ASYMMETRIC-LEAVES2 and an ortholog of eukaryotic NudC domain proteins repress expression of *AUXIN-RESPONSE-FACTOR* and class 1 *KNOX* homeobox genes for development of flat symmetric leaves in *Arabidopsis*

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Summary

Leaf primordia form around the shoot apical meristem, which consists of indeterminate stem cells. Upon initiation of leaf development, adaxial-abaxial patterning is crucial for appropriate lateral expansion, via cellular proliferation, and the formation of flat symmetric leaves. Many genes that specify such patterning have been identified, but regulation by upstream factors of the expression of relevant effector genes remains poorly understood. In *Arabidopsis thaliana,* *ASYMMETRIC LEAVES2* (*AS2*) and *AS1* play important roles in repressing transcription of class 1 *KNOTTED1*-like homeobox (*KNOX*) genes and leaf abaxial-determinant effector genes. We report here a mutation, designated *enhancer of asymmetric leaves2 and asymmetric leaves1* (*eal*), that is associated with efficient generation of abaxialized filamentous leaves on the *as2* or *as1* background. Levels of transcripts of many abaxial-determinant genes, including *ETTIN* (*ETT*)/*AUXIN RESPONSE FACTOR3* (*ARF3*), and all four class 1 *KNOX* genes were markedly elevated in *as2 eal* shoot apices. Rudimentary patterning in *as2 eal* leaves was suppressed by the *ett* mutation. *EAL* encodes BOBBER1 (BOB1), an *Arabidopsis* ortholog of eukaryotic NudC domain proteins. *BOB1* was expressed in plant tissues with division potential and *bob1* mutations resulted in lowered levels of transcripts of some cell-cycle genes and decreased rates of cell division in shoot and root apices. Coordinated cellular proliferation, supported by *BOB1,* and repression of all class 1 *KNOX* genes, *ETT/ARF3* by *AS2* (*AS1*) and *BOB1* might be critical for repression of the indeterminate state and of aberrant abaxialization in the presumptive adaxial domain of leaf primordia, which might ensure the formation of flat symmetric leaves.

Key words: ASYMMETRIC LEAVES2 (AS2), AUXIN RESPONSE FACTOR (ARF), *Arabidopsis thaliana*, KNOX, NudC, Leaf polarity

Introduction

Leaf primordia are clusters of cells in a determinate state at the periphery of the shoot apical meristem (SAM), which contains aggregates of indeterminate stem cells. As each leaf grows, its morphology becomes established along three axes, the proximal-distal, adaxial-abaxial and medial-lateral axes. Adaxial-abaxial patterning at the initial stage, occurring in regions adjacent to the SAM, is critical for the lateral expansion of the lamina along the medial-lateral axis for formation of flat symmetric leaves (Steeves and Sussex, 1989; Waites and Hudson, 1995; Tsukaya, 2006; Iwakawa et al., 2007; Szakonyi et al., 2010; Moon and Hake, 2010). Mechanisms that repress stem cell identity and control initial patterning for establishment of adaxial-abaxial polarity in the leaf primordium are obviously critical to plant development.

In *Arabidopsis thaliana,* three members of the family of class III homeodomain-leucine zipper (HD-ZIPIII) genes, namely *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) are expressed in the leaf adaxial domain and determine adaxial cell fate (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003) and levels of their transcripts are negatively regulated by microRNA165 (miR165) and miR166 (Bao and Barton, 2004; Mallory et al., 2004). Mutation of any *HD-ZIPIII* gene that confers resistance to miR165/166-mediated degradation of the corresponding transcript results in formation of radialized leaves with adaxial identity or trumpet-shaped leaves (McConnell et al., 2001; Emery et al., 2003; Mallory et al., 2004; Zhong and Ye, 2004). Genes in the *YABBY* (*YAB*) and *KANADI* (*KAN*) families promote the specification of leaf abaxial

fate (Sawa et al., 1999; Siegfried et al., 1999; Bowman and Smyth, 1999; Kerstetter et al., 2001; Eshed et al., 2001; Kumaran et al., 2002; Eshed et al., 2004; Wu et al., 2008; Sarojam et al., 2010). In addition, two functionally redundant genes, *ETTIN/AUXIN RESPONSE FACTOR3* (*ETT/ARF3*) and *ARF4*, whose transcripts are degraded by a *trans*-acting small interfering RNA (ta-siRNA), designated tasiR-ARF, in the adaxial domain of the leaf primordium to limit their specific expression to the inner region and the abaxial domain of leaves (Montgomery et al., 2008; Chitwood et al., 2009; Schwab et al., 2009), play important roles in lateral growth, as well as in specification of the abaxial fate and heteroblasty of leaves (Pekker et al., 2005; Hunter et al., 2006). The results of investigations of these effectors support the hypothesis that the specification of adaxial-abaxial polarity is tightly coupled with lateral expansion (Waites and Hudson, 1995). However, upstream factors that control the expression of such direct effectors in polarity-controlling pathways remain to be identified.

The *ASYMMETRIC LEAVES2* (*AS2*) and *AS1* genes of *A. thaliana* are key regulators of the formation of the flat symmetric leaves. *AS2* and *AS1* encode, respectively, a plant-specific nuclear protein with an AS2/LOB domain (Iwakawa et al., 2002; Shuai et al., 2002) and a nuclear protein with a myb domain (Byrne et al., 2000; Sun et al., 2002). The two proteins have been reported to form a complex (Xu et al., 2003; Yang et al., 2008) and effects of overexpression of AS2 are reported to depend on AS1 (Iwakawa et al., 2007). Mutations in these genes are associated with pleiotropic abnormalities in leaves along the three developmental axes (Rédei and Hirono, 1964; Tsukaya and Uchimiya, 1997; Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Iwakawa et al., 2002). AS2 and AS1 repress the transcription of class 1 *KNOTTED*-like homeobox (*KNOX*) genes, namely, *BREVIPEDICELLUS* (*BP*)/*KNAT1*, *KNAT2* and *KNAT6,* and with the exception of *SHOOT-MERISTEMLESS* (*STM*) (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001), which are exclusively expressed around the SAM and play roles in maintaining an indeterminate cell state (Hake et al., 2004). In addition, the AS1-AS2 complex directly represses the transcription of *BP* and *KNAT2* (Guo et al., 2008). Some of the pleiotropic abnormalities, including short leaves, of *as2* and *as1* plants have been attributed to ectopic expression of *BP, KNAT2* and *KNAT6* (Ikezaki et al., 2010). Furthermore, *AS2* and *AS1* repress levels of transcripts of *ETT/ARF3*, *KAN2* and *YAB5* genes in shoot apices (Iwakawa et al., 2007), suggesting that AS2 and AS1 might be involved in adaxial development. However, genetic evidence in support of this suggestion remains to be demonstrated.

Although it has been proposed that *AS2* and *AS1* are involved in the establishment of adaxial polarity, abnormalities related to adaxial defects in leaves are not obvious in *as2* and *as1* single mutants. However, defects in polarity do develop in *as2* and *as1* leaves under certain growth conditions and, also, in conjunction with mutation of members of certain groups of genes (Qi et al., 2004; Inagaki et al., 2009; see Introduction of Kojima et al., 2011). These genes include several that mediate the biogenesis of tasiR-ARF (see above). Other relevant genes belong to several different groups: those for ribosome biogenesis; chromatin modification; the genome stability; and cell proliferation. While these observations do suggest genetic interactions between *AS2* (also *AS1*) and each of these enhancer genes, our understanding of the mechanism of regulation of the expression of polarity-related effectors by AS2/AS1 is still limited.

In the present study, we report a novel enhancer mutation in *A. thaliana* that causes marked defects in adaxial development, with generation of abaxialized filamentous leaves, on the *as2* or the *as1* background. In doubly mutant in *AS2* (or *AS1*) and the enhancer gene, levels of transcripts of many abaxial-determining genes, including *ETT/ARF3* and all four class 1 *KNOX* genes rose markedly. Furthermore, introduction of mutation of *ETT/ARF3* into the double mutant significantly suppressed the formation of filamentous leaves, with formation of flat symmetric leaves. We propose that maintenance of low levels of transcripts of *ETT/ARF3* and of all class 1 *KNOX* genes by AS2/AS1 and the enhancer gene must be critical. AS2/AS1 and the enhancer might temporally and spatially control the developmental transition from the indeterminate meristematic state to the determinate state in the shoot apex that might be required for the formation of flat symmetric leaves.

Results

The establishment of adaxial cell fate in *as2-1 eal-1* and *as1-1 eal-1* double-mutant leaves was defective

In a genetic screening for enhancers of the effect of the *as1-1* mutation, we identified several mutations that generated filamentous leaves both on the *as1-1* and on the *as2-1* mutant background. We named one of these mutations *enhancer of asymmetric leaves2 and asymmetric leaves1-1* (*eal-1*). The *eal-1* single mutant formed flat, symmetric and pointed leaves, while the wild type and the *as1*, *as2* and *eal-1* single mutants did not have filamentous leaves (Fig. 1A–D, Table 1). In contrast to the single mutants, 18% and 2% of *as1-1 eal-1* double-mutant plants had filamentous leaves and lotus-like leaves, respectively. The *as2-1 eal-1* double-mutant plants produced filamentous leaves and lotus-like leaves at efficiencies of 76% and 14%, respectively

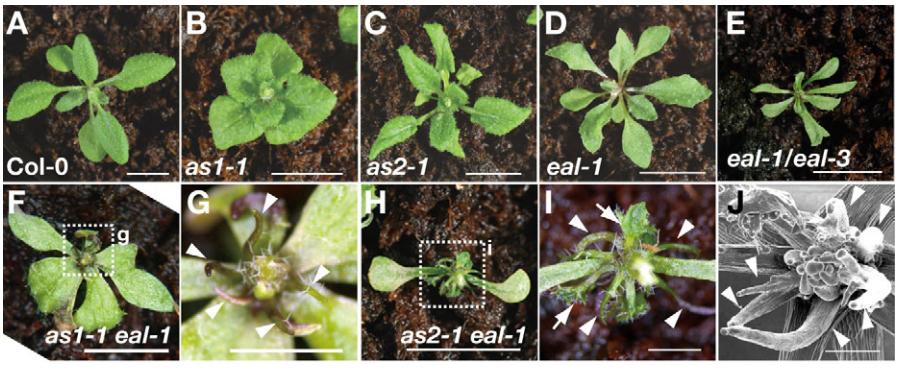

Fig. 1. The *as1 eal* and *as2 eal* double mutants generated filamentous leaves. (A) Wild-type Col-0, **(B)** *as1-1*, **(C)** *as2-1*, **(D)** *eal-1*, **(E)** *eal-1/eal-3 trans*-heterozygote (TH), **(F,G)** *as1-1 eal-1*, and **(H–J)** *as2-1 eal-1* plants. (G,I) High-magnification views of shoot apices, corresponding to boxed regions designated g and i in F and H, respectively. (J) SEM of *as2 eal*, showing filamentous leaves. Arrowheads indicate filamentous leaves and arrows indicate lotus-like leaves. Scale bars: 10 mm in A-E and H; 5 mm in F; 2 mm in G and I; 500 µm in J.

Table 1. Frequencies of plants with filamentous and lotus-like leaves

Genotype	Number of plants examined	Filamentous leaves	Lotus-like leaves
Col-0	64	0% (0)	0% (0)
as1-1	91	0% (0)	0% (0)
as2-1	91	0% (0)	0% (0)
eal-1	132	0% (0)	0% (0)
as1-1 eal-1	175	18% (32)	2% (0)
as2-1 eal-1	111	76% (84)	14% (16)

Frequency is defined as the ratio of the number of plants with more than one filamentous or lotus-like leaf to the total number of plants examined.
The numbers of plants with filamentous or lotus-like leaves are indicated in parentheses.
Plants were grown at 22℃.

(Fig. 1F–J, Table 1). Despite phenotypic similarity between the *as2-1 eal-1* and *as1-1 eal-1* double mutants, the frequency of formation of filamentous leaves by *as2-1 eal-1* plants was much higher than that by *as1-1 eal-1* plants. Therefore, as described bellow, we focused on the *as2-1 eal-1* double mutant.

Using transverse sections of leaves, we analyzed vascular patterns in *as2-1 eal-1* leaves and in corresponding single-mutant leaves (Fig. 2A–F). In wild-type, *as2-1* and *eal-1* plants, xylem and phloem tissues were similarly located on the adaxial and abaxial sides, respectively, of the vascular bundles (Fig. 2A–C). In the *as2-1 eal-1* filamentous leaves, neither phloem nor xylem cells were obvious (Fig. 2D,E). In *as2-1 eal-1* double-mutant lotus-like leaves, phloem tissue was observed around xylem tissue (Fig. 2F).

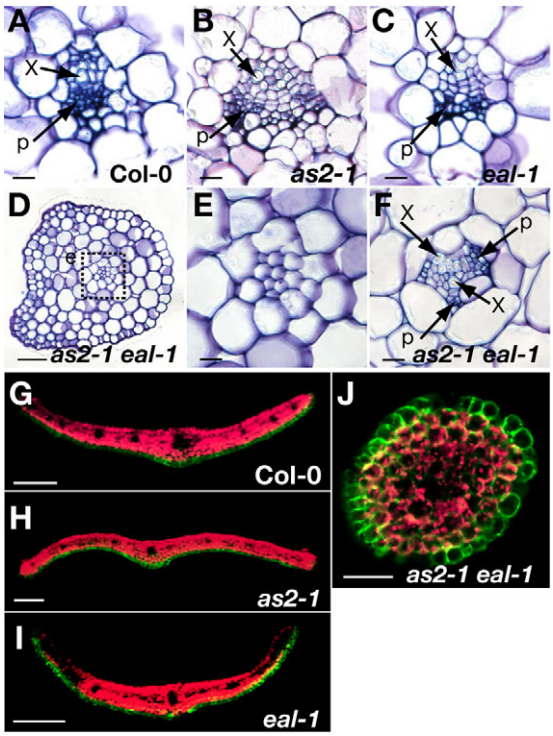

Fig. 2. Filamentous leaves were abaxialized. (A–F) Defects in vascular patterning in *as2 eal* leaves. Transverse sections of rosette leaves of (A) wild-type Col-0, (B) *as2-1*, (C) *eal-1* and (D-F) *as2-1 eal-1* plants. Sections from filamentous leaves (D,E) and lotus-like leaves (F) of the *as2 eal* double mutant. (E) High-magnification view of the central region of leaves, which corresponds to the boxed region designated e in D. x, xylem; p, phloem. **(G–J)** Expression of *FILp:GFP* in transverse sections of developing leaves of (G) Col-0, (H) *as2-1*, (I) *eal-1* and (J) *as2-1 eal-1* plants. Green, signals due to GFP; red, autofluorescence. Scale bars: 10 μm in A-D and F; 50 μm in E and J; 100 μm in G-I.

We investigated patterns of expression of cDNA for green fluorescent protein (GFP) under the control of the *FIL* promoter (the cDNA was designated *FILp:GFP*), which is expressed in abaxial cells of leaf primordia (Watanabe and Okada, 2003). We detected signals due to GFP only on the abaxial sides of wild-type, *as2-1* and *eal-1* leaves (Fig. 2G–I). By contrast, signals due to GFP were detected over the either surface of the filamentous leaves of the *as2-1 eal-1* double mutant (Fig. 2J), suggesting a defect in adaxialization.

Levels of transcripts of polarity-determining and class 1 *KNOX* genes were elevated in *as2-1 eal-1* shoot apices

We performed real-time RT-PCR using RNA from the shoot apices of wild type, *as2-1, eal-1* and *as2-1 eal-1* double-mutant plants. We quantified transcripts of three families of transcription-related genes that are involved in the determination of adaxial-abaxial polarity, namely, *KAN* genes; *FIL/YAB* genes; and *ARF* genes, which separately and redundantly specify abaxial cell fate; genes in the *HD-ZIP III* family (*PHB*, *PHV*, and *REV*), which specify adaxial cell fate; and all the genes in the class 1 *KNOX* gene family, which are expressed in the SAM and its periphery in wild-type plants.

As shown in Fig. 3A, levels of transcripts of a number of genes that are involved in the establishment of abaxial cell fate (*KAN1, KAN2, YAB5* and *ETT/ARF3*) were higher in the *as2-1 eal-1* double mutant than in the wild type and in the corresponding single mutants. Levels of transcripts of *ETT/ARF3, FIL* and *YAB5* were elevated in the *eal-1* mutant, while those of *KAN2, FIL, ETT/ARF3* and *YAB5* were elevated in the *as2-1* mutant. However, levels of *HD-ZIP III* transcripts in the double mutant were not significantly different from those in the wild type (Fig. 3B). These results suggest that the filamentous leaves of *as2-1 eal-1* plants had accentuated abaxialized features. Levels of transcripts of all class 1 *KNOX* genes (*BP, KNAT2, KNAT6* and *STM*) were also much higher in the *as2-1 eal-1* double mutant than in the wild type and in the *as2-1* and *eal-1* single-mutant plants.

Since *eal-1* is a weak allele and other *eal* alleles were embryonic-lethal (see below), we generated *trans*-heterozygotes (*eal-1/eal-3*; TH). The *eal-1/eal-3* plants had a dwarf phenotype, with pointed leaves that were smaller than those of *eal-1* plants (Fig. 1E). We examined the effects of the single mutation in the *EAL* gene on levels of transcripts of class 1 *KNOX* genes in shoot apices. As shown in Fig. 3C, levels of transcripts of all four class 1 *KNOX* genes were markedly elevated in *eal-1/eal-3* (TH) shoot apices.

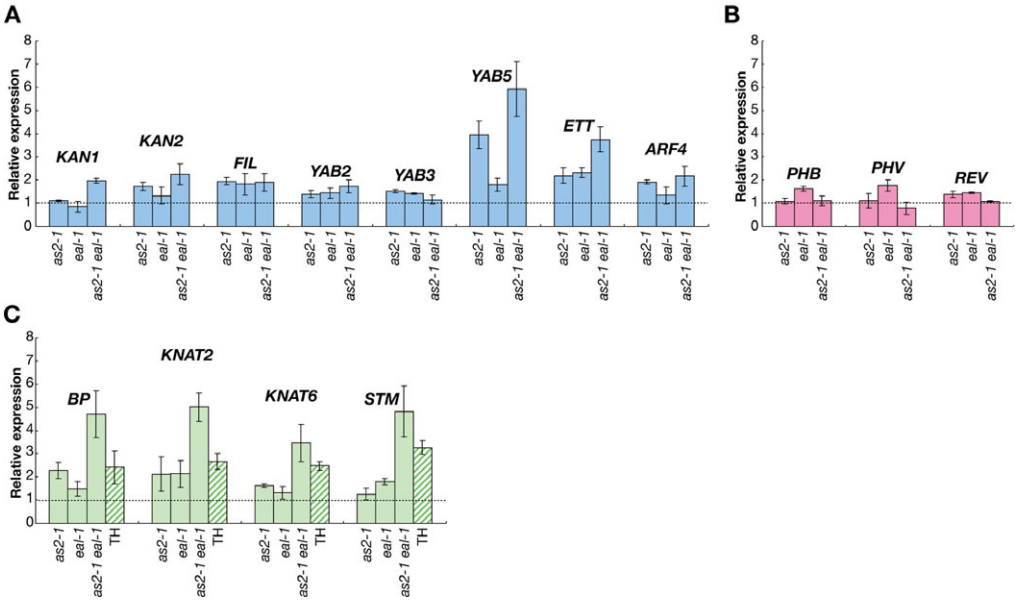

Fig. 3. Levels of transcripts of genes involved in the determination of leaf polarity and of class 1 *KNOX* genes. Levels of transcripts of genes that are involved in **(A)** abaxialization and **(B)** adaxialization of leaves; those of **(C)** class1 *KNOX* genes; and those of corresponding genes in wild-type Col-0 plants were measured by quantitative real-time RT-PCR (qRT-PCR). Total RNA was extracted from shoot apices of 18-day-old wild-type, *as2-1*, *eal-1* and *as2-1 eal-1* plants and of 15-day-old Col-0 and *eal-1/eal-3 trans*-heterozygotes, designated TH. Each value from 18-day-old plants was normalized by reference to the level of *POLYUBIQUITIN 10* (*UBQ10*, At4g05320) transcripts and those from 15-day-old plants were normalized by reference to the level of *ß-6-tubulin* (At5G12250) transcripts. The values from wild-type plants were set arbitrarily at 1.0. Bars indicate the s.d. among more than three biological replicates.

ETT/ARF3 was involved in the polarity defects in *as2-1 eal-1* leaves

Since the transcript level of *ETT/ARF3* was elevated in *as2-1 eal-1*, we examined effects of a mutation of *ETT/ARF3* on phenotypes of *as2-1 eal-1*. We introduced the *ett-13* mutation, into the *as2-1 eal-1* double mutant to generate the *as2-1 eal-1 ett-13* triple mutant. As shown in Figs 4A and 4B, the phenotype of *eal-1 ett-13* mutant plant was similar to that of the *eal-1* plant. Most of our *as2-1 eal-1 ett-13* triple mutants (79%) had symmetrically expanded leaves and no filamentous or lotus-like leaves (Fig. 4C,D). Thus, the polarity defects of the *as2-1 eal-1* double mutant were efficiently suppressed by the *ett-13* mutation, indicating that increase in the level of *ETT/ARF3* transcripts was responsible for the adaxial defect.

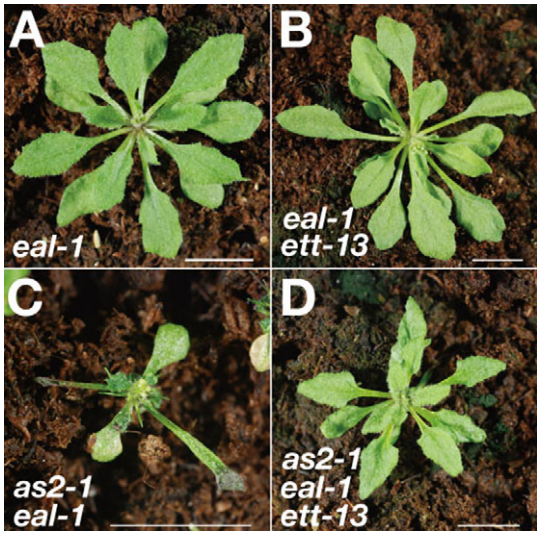

Fig. 4. The *ett* mutation efficiently suppressed the abnormal phenotype of *as2-1 eal-1* leaves. Gross morphology of **(A)** *eal-1*, **(B)** *eal-1 ett-13*, **(C)** *as2-1 eal-1* and **(D)** *as2-1 eal-1 ett-13* triple-mutant plants. Scale bars: 10 mm.

The *eal-1* mutation was located in the *BOBBER1 (BOB1)* gene that encodes a homolog of the NudC protein of *Aspergillus nidulans*

We identified the *eal* mutation as a mutation in the *BOB1* gene, which encodes 304 amino acid residues. *BOB1* is homologous to *nuclear distribution* gene *C* (*nudC*) of *Aspergillus nidulans* (Fig. 5A, supplementary material Fig. S1) (Jurkuta et al., 2009). The *eal-1* mutation, which was identical to the *bob1-3* mutation (Perez et al., 2009), was generated by a G-to-A base substitution that caused the replacement of Gly by Glu at position 141 (Fig. 5A).

We obtained two T-DNA insertion lines, *eal-2* (SALK_001125) and *eal-3* (GK_406_D03) (Fig. 5A, supplementary material Fig. S1). The *eal-2* mutation was the same as *bob1-2* (Jurkuta et al., 2009), while *eal-3* was a new allele of *BOB1*. The embryonic development of *eal-3* homozygotes was arrested at the globular stage, and this phenotype is similar to that of *bob1-1* and *bob1-2* homozygotes (supplementary material Fig. S2) (Jurkuta et al., 2009; Perez et al., 2009).

BOB1 was expressed in tissues with cell-division potential

BOB1 transcripts accumulated in tissues, such as shoot apices, developing rosette leaves and roots, that contain division-competent cells (Fig. 5B). To prepare a functional reporter construct, we cloned the genomic DNA that contained the 2,181-bp 5′-upstream region and the 1,796-bp coding region of BOB1. We fused this genomic DNA, in frame, to the *GFP* reporter gene at the last codon of the *BOB1* gene to create the fusion gene *pBOB1::BOB1::GFP*, which we then introduced into the eal-1 *mutant*. The fusion gene restored a normal phenotype and complemented the *eal-1* mutation in all transgenic lines (supplementary material Fig. S3).

In the aerial parts of the transgenic plants, signals due to GFP were most abundant in shoot apices with developing leaves (Fig. 5C), which contain strongly division-competent cells. We also examined patterns of expression of *BOB1::GFP* in transverse sections of shoot apices (Fig. 5D). Although the detected signals throughout leaf primordia at early stages (P1-P3), the intensities of

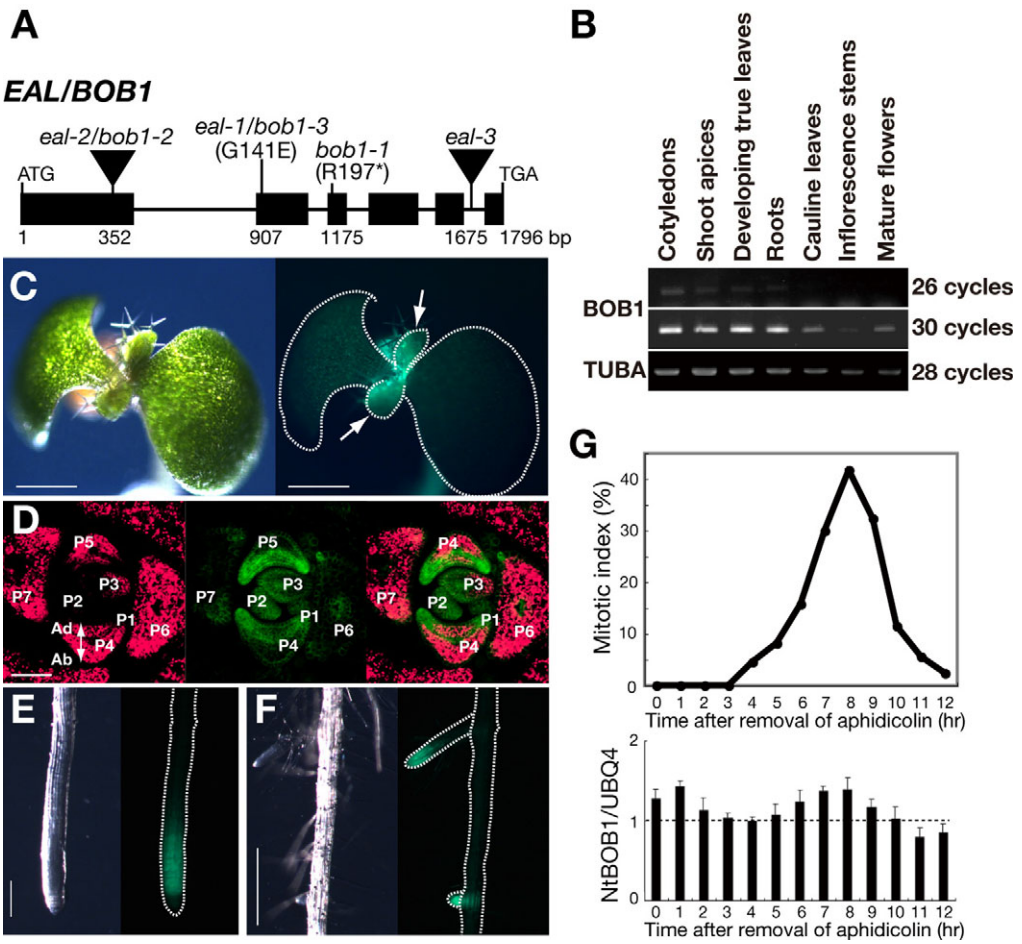

Fig. 5. The *EAL* gene encodes BOBBER1, a homolog of NudC. **(A)** Exon-intron (black rectangle-thin line) organization between the initiation and termination codons of the *EAL*/*BOB1* gene and sites of point mutations in *eal-1*/*bob1-3* and *bob1-1* and of T-DNA insertions in *eal-2*/*bob1-2* and *eal-3*. **(B)** Accumulation of *BOB1* transcripts in wild-type Col-0 as determined by RT-PCR. Transcripts were detected in the cotyledons, developing true leaves and shoot apices of 10-day-old plants; in the third and fourth rosette leaves and roots of 19-day-old plants; and in the cauline leaves, inflorescence stems and mature flowers of 40-day-old plants. **(C–F)** The expression of *BOB1::GFP*, driven by the *BOB1* promoter, in *eal-1* plants. (C,E,F) Panels include a bright-field image and a fluorescence image. (D) The expression of *BOB1::GFP* in a transverse section of an 11-day-old vegetative shoot apex. Expression in leaf primordia at different growth stages. P1-P7 indicate stages of development; the larger the number, the older the primordium. (E) Expression at the tip of a primary root and (F) a lateral root of 6- or 8-day-old plants. Green, signals due to GFP; red, autofluorescence. Ad, adaxial side; Ab, abaxial side. **(G)** Patterns of accumulation of the *NtBOB1* transcript during progression of the cell cycle in BY-2 cells that had been synchronized with aphidicolin. Mitotic indices are shown in the upper panel. Levels of transcripts at indicated times were determined by qRT-PCR (lower panel). Scale bars: 500 μm in C and F; 100 μm in D; 200 μm in E.

signals in central regions and the epidermis were stronger than those from other cells at later stages (P4 and P5) (Fig. 5D). As the primordia grew, the intensity of signals fell rapidly (P6 and P7). We also observed signals due to GFP at the tips exclusively of primary and lateral roots (Fig. 5E,F). These observations suggest a potential role for *BOB1* in cell-division ability and/or in maintenance of cells in an indeterminate state.

Using a *BOB1* homolog of *Nicotiana tabacum* (*NtBOB1*), we quantified levels of *NtBOB1* transcripts by real-time RT-PCR during progression of the cell cycle in synchronized tobacco BY-2 cells. Fig. 5G shows that accumulation of the *NtBOB1* transcript exhibited two peaks that effectively overlapped progression through the S and M phase, respectively. However, the relative heights of the peaks were small. Thus, it seems likely that transcription of *NtBOB1* is controlled, at least to a small extent, by the cell cycle.

BOB1 complemented defects in colony growth and the movement of nuclei in the *nudC3* mutant of *Aspergillus nidulans*

Colonies of the *nudC3* mutant of *A. nidulans* exhibit temperature-sensitive growth, and the migration of nuclei that normally occurs prior to the formation of cell plates during cytokinesis is also temperature-sensitive (Osmani et al., 1990; Chiu and Morris, 1995; Chiu and Morris, 1997). We examined whether *BOB1* might allow *nudC3* mutant cells to grow normally at an elevated temperature with normal movement of nuclei using *BOB1* cDNAs driven by the *alcA* promoter of *A. nidulans* (Fig. 6A).

Even at the permissive temperature (30℃), *nudC3* colonies were smaller than wild-type colonies (Fig. 6B, columns 1 and 2). At the restrictive temperature (42℃), the *nudC3* colonies were much smaller than the wild-type colonies. Wild-type *BOB1* cDNA fully reversed the defects in colony growth of *nudC3* cells at both temperatures (Fig. 6B, column 3). Moreover, *eal-1* cDNA also reversed the growth defects at 30℃ and 42℃, but *eal-1* cDNA was slightly less effective than *BOB1* cDNA (Fig. 6B, column 4). As anticipated, *bob1-1* cDNA failed to reverse the growth defects at 30℃ and 42℃ (Fig. 6B, columns 2, 5 and 6).

As shown in Fig. 6C,D, *BOB1* cDNA rescued the defect in nuclear migration in *nudC3* cells at 42℃, and 84% of nuclei in *nudC3* cells that had been transformed with *BOB1* cDNA were normally distributed. By contrast, *eal-1* cDNA yielded only the background level of movement of nuclei.

Mutations in *BOB1* affected the progression of the cell cycle in shoot and root apices

Using cross sections of shoot apices, we investigated the structure of the shoot apical meristem (SAM) in wild-type, *as2-1, eal-1*, *as2-1 eal-1* and *eal-1/eal-3 trans*-heterozygous (TH) plants. The number of cells in the L1 and L2 layers in the region of the SAM was depressed in the *eal-1* and *as2 eal-1* mutants (Fig. 7A,B). In

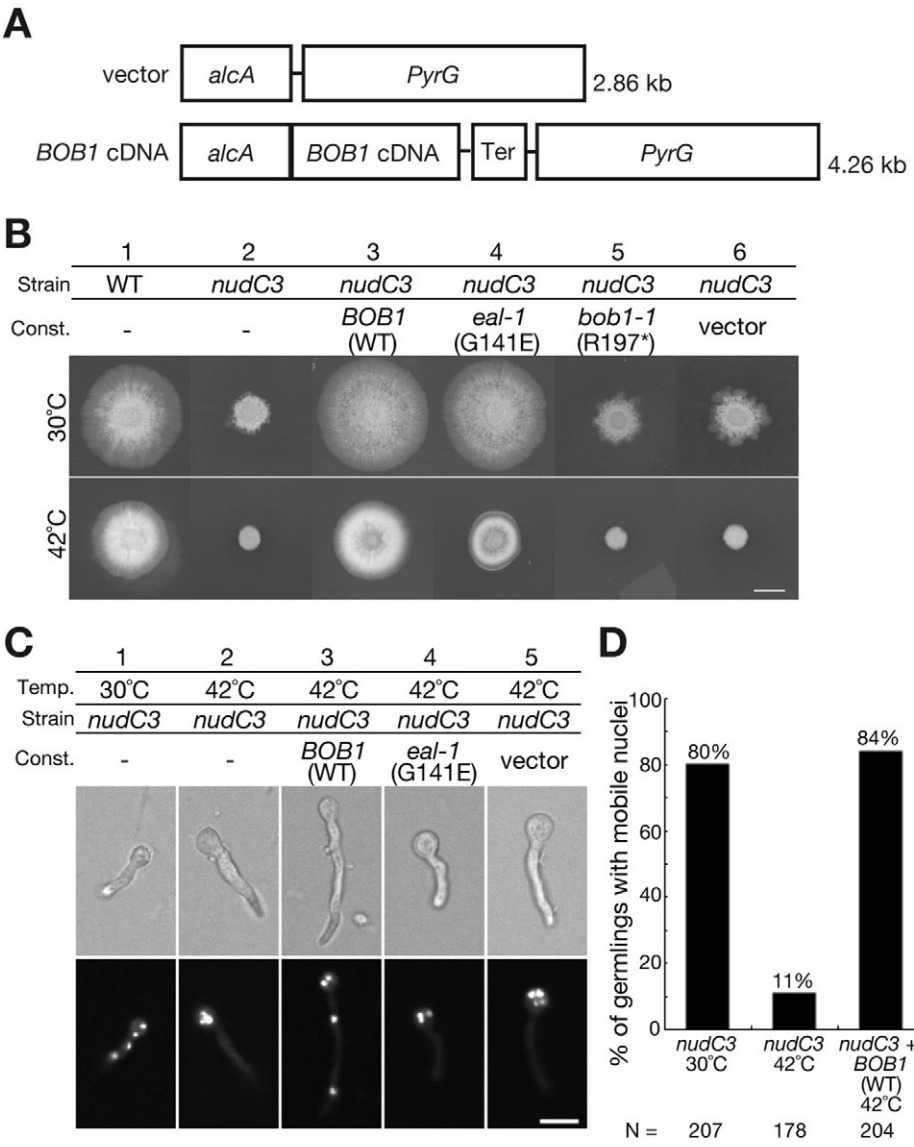

Fig. 6. The *BOB1* gene of *Arabidopsis* rescues the temperature-sensitive *nudC3* mutation of in *A. nidulans*. **(A)** Schematic representation of the DNA constructs used for complementation tests. The vector included the inducible/repressible *alcA* promoter, the *pyrG* gene of *A. nidulans* and the terminator sequence (Ter) of *Aspergillus oryzae Taka-amylase A*. The wild-type and mutant *BOB1* cDNA (*eal-1* or *bob1-1*) were cloned, separately, downstream of the *alcA* promoter. The unmodified vector was used as a negative control. **(B)** Complementation of the growth defect of the *nudC3* mutant by *BOB1*. Cells of non-transformed wild-type *A. nidulans* (column 1), the *nudC3* mutant (column 2) and *nudC3* harboring the *BOB1* (column 3), *eal-1* (column 4), *bob1-1* (column 5) or the vector (column 6) construct were incubated at 30℃ or 42℃ for 4 days. Scale bar: 10 mm. **(C)** Complementation of the defect in the movement of nuclei by *BOB1*. Spores from the *nudC3* mutant were germinated and the mutant was grown at 30℃ (column 1) and at 42℃ (columns 2-5) for 10 hours. The germlings were stained with DAPI. Scale bar: 10 µm. **(D)** The overall rates of movement of nuclei. Germlings into which nuclei had moved under the indicated growth conditions were counted. N represents the number of germlings scored under each growth condition.

addition, the structure of the *as2-1 eal-1* SAM was disorganized. The number of cells in the SAM was reduced still further in the *eal-1/eal-3* SAM, suggesting reduced cell-division ability in the *eal-1/eal-3* SAM.

We quantified transcripts of genes that are involved in progression of the cell cycle in shoot apices of wild-type, *eal-1*, and *eal-1/eal-3* plants. As shown in Fig. 7C, in *eal-1*, levels of transcripts of *histone H4*, *MINICHROMOSOME MAINTENANCE-7* (*MCM7*), *CYCLIN B1;2* (*CYCB1;2*), *HINKEL* and *KNOLLE* genes were slightly depressed, and the extent of such depression was much greater in *eal-1/eal-3* plants. The *eal-1* mutant had short roots and the *eal-1/eal-3* mutant had even shorter roots (Fig. 7D). The extent of root elongation in *eal-1* (60%) and *eal-1/eal-3* (30-40%) plants was significantly lower than in wild-type plants (Fig. 7E). To examine the efficiency of cell division, we monitored the expression of *CYCB1;2-ß-glucuronidase* (*GUS*), in which the *CYCB1;2* genomic sequence containing the promoter region was fused to the *GUS* gene, which we used as a marker of the G2-M transition. In *eal-1* and *as2-1 eal-1* seedlings, the intensity and number of signals due to GUS were markedly lower than those in the wild type (Fig. 7F). These results suggest that cell-division ability is reduced in the *eal-1* mutant.

We measured the ploidy of nuclei in the first two leaves of 18-day-old wild-type, *as2-1*, *eal-1* and *as2-1 eal-1* plants by flow cytometry (Fig. 7G). In wild-type and *as2-1* leaves, patterns of ploidy from 2C to 16C were similar. However, the *eal-1* mutant contained nuclei with higher ploidy (from 32C and 64C). The ploidy in the *as2-1 eal-1* double mutant was even higher. Palisade cells of the *eal-1* mutant were also more than twice the size of those of wild-type plants (Fig. 7H,I). These results indicate that mutations in *BOB1* resulted in early entry into the endocycle and that increases in ploidy were exacerbated by the *as2-1* mutation.

Discussion

Our present study showed that the *eal-1* mutation in the *BOB1* gene enhances defects in the adaxial development of *as2* leaves, converting flat leaves to abaxialized filamentous leaves. This effect is attributable to elevated levels of transcripts of the *ETT/*

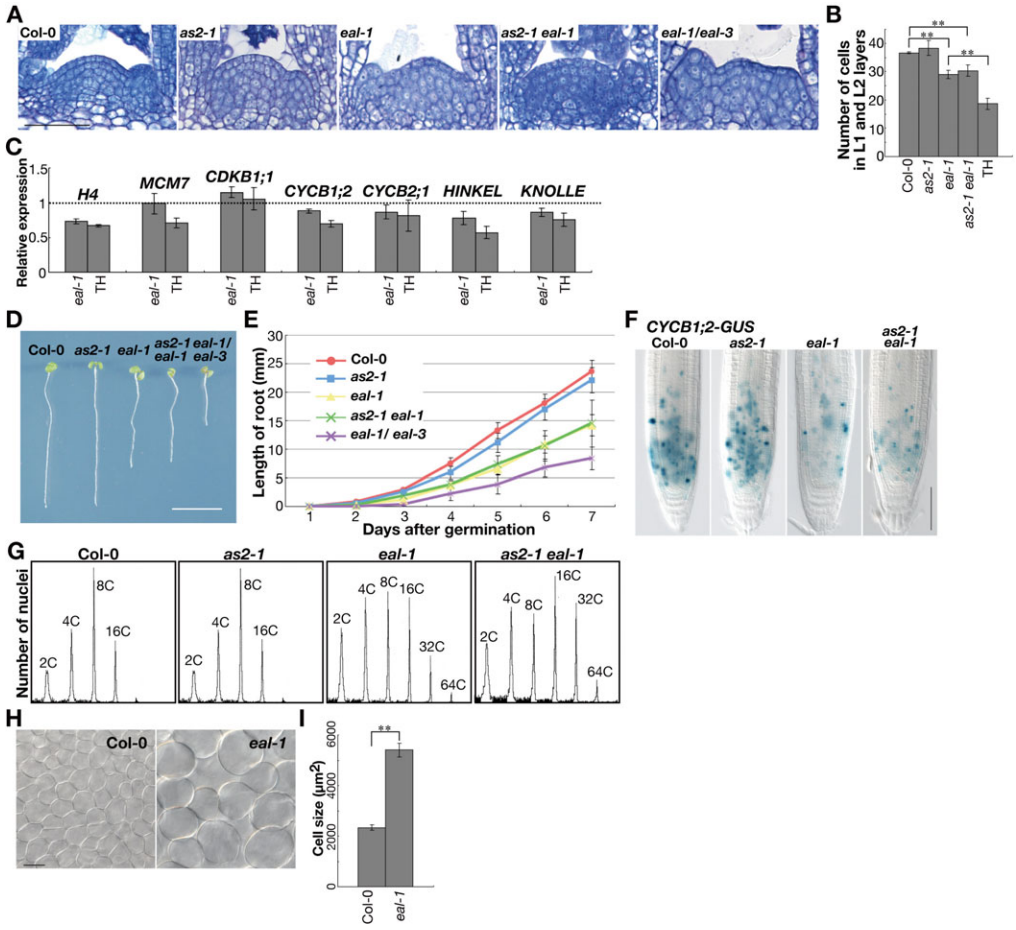

Fig. 7. Mutation of the *BOB1* gene negatively affects cell division. (A) Toluidine blue-stained sections of shoot apices from 9-day-old wild-type Col-0, *as2-1*, *eal-1*, *as2-1 eal-1* and *eal-1/eal-3 trans*-heterozygote (TH) plants. **(B)** Number of cells in L1 and L2 layers of meristem domes in 9-day-old wild-type, *as2-1*, *eal-1*, *as2-1 eal-1* and *eal-1/eal-3 trans*-heterozygote plants. Bars indicate the s.e.m. among three plants. **(C)** Expression of transcripts, as determined by qRT-PCR. Relative levels of expression of cell cycle-related genes in the shoot apices of 15-day-old *eal-1/eal-3* (TH) plants, relative to those of Col-0. Each value was normalized by reference to levels of transcripts of *ß-6-tubulin* (At5G12250). Bars indicate the s.d. among more than three biological replicates. **(D)** Seedlings of 7-day-old Col-0, *as2-1*, *eal-1, as2-1 eal-1* and *eal-1/eal-3* (TH) plants. **(E)** Root growth of Col-0, *as2-1*, *eal-1* and *as2-1 eal-1* plants from 1 to 7 days after germination. Averages ± s.d. for roots of more than 10 plants of each genotype are shown. The root length was measured using ImageJ software (http://rsb.info.nih.gov/ij/). **(F)** Patterns of staining for GUS activity in primary root tips of 6-day-old Col-0, *as2-1*, *eal-1* and *as2-1 eal-1* plants that harbored *CYCB1;2-GUS*. **(G)** Flow cytometric analysis of ploidy using nuclei prepared from first and second leaves of 18-day-old Col-0, *as2-1*, *eal-1* and *as2-1 eal-1* plants. **(H)** Palisade cells in first leaves of 21-day-old Col-0 and *eal-1* plants. **(I)** Quantitative analysis of cell size in the first leaves of Col-0 and *eal-1* plants. Bars indicate the s.d. Scale bars: 50 µm in A; 10 mm in D; 100 µm in F; and 10 µm in H. Significant differences were determined by Student's *t*-test. $^{**}P<0.01$ (B,I).

ARF3 gene (Figs 1–5). The *BOB1* gene, in cooperation with the *AS2* (*AS1*) gene, plays a crucial developmental role via repression of both the indeterminate state and the abaxial fate of cells in the presumptive adaxial domain of leaf primordia after the commitment to leaf initiation around the shoot apical meristem, promoting the establishment of the adaxial polarity of leaves (Fig. 8). Thus, repressive activity is required for the formation of flat symmetric leaves with appropriate adaxial-abaxial polarity.

BOB1 is involved in the repression of transcription of genes involved in leaf abaxialization via an unknown pathway that is independent of the *AS2* (*AS1*) pathway

How do *BOB1* and *AS2* (*AS1*) act together to regulate the repression of levels of *ETT/ARF3* transcripts? *AS2* transcripts are detected in the adaxial region of leaf primordia and *AS1* transcripts are detected in the inner region of leaves that includes the vasculature (Iwakawa et al., 2007). Regions of expression of *BOB1* in leaf primordia are overlapped sites at which *AS2* and *AS1* transcripts were found (Fig. 5). The levels of the *ETT/ARF3* transcript in shoot apices of *as2-1* and *eal-1* were higher than in the wild type, while that in the *as2-1 eal-1* double mutant was even higher still (Fig. 3A). Let us consider the following two possibilities; *BOB1* might act on the repression of transcription of the *ETT/ARF3* gene via an unidentified pathway that might be independent of the *AS2* (*AS1*)-mediated pathway (Fig. 8). Alternatively, both BOB1 and AS2 (AS1) might function in the same pathway. The overlap among regions of expression of the *BOB1*, *AS2* and *AS1* genes in the shoot apex (Fig. 5C,D) (Byrne et al., 2000; Iwakawa et al., 2007) supports both possibilities. However, our *bob1* and *as2* (*as1*) mutants had completely different morphology (Fig. 1), suggesting that the second possibility is unlikely. Similarly, it seems plausible that *AS2* and *BOB1* might repress the expression of class 1 *KNOX* genes via two independent pathways.

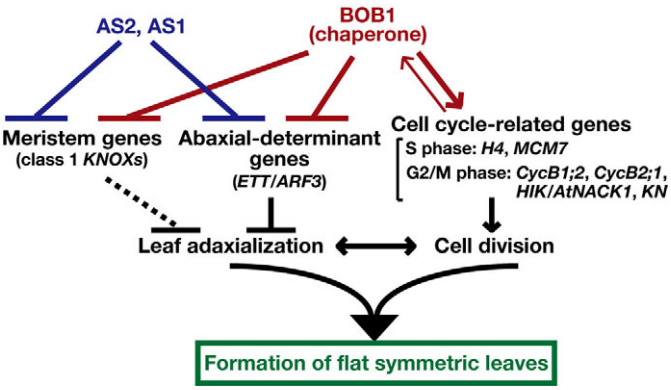

Fig. 8. Roles of AS1, AS2 and BOBBER1 in shoot apices during the formation of flat symmetric leaves. The AS1-AS2 complex and BOB1 act independently to repress the levels of transcripts of leaf abaxial-determinant genes, which include *ETT/ARF3,* and of "meristem" genes, namely class1 *KNOX* genes. The relationship between class 1 *KNOX* genes and the differentiation of the leaf adaxial side of leaves remains to be clarified. BOB1 acts positively to regulate cell division for the formation of flat symmetric leaves via regulation of the transcription of cell cycle-related genes. The balance between leaf adaxialization and cell division is critical for the formation of flat symmetric leaves.

BOB1 is involved in progression of the cell cycle and in the extent of expression of class 1 *KNOX* genes in the shoot apex, acting in cooperation with *AS2* (*AS1*)

We have described six lines of evidence in support of the hypothesis that *BOB1* might be involved in cell division. Mutations in *BOB1* caused (1) a reduction in the number of cells in shoot apices; (2) a delay of root growth, due, perhaps, to slow cell division; (3) a decrease in levels of transcripts of genes involved in the cell cycle; and (4) early transition to the endocycle, which might occur during the progression of G2 phase (Fig. 7). In addition, we observed (5) two peaks in levels of transcripts of a tobacco homolog of *BOB1,* during the cell cycle, namely, during the S and M phases; and (6) elevated levels of expression of *BOB1* in leaf primordia at early developmental stages and in primary and lateral root tips, where the potential for cell division is high (Fig. 5). These results imply that mutations in *BOB1* result in a decrease in the rate of progression of the cell cycle and/or in the frequency of onset of cell division around shoot and root apical meristems. Such impaired cell division in *bob1* tissues might be due to lower levels of the transcripts of cell cycle-related genes, as described above. Although the wild-type *BOB1* gene plays an inhibitory role in the expression of *ETT/ARF3* and class 1 *KNOX* genes (Fig. 3A), it acts positively to maintain appropriate levels of transcripts of cell cycle-related genes to ensure the proper progression of the cell-division cycle in shoot and root apices (Figs 7C, 8).

Our observation that levels of transcripts of all four class 1 *KNOX* genes were significantly elevated in the *eal-1/eal-3* shoot apex (Fig. 3C; TH) are consistent with previous reports of the expansion of the domain of expression of the *STM* gene in *bob1* embryos (Jurkuta et al., 2009). In addition, levels of transcripts of all class 1 *KNOX* genes were even higher and the expression domain of the *BP* gene was even more extensive in petioles at the *as2-1 eal-1* shoot apex than at the shoot apex of the corresponding single mutants (Fig. 3C, supplementary material Fig. S4). These results predict that the size of the population of cells in the indeterminate state is enhanced in the shoot apex of the double mutant. Effects of the expansion of the expression domain of class 1 *KNOX* genes in the shoot apex on development of the adaxial domain remains to be examined.

Relationship between the molecular function and developmental role of *BOB1*

In *A. nidulauns*, the *nudC* gene is required for regulation of the movement of nuclei during the asexual reproductive cycle, as well as for deposition of the cell wall, colony growth and viability (Osmani et al., 1990; Chiu et al., 1997). Homologs of NudC from mammals, *Drosophila*, *Caenorhabditis elegans* and *Arabidopsis* complement *nudC3* mutation of *A. nidulans*, restoring the normal movement of nuclei and colony growth (Fig. 6) (Miller et al., 1999; Morris et al., 1997; Cunniff et al., 1997; Dawe et al., 2001). These observations suggest that the function of NudC in the movement of nuclei is evolutionarily conserved in eukaryotes.

Cappello et al. (2011) showed that mammalian NudC is required for the migration of nuclei and of neurons during neocortical development of the brain. In addition, suppression of the expression of genes homologous to *nudC* led to defects in cytokinesis and chromosome congression during karyokinesis in cultured non-neural cells (Aumais et al., 2003; Nishino et al., 2006; Zhang et al., 2002; Zhou et al., 2003; Zhou et al., 2006). Thus, mammalian homologs of NudC appear to be involved both in organogenesis and in cell division. BOB1 might play similar roles in both phenomena.

Several homologs of NudC, including BOBBER1, have been shown to act as molecular chaperones in vitro, and a role as chaperone is the only known molecular function of these homologs (Faircloth et al., 2009; Perez et al., 2009; Zhu et al., 2010). The *bob1-3* (identical to *eal-1*) mutation does not affect such chaperone activity in vitro (Perez et al., 2009), but this mutation did abolish the migration of nuclei, in *A. nidulans* (Fig. 6). The mutation also acted as an effective enhancer of defects in adaxial development in *as2* leaves and depressed the efficiency of cell division in shoot and root apices (Figs 1, 2). Thus, we found no obvious correlation between the chaperone activity of eal-1 protein and the developmental and cellular abnormalities in *eal-1* mutant plants. These observations suggest that BOBBER1 not only acts as a molecular chaperone but also has some other unidentified function. However, the observed absence of a correlation between the activity as a chaperone and defects in leaf development and cell division in *eal-1* plants might be explained by the assumption that the developmental effects of the *eal-1* mutation are greater *in vivo* than the effects of the *eal-1* mutation on chaperone activity in vitro.

BOB1 acts together with AS2 (AS1) as a modulator in the formation of flat leaves

As discussed above, *BOB1* plays positive roles in cell division, as well as in the establishment of adaxial polarity in leaves. Waites and Hudson (1995) proposed that the cell proliferation required for the lateral growth of the leaf lamina might be tightly coupled with the establishment of adaxial-abaxial polarity in the leaf primordium. As proposed in Fig. 8, the balance between leaf adaxialization and cell division might be coordinately controlled to ensure the development of flat symmetric leaves. Since BOB1 is involved in both the stimulation of cell division and the establishment of adaxialization, it might act, together with AS2

(AS1), as a crucial factor that appropriately modulates cell division and development.

Materials and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Col-0 (CS1092), *as2-1* (CS3117), *as1-1* (CS3374) and *eal-2* (SALK_001125) were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus, OH, USA). Seeds of the *eal-3* (GK_476D03) mutant were obtained from the Nottingham Arabidopsis Stock Center (NASC; University of Nottingham, Loughborough, UK). Seeds of *ett-13* (SALK_040513) mutant and CYCB1;2-GUS were kindly provided by Dr Yuval Eshed (Weizmann Institute of Science, Israel) and Dr Masaki Ito (Nagoya University, Japan), respectively. Growth conditions for plants were described previously (Semiarti et al., 2001).

Isolation and mapping of the *EAL* gene

To isolate the *eal-1* mutant, seeds of *as1-1* were mutagenized by 0.03% ethyl methanesulfonate and approximately 5,500 M2 plants from 201 M1 stocks were screened on the basis of leaf phenotype. We crossed the *eal-1* mutant with wild-type Col-0 and the segregation rate of F2 plants suggested that the enhancement of the *as1* phenotype was due to single recessive locus. To map the *EAL* locus, we crossed the *as1-1 eal-1* double mutant with wild-type Landsberg *erecta* (L*er*). Samples of genomic DNA from 1,306 F2 plants were analyzed in terms of simple sequence length polymorphism and cleaved-amplified polymorphism markers, which were generated on the basis of polymorphism data provided by Cereon (http://www.Arabidopsis.org/Cereon). The *EAL* locus was mapped to an approximately 30-kb region on chromosome 5, which contains eleven predicted genes. We compared the genomic sequences of the wild type and the *eal-1* mutant and found that the *eal-1* mutant harbored a mutation at the 19th nucleotide of the second exon of At5g53400. For genotyping of *eal-1*, we amplified genomic DNAs by the polymerase chain reaction (PCR) using the primers listed in supplementary material Table S1 and digested the DNA products with the restriction enzyme *Xmn*I (New England Biolabs, Beverly, MA, USA).

Construction of *pBOB1::BOB1::GFP*

A 4.1-kb fragment of genomic DNA from a wild-type plant (Col-0), encompassing the 2.3-kb upstream sequence of *BOB1* and the 1.8-kb coding region of *BOB1*, was amplified by PCR with a pair of primers (supplementary material Table S2). The resultant DNA fragment was inserted into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA), and then the product was introduced into the binary vector pGWB4 (Nakagawa et al., 2007) with the GFP coding sequence. We transformed the *eal-1* mutant using *Agrobacterium tumefaciens* strain GV3101 that harbored the plasmid with the incorporated DNA fragment, as described by Bechtold and Pelletier (1998).

RT-PCR

Manipulation of DNA and qRT-PCR were performed as described by Iwakawa et al. (2007). Primer sets for RT-PCR and qRT-PCR are listed in supplementary material Table S3. Results were normalized by reference to results for *UBQ10* (At4g05320), *ß-6-tubulin* (At5G12250) or *α-tubulin* (At5G19770).

Fluorescence microscopy

We crossed *as2-1 eal-1* and *FILp:GFP* (Watanabe and Okada, 2003; Ueno et al., 2007). Shoot apices containing leaf primordia were embedded in 5% agar and then agarose blocks were sliced into sections with a vibratome. Fluorescence was observed with a confocal laser scanning microscope (LSM510 META; Carl Zeiss Inc., Oberkochen, Germany). We observed fluorescence images of tissues derived from plants that harbored *pBOB1::BOB1::GFP* with a stereomicroscope (SteREO Lumar.V12; Carl Zeiss Inc., Jena, Germany).

Detection of GUS activity and histological analysis

The activity of GUS in roots was detected as described by Iwakawa et al. (2007). Plant tissues were prepared for thin sectioning as described by Ueno et al. (2007). Scanning electron microscopy (SEM) was performed as described previously (Semiarti et al., 2001).

Measurements of ploidy

Ploidy analysis was performed as described by Suzuki et al. (2005).

Strains of *Aspergillus nidulans*, growth conditions and transformation

Aspergillus nidulans strains FGSC A773 (*pyrG89; wA3; pyroA4*) and FGSC A779 (*nudC3 pyrG89 pabaA1; wA2; nicA2*) were obtained from the Fungal Genetics Stock Center (FGSC; University of Missouri, Kansas City, MO, USA). The strains were grown at a permissive temperature of 30℃ or at a restrictive temperature of 42℃ in standard minimal medium (Rowlands and Turner, 1973) supplemented with appropriate requirements. The minimal medium for A773 was supplemented with 0.0002% pyridoxine HCl, 0.12% uridine and 0.11% uracil. The minimal medium for A779 was supplemented with 0.0002% niacin, 0.0002% *p*-aminobenzoic acid, 0.12% uridine and 0.11% uracil (Morris et al., 1997). The promoter of *alcA*, the gene for alcohol dehydrogenase I of *A. nidulans*, was fused to full-length *BOB1* cDNA and the fused construct and the *PyrG* coding sequence were subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA, USA). The *eal-1* and *bob1-1* mutations in *BOB1* cDNA were induced with a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). *A. nidulans* was transformed as described by Makita et al. (2009). Transformants were grown in liquid medium or on 1.4% agar-solidified medium, which were the minimal medium for A779 without uridine and uracil.

DAPI staining of nuclei

To stain nuclei of developing germlings, conidia of A779 and the transformants were incubated in 10 ml of each liquid standard minimal medium for 10 hours. Then, we added 4′,6-diamidino-2-phenylindole (DAPI) and Triton X-100 to final concentrations of 0.1 µg/ml and 0.1%, respectively, and incubated cells for a further 10 minutes. We observed DAPI fluorescence under an Olympus microscope (BX51TRF; Olympus, Tokyo, Japan).

BY-2 cells and synchronization

Maintenance of tobacco suspension-cultured BY-2 cells and synchronization of the BY-2 cell cycle at the G_1/S boundary were performed as described previously (Nishihama et al., 2002). RNA was extracted from BY-2 cells with an RNeasy kit (QIAGEN, Hilden, Germany) and poly(A)$^+$ RNA was isolated with Dynabeads® (Dynal Biotech, Lake Success, NY, USA). Reverse transcription was performed with a First-Strand cDNA Synthesis Kit (GE Healthcare, Buckinghamshire, UK). Primer sets for qRT-PCR are listed in supplementary material Table S1. Results were normalized by reference to results for *NtUBQ4*.

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