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.

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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), PHAYOLUTA (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 Arabidopsis 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 (Rédéi 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.](http://bio.biologists.org/Downloaded from http://bio.biologists.org/)
(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 below, 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).

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 \( HD-ZIP \) 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 \( HD-ZIP \) 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of plants examined</th>
<th>Filamentous leaves</th>
<th>Lotus-like leaves</th>
</tr>
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<tbody>
<tr>
<td>Col-0</td>
<td>64</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>as1-1</td>
<td>91</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>as2-1</td>
<td>91</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>eal-1</td>
<td>132</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>as1-1 eal-1</td>
<td>175</td>
<td>18% (32)</td>
<td>2% (0)</td>
</tr>
<tr>
<td>as2-1 eal-1</td>
<td>111</td>
<td>76% (84)</td>
<td>14% (16)</td>
</tr>
</tbody>
</table>

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°C.
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.

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. 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 bar: 10 mm.

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) class 1 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.

Fig. 2. ETT/ARF3 was involved in the polarity defects in as2-1 eal-1 leaves.
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 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˚C), nudC3 colonies were smaller than wild-type colonies (Fig. 6B, columns 1 and 2). At the restrictive temperature (42˚C), 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˚C and 42˚C, 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˚C and 42˚C (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˚C, 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).
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/
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 class 1 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 cal-1/cal-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 cal-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.

Fig. 6. (A) The molecular function and developmental role of BOB1

In A. nidulans, 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 nudC 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 boh1-3 (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...
were grown at a permissive temperature of 30°C or at a restrictive temperature of 42°C in standard minimal medium (Rowlands and Turner, 1973) supplemented with appropriate requirements. The minimal medium for A779 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-amino benzoic acid, 0.12% uridine and 0.11% uracil (Morris et al., 1997). The promoters of At5g53400 and At5g12250 were cloned into the binary vector pGWB4 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.

DAPI staining of nuclei
To stain nuclei of developing gernlings, 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 G1/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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References
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