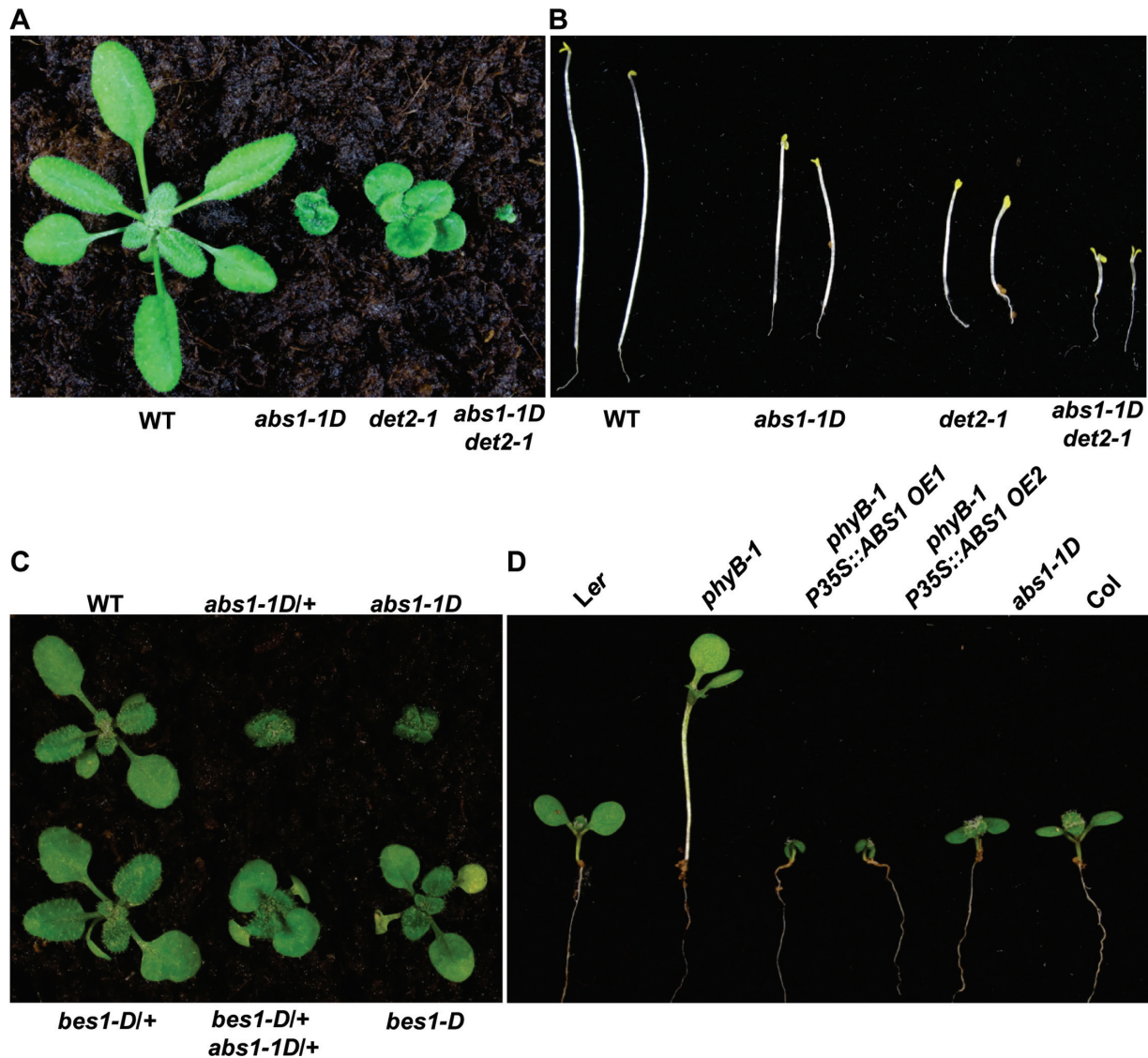
Fig. 6. Genetic interactions between *abs1-1D* and various mutants with altered photomorphogenic growth. (A) Phenotypes of representative 3-week-old wild-type, *abs1-1D*, *det2-1* and *abs1-1D det2-1* double mutant plants. (B) Comparison of hypocotyl elongation of 1-week-old wild-type, *abs1-1D*, *det2-1*, and *abs1-1D det2-1* double mutant plants grown in the dark. (C) Phenotypes of representative 2-week-old wild-type, *abs1-1D*/+ heterozygote, *abs1-1D* homozygote, *bes1-D*/+ heterozygote, *bes1-D*/+ *abs1-1D*/+ double heterozygote and *bes1-D* homozygote plants. (D) Comparison of hypocotyl elongation of 1-week-old *Arabidopsis* Landsberg *erecta* ecotype (L*er*), *phyB-1* mutant, two independent transgenic lines overexpressing *ABS1* in a *phyB-1* mutant background, and *Arabidopsis* Columbia ecotype (Col) grown in the light.

been shown to be the catalytic centre (Ma *et al.*, 2005). With our genetic data, it is obvious that ABS1 is capable of regulating plant growth and development. To our knowledge, this is the first case where the overexpression of a BAHD family acyltransferase was shown to induce such dramatic dwarf phenotypes in the light.

Although defects in many regulatory pathways can lead to dwarf mutant phenotypes, three lines of evidences suggest that *abs1-1D* is intimately associated with BR-deficient phenotypes. Firstly, *abs1-1D* mutants demonstrate many phenotypes of BR-deficient or -signalling mutants such as *det2* and *bri1*. These features include extreme dwarfism when grown in the light, de-etiolation in the dark, higher accumulation of chlorophyll

in the leaves, and aberrant cell elongation in leaf petioles. One distinction between hormone-deficient and -signalling mutants is whether exogenously applied plant hormone can rescue the mutant phenotype. We found that exogenously applied BR could rescue the *abs1-1D* mutant phenotypes, and this certainly indicates that the BR-signalling pathway is normal in *abs1-1D*. Although we did not measure the endogenous level of BR, our data suggested that the dwarf phenotype caused by the overexpression of *ABS1* is probably associated with the lack of active endogenous BR.

Secondly, genetic evidence further strengthens the intimate relationship between ABS1 and plant growth, and developmental programmes regulated by BR homeostasis and signalling.
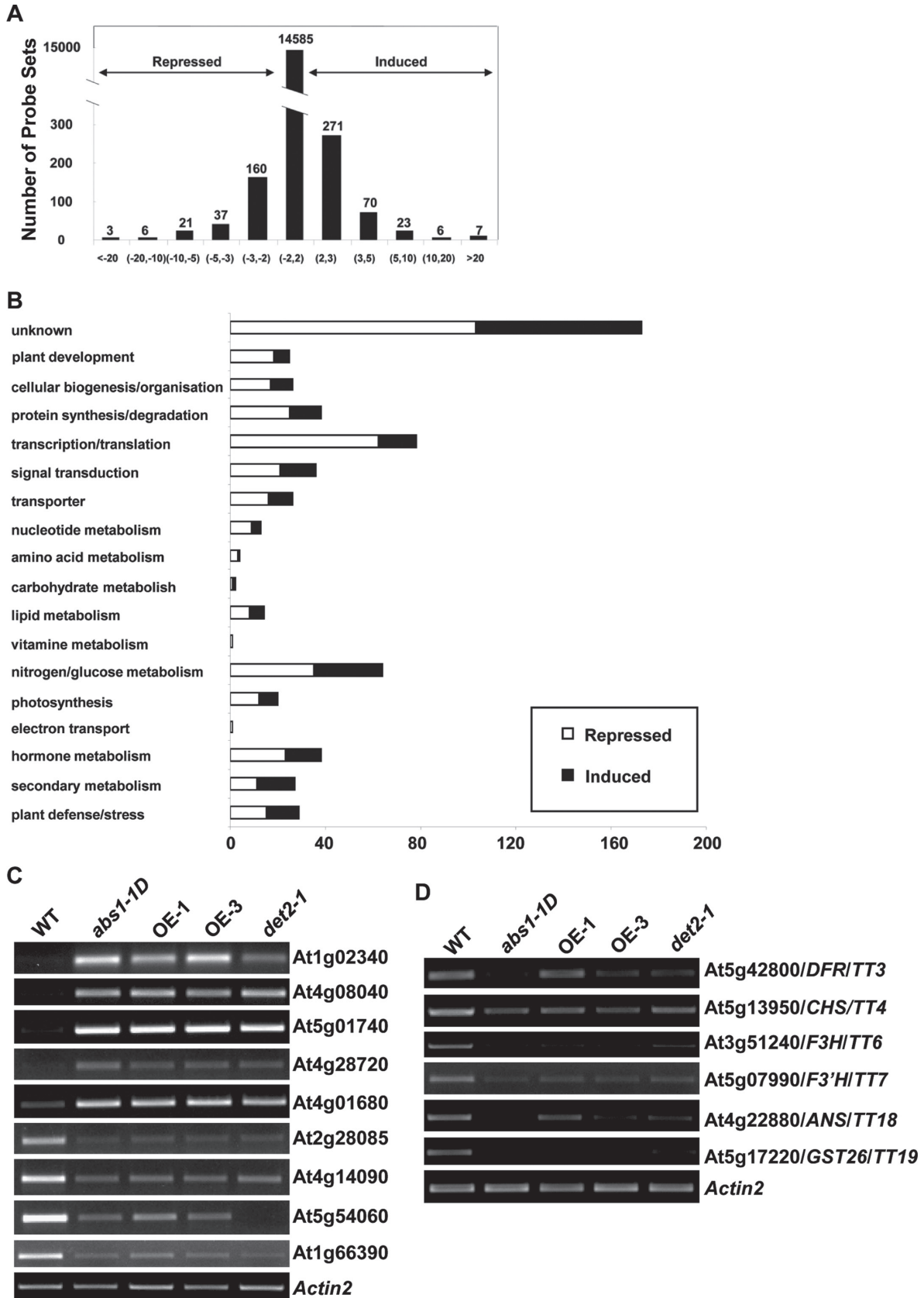
Fig. 7. Global transcriptome profiling of *abs1-1D*. (A) Distribution of genes with altered expression in *abs1-1D* compared with the wild type. (B) Functional classification of genes that are significantly repressed or induced (by at least twofold) in *abs1-1D* versus wild-type plants. (C) Semi-quantitative RT-PCR analysis of expression of the major up- and downregulated genes shown in microarray data in wild type, *abs1-1D*, two *ABS1* overexpression lines (OE-1 and OE-3), and *det2-1*. (D) Semi-quantitative RT-PCR analysis of the expression of indicated flavonoid biosynthesis genes from the same samples as in (C). *Actin2* expression was used as a control.

Double mutants of *abs1-1D* and *det2-1* are extreme dwarf and sterile, suggesting that ABS1 and DET2 may contribute to the same genetic pathway. In addition, when the *abs1-1D* allele was introduced into a *bes1-D* mutant background, in which the BR-signalling pathway is constitutively active, the dwarf phenotype conferred by the *abs1-1D* allele was effectively suppressed. This suggested that the constitutively active BR-signalling pathway in *bes1-D* clearly suppresses the *abs1-1D* dwarf phenotype. Moreover, overexpressing *ABS1* in a *phyB-1* mutant background reversed the long-hypocotyl phenotype of the *phyB-1* mutant. In essence, *abs1-1D* can be seen as a dominant genetic suppressor of the *phyB-1* mutant, similar to what has been observed in other *phyB-1* suppressor genes, *BAS1* and other *SUPPRESSOR OF PHYB-4* (*SOB*) genes (Neff *et al.*, 1999; Turk *et al.*, 2005). Interestingly, *BAS1* encodes a cytochrome P450 that inactivates BRs, representing one of the few examples of BR-inactivating mechanisms (Neff *et al.*, 1999; Poppenberger *et al.*, 2005).

Lastly, we also established through semi-quantitative RT-PCR and gene chip analysis that key BR biosynthesis genes are upregulated in *abs1-1D*, reminiscent of the feed-back upregulation of these genes in BR-deficient mutants such as *det2-1* (Choe *et al.*, 2001; Tanaka *et al.*, 2005; Song *et al.*, 2009). Both *ABS1*/At4g15400 and its close homologue At5g47980 were also induced by exogenous BR treatment, providing further evidence for the linkage between BAHD acyltransferases and BR actions. There are at least two possibilities. One is that both genes are downstream targets that are involved in the response of the plant cell to BR. It is known that some BAHD family members may be involved in cell-wall biosynthesis, and many BR-responsive genes are involved in cellular processes such as cell-wall biosynthesis (Goda *et al.*, 2002; Müssig *et al.*, 2002; Hoffmann *et al.*, 2004; Besseau *et al.*, 2007; Gou *et al.*, 2009). Another scenario is that the induction of these genes may represent a mechanism for BR inactivation, much like the effect of GA treatment on the expression of GA-inactivating oxidases (Thomas *et al.*, 1999). The dominant nature of *abs1-1D* and its phenotype do show that it is probably a negative regulator of plant growth and development, lending more support to the latter hypothesis. However, in either case, these genes are linked with BR regulation. We found that *ABS1* expression was elevated in the *bes1-D* mutant, consistent with what was observed previously with *bes1-D* genome-wide transcriptome analysis (Yu *et al.*, 2011). This seems to suggest that *BES1* positively regulates *ABS1*, directly or indirectly. However, two recent reports profiling direct target genes for BZR1 or BES1 did not identify *ABS1* as a direct target of either BZR1 or BES1 (Sun *et al.*, 2010; Yu *et al.*, 2011). This seems to suggest that there may be additional regulatory components between *BZR1* and/or *BES1* and *ABS1*.

While this work was under review, At4g15400 was identified as *BIA1* (*BRASSINOSTEROID INACTIVATOR1*) in an independent study and was shown to be involved in BR homeostasis (Roh *et al.*, 2012). Results from this new report, particularly the reduced levels of active BRs in At4g15400 overexpression plants, and our genetic data, certainly argue a role for At4g15400/*BIA1*/*ABS1* in regulating BR homeostasis.

Genome-wide transcriptome profiling of *abs1-1D* revealed clear reprogramming of many metabolic and regulatory genes in *abs1-1D*. The most prominent changes were upregulation of the BR biosynthesis pathway and downregulation of the flavonoid biosynthesis pathway. Intriguingly, the downregulation of flavonoid biosynthesis genes could also be observed in the BR-deficient mutant *det2-1*, establishing a link between flavonoid biosynthesis and BR actions. Although members of the BAHD family of acyltransferases have been shown previously to be involved in the flavonoid biosynthesis pathway, there are few reports regarding the link between BR homeostasis and the flavonoid pathway. In a genetic screen for dominant *bri1* enhancers, a mutant *ben1-1D* was isolated (Yuan *et al.*, 2007). Interestingly, *BEN1* encodes a dihydroflavonol 4-reductase-like protein, an enzyme presumably involved in the flavonoid pathway (Yuan *et al.*, 2007). Although the precise mechanism is still unclear regarding how BEN1 is involved in BR homeostasis or how the overexpression of ABS1 is associated with the flavonoid pathway, there are certainly increasing data suggesting the existence of a link between BR and the flavonoid pathway.

Supplementary data

Supplementary Fig. S1. Phenotypes of 3-week-old wild-type, *abs1-1D* homozygote, and *abs1-1D*/+ heterozygote plants.

Supplementary Fig. S2. Identification of a loss-of-function allele of *ABS1*.

Supplementary Fig. S3. Multiple alignments of amino acid sequences of *ABS1*, At5g47980, At5g23970, and four canonical BAHD enzymes.

Supplementary Fig. S4. Phenotypes of At5g47980 overexpression lines.

Supplementary Fig. S5. Phenotypic analysis of *ABS1* overexpression lines.

Supplementary Fig. S6. Hypocotyl elongation measurements of wild-type, *abs1-1D*, and *det2-1* following epiBL treatment in the light and dark, and genotyping of the *abs1-1D det2-1* double mutant.

Supplementary Table S1. Primers and PCR conditions used in this study.

Supplementary Table S2. List of genes that showed significant expression changes (at least twofold) in *abs1-1D* compared with wild-type plants.

Acknowledgements

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