Common regulatory networks in leaf and fruit patterning revealed by mutations in the *Arabidopsis ASYMMETRIC LEAVES1* gene

Hugo Alonso-Cantabrana¹*, Juan José Ripoll¹‡, Isabel Ochando¹‡, Antonio Vera¹, Cristina Ferrándiz² and Antonio Martinez-Laborda¹§

Carpels and leaves are evolutionarily related organs, as the former are thought to be modified leaves. Therefore, developmental pathways that play crucial roles in patterning both organs are presumably conserved. In leaf primordia of *Arabidopsis thaliana*, the *ASYMMETRIC LEAVES1* (AS1) gene interacts with AS2 to repress the class I *KNOTTED1*-like homeobox (KNOX) genes *BREVIPEDICELLUS* (BP), *KNAT2* and *KNAT6*, restricting the expression of these genes to the meristem. In this report, we describe how AS1, presumably in collaboration with AS2, patterns the *Arabidopsis* gynoecium by repressing BP, which is expressed in the replum and valve margin, regulates the expression of this gene. Misexpression of AS1 is slightly reduced, and enhances the effect of mutations in BP and valve margin, interacts in the replum with *REPLUMLESS* (RPL), a gene with an important function in valve development. Altogether, these findings strongly suggest that BP plays a crucial role in replum development. We propose a model for pattern formation along the mediolateral axis of the ovary, whereby three domains (replum, valve margin and valve) are specified by the opposing gradients of two antagonistic factors, valve factor and replum factor, the class I KNOX genes working as the latter.

KEY WORDS: *Arabidopsis*, Fruit development, Pattern formation, *ASYMMETRIC LEAVES 1*, *BREVIPEDICELLUS (KNAT1)*, Class I KNOX genes

INTRODUCTION

The gynoecium of *Arabidopsis thaliana* consists of two congenitally fused carpels, which give rise to a basal ovary topped with a solid style and an apical stigma (Ferrándiz et al., 1999; Bowman et al., 1999; Dinneny and Yanofsky, 2005; Balanzá et al., 2006). The ovary is composed of two valves connected on both sides by the replum (Sessions and Zambryski, 1995). After pollination, *Arabidopsis* develops a silique, the characteristic dehiscent fruit shared by all Brassicaceae (Ferrándiz et al., 2004). The valve margin, a thin region of small cells located between replum and valve tissues, plays a crucial role in dehiscence (Ferrándiz, 2002). The MADS-box gene *FRUITFULL* (FUL) represses the expression of genes involved in valve margin development (Ferrándiz et al., 2000a; Liljegren et al., 2004), the MADS-box genes *SHATTERPROOF* (SHP1 and SHP2) (Liljegren et al., 2000), and their downstream genes *ALCATEARZ* (ALC) and *INDEHISCENT* (IND), both of which code for basic helix-loop-helix (bHLH) domain proteins (Rajani and Sundaresan, 2001; Liljegren et al., 2004). In this way, FUL prevents valves from adopting a valve margin fate (Ferrándiz et al., 2000a; Liljegren et al., 2004). The *FUL* and *SHP* genes are induced by the cooperating activities of *FILAMENTOUS FLOWER* (FIL) (Chen et al., 1999; Sawa et al., 1999a; Sawa et al., 1999b) and *YABBY3* (YAB3) (Siegfried et al., 1999), two genes belonging to the YABBY family involved in abaxial tissue specification, and *JAGGED* (JAG), a gene that encodes a putative transcription factor with a single C2H2 zinc-finger domain and promotes growth in lateral organs (Dinneny et al., 2004; Ohno et al., 2004). These genes probably act in a concentration-dependent manner, in such a way that activation of FUL would require high levels of their products, while SHP expression would be induced by lower levels (Dinneny et al., 2005). The homeobox gene *REPLUMLESS* (RPL) downregulates valve margin genes in the replum (Roeder et al., 2003; Liljegren et al., 2004) by repressing the expression of *FIL*, *YAB3* and *JAG* (Dinneny et al., 2005). This gene, also designated *BELLRINGER* (BLR), *PENNYWISE* (PNY) and *VAAMANA* (VAN), interacts with the class I *KNOTTED1*-like homeobox (KNOX) genes *SHOOTMERISTEMLESS* (STM), *BREVIPEDICELLUS* (BP, also known as *KNAT1*) and *KNAT6* to regulate meristem function (Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004).

The mechanism by which leaf founder cells are distinguished from stem cells of the meristem involves downregulation at positions of leaf initiation of class I KNOX genes (Lincoln et al., 1994; Dockx et al., 1995; Long et al., 1996; Semiarti et al., 2001), and the subsequent expression of the *ASYMMETRIC LEAVES* genes (AS1 and AS2) (Byrne et al., 2000; Byrne et al., 2002). AS1 codes for a myb transcription factor (Byrne et al., 2000; Sun et al., 2002), and AS2 encodes a protein containing the LATERAL ORGAN BOUNDARIES domain (Iwakawa et al., 2002; Shuai et al., 2002). Both AS genes interact in the same pathway to promote the differentiation of leaf cells by maintaining the repression of BP, KNAT2 and KNAT6. Thus, in the absence of any of the AS products, these three KNOX genes are misexpressed in leaves (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Xu et al., 2003).

¹División de Genética, Universidad Miguel Hernández, Campus de San Juan, Ctra. de Valencia s/n, 03550-San Juan de Alicante, Spain. ²Instituto de Biología Molecular y celular de Plantas (CSIC-UPV), Avda. de los Naranjos s/n, 46022-Valencia, Spain.

*Present address: Instituto de Biología Molecular y celular de Plantas (CSIC-UPV), Avda. de los Naranjos s/n, 46022-Valencia, Spain.

†Present address: Unidad de Reproducción Asistida, Clínica Vistahermosa, Avda. de Denia 103, 03015-Alicante, Spain.

§Author for correspondence (e-mail: laborda@umh.es)

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As early as 1790, Goethe advanced the hypothesis that floral organs are modified vegetative leaves (Coen, 2001). This hypothesis has found strong support in genetic and molecular research carried out during the last 15 years, such as the transformation of floral organs into carpelloid leaf-like organs in a triple mutant lacking the ABC homeotic functions (Bowman et al., 1991), and the transformation of vegetative leaves into floral organs by the ectopic expression of SEPALLATA and floral homeotic genes (Honna and Goto, 2001; Pelaz et al., 2001). Interestingly, AS1 genes are also expressed in carpels (Byrne et al., 2000; Sun et al., 2002). However, despite the probable foliar evolutionary origin of this organ (Friedman et al., 2004), the role of AS1 and AS2 in the downregulation of class I KNOX genes in carpels remains unclear. In this report, we show that AS1 also negatively regulates BP in ovary tissues. Thus, in as1 mutants, BP is misexpressed in the ovary, causing an increase in replum size and a slight reduction in valve width. We also show a strong interaction between loss-of-function alleles in AS1 and FUL, so that double mutants exhibit very large repla and a small valve region. These phenotypes can be interpreted as a shift of valve margins to more lateral positions in the ovary. Our results, showing that BP is expressed in the replum but not in valves and that this gene positively regulates RPL expression, together with the phenotype caused by BP misexpression, strongly suggest that class I KNOX genes play a crucial role in replum development. A model is presented that accounts for the function of these and other genes in patterning the ovary.

MATERIALS AND METHODS

Plant material and growth conditions

Several lines were obtained from NASC (The European Arabidopsis Stock Centre, Nottingham, UK): as1-1 (Redei, 1965) and rpl-2 (pny-40126) (Roeder et al., 2003; Smith and Hake, 2003), both in Col background; as2-1 (Redei, 1965), in An background; bp-1 (Koornneef et al., 1983; Venglat et al., 2002), in Ler background; and transgenic lines 3SS::BP (Chuck et al., 1996) in No-0 background, and KNAT1::GUS-1 (Ori et al., 2000) in Col background. The KNAT1::GUS-18 and as1-1 KNAT1::GUS-18 lines (Ori et al., 2000), both in Col background, were provided by Sarah Hake (Plant Gene Expression Center, Albany, CA) and Naomi Ori (The Hebrew University of Jerusalem, Israel) and the bp-9 rpl-2 (bp-9 pny-40126) double mutant (Smith and Hake, 2003), in Col background, was provided by Sarah Hake. The knat2 gene trap line GT7953 (Byrne et al., 2002) and the BLR::GUS line (Byrne et al., 2003), both in Ler background, were provided by Robert Martienssen (Cold Spring Harbor Laboratory, NY). The rpl-1 mutant (Roeder et al., 2003), in Ler background, and the transgenic line SHP2::GUS (Savidge et al., 1995; Roeder et al., 2003), in No-0 background, were provided by Martin Yanofsky (University of California at San Diego, La Jolla, CA). The ful-1 mutation is in Ler background (Gu et al., 1998). The as1-104 allele has been isolated during a genetic screen of ethyl methanesulphonate (EMS) mutagenized ful-1 plants (Roeder et al., 2003). Plants were grown at 20-22°C with continuous cool-white fluorescent light as previously described (Ripoll et al., 2006).

Plant genetics

Multiple mutants were identified among the F2 from the characteristic mutant phenotype caused by individual mutations: leaf phenotype for as alleles, inflorescence phenotype for rpl alleles, fruit phenotype for ful-1, and the downward-pointing fruit phenotype for bp-1. The wild-type KNAT2 allele was genotyped using two primers, KNAT2-1F (GAGCTGT-CAGTGTCGTACTGG) and KNAT2-1R (CAAGCCTCTTGCGC-CATCAAGC), flanking the Ds transposon. The knat2 homozygous plants did not yield any PCR product, and all their offspring showed resistance to kanamycin. Partial introgression in Col background of the 3SS::BP construct and the as1-1 bp-1 genotype was achieved by crossing twice to Col and as1-1, respectively, to obtain 3SS::BP (2xCol) and as1-1 bp-1 (2xCol) plants.

Student t-tests were performed on the data set of Fig. 2. In every case, the null hypothesis (H0) to be tested was that the lines being compared showed the same phenotype. Tests of statistical significance are included in the supplementary material (see Table S1 in the supplementary material).

Microscopy

Light microscopy and scanning electron microscopy (SEM) were performed as previously described (Ripoll et al., 2006). For GUS staining, samples were treated for 15 minutes in 90% ice-cold acetone, and then washed for 5 minutes with washing buffer (25 mM sodium phosphate; 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide; 1% Triton X-100), vacuum infiltrated for 10 minutes in staining buffer (washing buffer with 2 mM X-Gluc) and incubated overnight at 37°C. Phloroglucinol staining was done as previously described (Liljegren et al., 2000).

In situ hybridization was carried out as described by Ferrándiz and co-workers (Ferrándiz et al., 2000b). A 369 bp fragment from AS1 was amplified by PCR with the primers AS1-7 (GTAGCGAGAGTGTGT-TCTTGTCG) and AS1-8 (CAGGGGCGGTCTAATCTGC) and cloned into the pGEM-T vector (Promega). Two micrograms of Ncol-linearized plasmid were used to generate a DIG-labeled antisense riboprobe. A sense DIG-labeled riboprobe was generated after digestion with SpeI.

Quantitative real-time PCR

RNA was extracted using the Qiagen RNeasy Plant MiniKit, and DNA contamination was removed using the Qiagen RNase-free DNase Set. Reverse-transcription was performed from 1 μg RNA using the SuperScript First Strand kit (Invitrogen). Real-time PCR was performed using the Sybr Green PCR Master Mix (Applied Bioscience) in a volume of 20 μl on an ABI Prism 7000 System (Applied Bioscience). ELONGATION FACTOR 1-α (EF1-α, AT5G60390) was used as an internal control to normalize for variation in the amount of cDNA template (Frigerio et al., 2006). Primers RPL-F (AAGGCCGTTGCTCTCTTGAC) and RPL-R (TCTGTATCTGT-TGGATAAGGATGCA) were used to amplify a 51 bp fragment from RPL cDNA. The reported values are averages of two biological replicates, each one composed of three technical replicates. To calculate relative expression levels, RPL transcript levels were normalized relative to the standard EF1-α using the equation \( \Delta \Delta C_t = C_t(\text{RPL}) - C_t(\text{EF1-α}) \). Relative expression levels were calculated by applying the formula 2^\(-\Delta \Delta C_t\).
a null allele of AS1 in Ler background (see Fig. S1 in the supplementary material), also showed large replum and a reduction in valve width (Fig. 1G-L; see Table S1 in the supplementary material). In addition, fruits of as2-1 displayed the same phenotype as seen in those of as1-1 (Fig. 1M), suggesting that both genes collaborate in the same pathway of fruit patterning.

It has been previously reported that siliques of some as1 mutants, like as1-101, hardly dehisce (Sun et al., 2002). However, we observed no defects in the dehiscence of as1-1 siliques, and phloroglucinol staining of cross sections of these fruits showed normal valve margins and unaffected lignification patterns (Fig. 1Q,R). Consistent with these phenotypes, GUS staining from a SHP2::GUS reporter in the as1-1 background exhibited a pattern similar to that seen in the wild type, although the increased size of the replum was clearly highlighted (Fig. 1S-U).

**BP is involved in the fruit phenotype of as1 mutants**

Misexpression of class I KNOX genes appears as a possible cause for the fruit phenotypes observed in as1-1 and as2-1 mutants. Thus, we examined the effect on the fruit of the 35S::BP construct, in the original No-0 background and after its partial introgression in Col [35S::BP (2xCol) plants]. These plants displayed a phenotype similar to that seen in as1-1 fruits (Fig. 1N; see Table S1 in the supplementary material). Replum were wider than those of wild-type plants and showed an increased cell number, while the valves exhibited a small reduction in size because of the lower number of cells (Fig. 2; see Table S1 in the supplementary material). Numbers of cells in valves and replum of wild-type segregants of the introgression in Col of the 35S::BP construct (valve=63±3.9, n=20; replum=8±0.9, n=20) were also clearly different from those seen in 35S::BP and 35S::BP (2xCol) plants (see Table S1 in the supplementary material).

To further investigate the role of BP in the mutant phenotype, we obtained double mutants carrying as1-1 and the null allele bp-1, which were inspected after two backcrosses with Col [as1-1 bp-1 (2xCol) plants], as well as as1-104 bp-1 plants in Ler background. The alteration produced in fruits by both as1 mutations was partially alleviated in the double mutants. Replum were narrow, practically reverting to the appearance of the wild type (Fig. 1O,P), and numbers of cells in both replum and valves were different from those of the as1 mutants (Fig. 2; see Table S1 in the supplementary material). This partial rescue suggests that factors redundant with BP are also involved in the fruit
mutant phenotype of *as1-1*, and the other class I KNOX genes can be considered good candidates for such a redundant activity. A possible candidate is the *KNAT2* gene, which is expressed in the wild-type replum (Ori et al., 2000; Pautot et al., 2001). However, the *knat2* allele did not modify the phenotypes of *as1-1* and *as1-1 bp-1* fruits (see Fig. S4E-H in the supplementary material). This suggests that either *KNAT2* does not participate in the fruit mutant phenotype conferred by the *as1-1* allele or that *KNAT2* is completely redundant with another class I KNOX family gene.

**BP is overexpressed in *as1* ovaries**

Previous reports have shown the expression of *AS1* in carpels and fruits, although such studies have not addressed the expression of the gene in specific tissues of these organs (Byrne et al., 2000; Sun et al., 2002). Therefore, we first studied the expression of *AS1* in wild-type fruits by in situ hybridization. High levels of *AS1* transcripts were detected in valves, and low levels in the replum (Fig. 3A,B; see Fig. S2A-D in the supplementary material). This result is consistent with the phenotype of *as1-1* fruits, where both replum and valve tissues were altered.

![Fig. 2.](image) **Fig. 2.** Histograms indicating the number of outer epidermal cells in the valve and replum of several wild-type and mutant *Arabidopsis* lines. (A) Replum; (B) valve. Numbers inside the bars represent the mean number of cells, and lines on top represent standard deviations. Fruits from at least five plants for each genotype were collected for cell counting. At least 20 valves and 20 repla were counted for each line.

![Fig. 3.](image) **Fig. 3.** *AS1* represses the expression of *BP* in the *Arabidopsis* gynoecium. (A) In situ localization of *AS1* mRNA in a cross section of a Col pistil (stage 10), showing strong expression in valves and lower levels in replum. (B) A control section of a Col pistil (stage 10) hybridized with a sense probe, showing no signal. (C-G) Staining from the *KNAT1*:GUS-18 reporter in Col and *as1-1*. In the wild-type background, staining is restricted to the replum, valve margin and style of stage 12 gynoecia (C,E), and the same staining is seen in a fruit at stage 15 (G). In the *as1-1* background, valves of stage 12 gynoecia show ectopic expression of *BP* (D,F). (H,I) Staining from the *KNAT1*:GUS-18 reporter allows the detection of variations in expression intensity, and shows that *BP* expression in the replum of stage 15 fruits is more intense in the *as1-1* mutant (I) than in the wild-type (H) background. All gynoecia and fruits are in the *ER* background. Scale bars: 1 mm in G; 0.5 mm in H,I; 200 μm in C,D; 100 μm in A,B,E,F r, replum; st, style; v, valve.
As previously mentioned, the As1- fruit phenotype could probably be explained by misexpression of BP, and perhaps other class I KNOX genes. To test this hypothesis, we used two independent transgenic lines carrying the same construct that allows expression of the GUS reporter gene under control of the BP promoter, the KNAT1::GUS-1 and KNAT1::GUS-18 lines (Ori et al., 2000). During wild-type gynoecium development, the KNAT1::GUS-18 reporter was expressed in a stripe of cells that would develop into the replum and valve margin, with the highest levels detected at stage 12 (Fig. 3C,E). This expression was conserved during later stages as fruits developed (Fig. 3G). Beginning at stage 12, strong expression was also detected in the style (Fig. 3C; see Fig. S2E in the supplementary material). Examination of KNAT1::GUS-18 expression in as1-1 showed that the reporter activity occurred in a broader domain in the presumptive replum (Fig. 3D) and was ectopically observed, being detected in all tissues of as1-1 ovaries, including the valves (Fig. 3D,F). This result accounts for the participation of BP in the mutant phenotype of as1-1 fruits, and the similarity of the fruit phenotype observed between as1-1 and 35S::BP plants. In addition, the use of the KNAT1::GUS-1 transgene revealed a conspicuous increase in GUS staining intensity in the replum of as1-1 fruits (Fig. 3H,I). This finding strongly suggests that BP expression in the replum is higher in the as1-1 mutant than in the wild type.

**BP and RPL interact in the replum.**

Our results show that BP is mainly expressed in the presumptive replum and that its overexpression produces large repla and valves slightly reduced in size, as might be expected for a gene that functions to promote replum development. Moreover, recent reports have shown that the RPL protein, which is required in the replum to negatively regulate the expression of valve margin genes (Roeder et al., 2003), binds the class I KNOX transcription factors BP, STM and KNAT6 to form heterodimers that regulate meristem function (Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004). Therefore, a reasonable hypothesis is that BP and RPL also interact in the replum.

We used a reporter line for RPL, the 35S::BP Arabidopsis (Byrne et al., 2003), to examine the expression of the gene in wild-type and 35S::BP plants. In order to compare both expressions in homogeneous genetic backgrounds, we studied GUS staining in two kinds of F1 individuals carrying the 35S::BP/BLR::GUS line in heterozygosis, those resulting from a cross between 35S::BP/+ and BLR::GUS/+ plants and those resulting from a cross between BLR::GUS and No-0 plants (+/+;BLR::GUS/+ plants). RPL expression in the wild type was confined to the replum, with the strongest signal at stage 12 (Fig. 4A), as previously reported (Roeder et al., 2003). Plants containing
the 35S::BP construct exhibited ectopic expression of BLR::GUS in cotyledons, leaves, valves and valve margins (Fig. 4A-D), which indicates that BP positively regulates the expression of the RPL promoter. This activation was confirmed by quantitative real-time PCR (qRT-PCR), which showed an increase of RPL transcripts in 35S::BP plants compared with the No-0 accession (see Fig. S3 in the supplementary material).

In rpl mutants, replum cells express valve margin identity genes. Consequently, rpl-1 and rpl-2 repla exhibit stripes of narrow cells very similar to those of the valve margin (Fig. 4E,F,K) (Roeder et al., 2003). In accordance with the strong mutant phenotype for meristem function previously reported in bp-9 rpl-2 plants (Smith and Hake, 2003), fruits of this double mutant exhibited a more severe replumless phenotype than rpl-2 (Fig. 4G), similar to that shown by the strong rpl-3 mutant (Roeder et al., 2003), suggesting that BP and RPL also interact in the replum. However, fruits produced by bp-1, knat2 and bp-1 knat2 plants showed a wild-type aspect, both in replum and valves (see Fig. S4A-D in the supplementary material), which indicates that the activity of these genes is not indispensable for RPL function and replum development, probably due to the redundant activities of other class I KNOX genes (Byrne at al., 2002).

We then obtained as1 rpl double mutants to investigate whether the overexpression of class I KNOX genes affects the replumless phenotype caused by rpl alleles. Thirteen out of 24 repla of the as1-1 rpl-2 double mutant exhibited a wild-type phenotype (Fig. 4H), whereas the remaining 11 showed a moderate mutant phenotype (not shown), indicating a partial rescue of rpl-2 repla by as1-1. The as1-1 allele also rescued replum development when combined with rpl-1. Thus, outer repla of the as1-1 rpl-1 double mutant, both in ER and ER backgrounds, displayed either a wild-type (Fig. 4I) or a moderate mutant (Fig. 4J) phenotype. This suggests that, in the absence of RPL, an excess of class I KNOX products may prevent, either directly or indirectly, the expression of valve margin identity genes in the replum. In addition, the number of outer epidermal cells in valves of as1-1 rpl-2 fruits (48.3±5.2; n=18) was similar to those of as1-1 and 35S::BP plants (Fig. 2), indicating that RPL plays no role in the reduced valve width of these plants.

**Synergistic interaction between as1 and ful mutant alleles**

BP is expressed in the presumptive replum and valve margin, and might have a role in controlling pattern formation in these tissues. In this sense, the fruit phenotype caused by the as1-1 mutation and the resulting overexpression of BP could be interpreted as a lateral shift of the borders between the territories of the replum and the valves in the ovary, which would result in replum expansion, a consequent change in the positions of the valve margins, and a modest reduction in valve size. According to this hypothesis, eliminating FUL, a gene important for valve development, in a background that overexpresses BP should result in a synergistic interaction, severely affecting both replum and valves, owing to a greater shift of the borders.

After pollination, ful-1 fails to appropriately differentiate and elongate its valve cells, because of the ectopic expression of valve margin identity genes (Ferrándiz et al., 2000a; Liljegren et al., 2004). Consequently, mutant siliques are small in size and show compressed and creased repla. The reporter is expressed only in the valves of as1-104 (E), ful-1 (F) and as1-104 ful-1 (G) fruits. Note the very reduced width of valves and the large size of repla in the double mutant (G). The as1-104 fruit in E is heterozygous for the ful-1 reporter. (H,I) Stage 17 fruits from the as1-1 ful-1 rpl-1 triple mutant showing a reduced mutant phenotype compared with the as1-104 ful-1 double mutant. SEM (H) and transverse section (I) displaying GUS staining from the ful-1 reporter in the aberrant valves. All fruits are in the er background. Scale bars: 1 mm in A,B; 400 μm in C,D,H; 200 μm in E-G,I.

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As the ful-1 mutation is caused by a Ds transposon carrying a GUS enhancer trap element that has a transcription pattern that mimics the expression domain of the FUL gene, we studied the GUS
expression pattern driven by the FUL promoter in ful-1, as1-104 and as1-104 ful-1 fruits. Expression of the FUL enhancer trap in the ful-1 single mutant was restricted to the valve region (Fig. 5F), as previously reported (Gu et al., 1998), and the same expression pattern was detected in as1-104 plants that carried the ful-1 allele in heterozygosis (Fig. 5E). This expression remained unchanged in double mutant siliques in which GUS staining was also detected in the aberrant valves (Fig. 5G). Interestingly, the comparison of GUS staining in the single and double mutants clearly showed the different sizes of valves and repla. The valve region was much more reduced in as1-104 ful-1 than in the single mutants, while the opposite occurred with the replum, which was much larger in the double mutant (Fig. 5E-G).

As shown above, RPL does not contribute to the reduction in cell numbers in the valves of as1-1 and 35S::BP siliques, as the number of outer epidermal cells in valves of these fruits is similar to those of as1-1 rpl-2 plants. However, this observation does not exclude the possibility that RPL might participate in the fruit phenotype caused by as1 null alleles in the absence of FUL function. To examine this, we crossed the as1-1 rpl-1 double mutant, in ER background, with ful-1 to obtain the as1-1 ful-1 rpl-1 triple mutant in both ER and er backgrounds. Siliques from these plants exhibited a more moderate mutant phenotype, both in valves and replum, than those of the as1-104 ful-1 double mutant (Fig. 5H,L). This result indicates that RPL participates in the strong phenotype of as1 ful-1 and 35S::BP ful-1 siliques, along with BP and other class I KNOX genes.

**DISCUSSION**

Taking into consideration the evolutionary relationship between leaves and carpels, in an attempt to understand how the genes involved in leaf development participate in fruit formation, this study reports on the roles of AS1 and BP in fruit development. BP is expressed in the replum and valve margin, while AS1 transcripts are detected at high levels in valves and at low levels in the replum. In the absence of the AS1 function, BP is misexpressed, causing a conspicuous increase in replum size and a slight reduction in valve territory. This phenotype can be explained by a small shift of the valve margins to more lateral positions. We discuss below a model that integrates the function of these and other genes in patterning the mediolateral axis of the ovary.

**AS function represses BP in the gynoecium**

The mechanism involved in patterning the ovary shows interesting similarities to events that occur at the shoot apex to pattern the apical meristem and lateral organs. In the gynoecium, the activities of FIL, YAB3 and JAG promote valve and valve margin development, while RPL represses the expression of these genes in the replum, ensuring the formation of this tissue (Dinneny et al., 2005). In the shoot apex, the antagonistic activities of meristematic genes and lateral organ-expressed genes allow meristem maintenance, restricting organogenesis to the organ primordium. Thus, RPL is expressed in the meristem, where its product binds most class I KNOX proteins (STM, BP and KNAT6) to regulate developmental processes (Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004), whereas FIL, YAB3 and JAG are exclusively transcribed in lateral organs. Interestingly, several class I KNOX genes are expressed in the replum (this work) (Long et al., 1996; Pautot et al., 2001), a tissue that seems to have meristematic properties, because it gives rise to the placenta, where ovules are produced. All this suggests that the replum displays some kind of meristematic attributes (Roeder and Yanofsky, 2006), while the valves are more related to leaf blades.

The AS genes are expressed in leaf primordia, where they repress BP, KNAT2 and KNAT6 (Byrne et al., 2000, 2003; Ori et al., 2001), but not the related STM gene, which, in turn, negatively regulates AS1 and AS2, so that neither of these genes is transcribed in the meristem (Byrne et al., 2002). Similarly, in the gynoecium, AS1 is expressed in valves, where it also represses BP, which is transcribed only in the replum and valve margin. Thus, as1 alleles cause ectopic expression of BP in valves, giving rise to an abnormal fruit phenotype. Accordingly, plants carrying the 35S::BP transgene display this same phenotype, in such a way that overexpression of the BP gene alone could account for the AS1 fruit phenotype. Nevertheless, removal of BP function in the as1 background does not completely rescue the mutant phenotype, suggesting that other class I KNOX genes may also be misexpressed in as1 pistils. Mutations in AS2 produce the same fruit phenotype as as1 alleles, suggesting that this gene interacts with AS1 in the pistil to repress class I KNOX genes, as it does in leaves (Byrne et al., 2002, Xu et al., 2003). Moreover, AS1 is also expressed in the replum, although at lower levels, and this seems to be necessary to restrain BP transcripts below certain levels, as the intensity of GUS staining in the replum of plants carrying KNAT1::GUS-1 clearly increases in an as1 background. Although the overlapping expression of AS1 and BP in the replum may appear contradictory with previous studies carried out in leaves (Byrne et al., 2000; Ori et al., 2000), the activities of these two genes are not necessarily exclusive, as both are expressed in the leaves of several mutants (Kumaran et al., 2002; Hay et al., 2006).

**Do class I KNOX genes confer replum identity?**

Along the mediolateral axis, RPL is the only gene that has so far been shown to play a role in replum differentiation. However, several multiple mutant backgrounds lacking RPL function, such as as1 rpl, shp1 shp2 rpl, jag rpl and fil rpl, develop basically normal repla (this work) (Roeder et al., 2003; Dinneny et al., 2005), indicating that this gene is not indispensable for replum formation. Therefore, there must be other gene function(s) involved in the elaboration of the basal pattern for replum identity.

Although there are no conclusive data, several lines of argument support the idea that class I KNOX genes might play this role. First, these genes are transcribed in the replum, but not in valves. This is the case for STM (Long et al., 1996), KNAT2 (Pautot et al., 2001) and BP (this work). Second, the overexpression of BP, both in as1 mutants and 35S::BP plants, increases the size of the replum, whereas the valve territory appears slightly reduced, suggesting that BP promotes replum development and has an opposing role in valve formation. Third, BP activates the expression of RPL, a gene that plays a crucial role in the replum. This function of BP may be redundantly carried out by other class I KNOX genes, as the expression of RPL is not affected in a bp mutant background (Smith and Hake, 2003). And fourth, BP interacts with RPL in the replum, as the bp-9 rpl-2 double mutant shows a stronger replumless phenotype than rpl-2.

Despite this putative function of BP in replum development, no mutant phenotype in this tissue has been found to be caused by a null bp allele. A likely reason for this behavior is the known functional redundancy among class I KNOX genes (Byrne et al., 2002). Thus, BP function in the replum of bp mutants could be assumed by STM, as their products share high homology (Byrne et al., 2002), or by KNAT6, which acts redundantly with STM in the shoot apical meristem (Belles-Boix et al., 2006). Regrettably, the redundancy among the members of this gene family precludes a functional
analysis with loss-of-function mutations, since this strategy should require the isolation of multiple mutant lines lacking a shoot apical meristem.

**Patterning along the mediolateral axis of the ovary requires the antagonistic activities of valve and replum genes**

A recent work has proposed a cogent model that accounts for regionalization and differentiation of tissues along the mediolateral axis of the ovary (Dinneny et al., 2005). According to the model, the cooperating activities of FIL, YAB3 and JAG (FILJAG activity) promote the expression of FUL and SHP genes in the valve and the presumptive valve margin, respectively, in such a way that high levels of FILJAG activity in the valve would activate FUL expression, whereas the transcription of SHP genes would require only a weak FILJAG activity present in the valve margin. The FUL product, in turn, prevents the expression of SHP genes in valves. This same function is carried out by RPL in the replum through the negative regulation of FIL, YAB3 and JAG. Thus, by the action of FUL and RPL, SHP activity is restricted to the presumptive valve margin (Dinneny et al., 2005). Moreover, it has been suggested that an unknown replum factor is involved in the negative regulation of FUL expression (Liljegren et al., 2004), and it has been shown that the ectopic expression of FUL inhibits the differentiation of the outer replum (Ferrándiz et al., 2000a), suggesting that FUL negatively regulates replum genes and/or their products. These data, together with the downregulation of class I KNOX genes by FIL and YAB3 (Kumaran et al., 2002), support the notion that there are antagonistic gene activities in replum and valves.

We now add AS and class I KNOX genes to the model (Fig. 6). AS1 is expressed at high levels in valves and at lower levels in the replum, thus preventing the expression of class I KNOX genes in valves while maintaining the products of these genes below certain levels in the replum. This function (AS function in Fig. 6) would be brought about in collaboration with AS2, as as2 alleles produce the same fruit phenotype as as1 mutations. We propose that the territories of valve and replum become established by the opposing activities of valve factors (FILJAG activity) and replum factors (class I KNOX genes), while the valve margin forms in a narrow stripe in which both valve and replum factors are expressed. Valve factors should be working through a gradient, with the strongest activity in the middle of the valve, coinciding with the lateral plane of the ovary, in strong agreement with the role of the FILJAG activity in inducing, by means of a concentration-dependent mechanism, the expression of FUL and SHP genes in adjacent domains, valve and valve margin, respectively (Dinneny et al., 2005). In addition, we hypothesize that class I KNOX genes would be expressed at the highest level in the replum, while low levels of FIL and YAB3 proteins should exert a partial downregulation on this family of genes in the valve margin, because this repression is known to occur in leaves (Kumaran et al., 2002). This model is a variation of the basic French flag model for pattern formation (Wolpert, 1969), whereby three territories would be determined by the contribution of the opposing gradients of two antagonistic factors.

According to our model, in as1 and 35S::BP fruits, class I KNOX genes become overexpressed in the replum region and are ectopically transcribed in valves, where they antagonize the FILJAG activity, resulting in a shift in the position of the valve margin along the mediolateral axis. Moreover, lack of outer replum in 35S::FUL fruits (Ferrándiz et al., 2000a), the synergistic relationship between as1 and ful alleles (this work), and the reduction of the mutant phenotype in the triple as1 ful rpl with respect to the as1 ful double mutant (this work) suggest that FUL has an inhibitory role on RPL, and perhaps on class I KNOX genes as well. The model also accounts for previous results. For instance, fil mutants show a large replum, yet FIL is not expressed in this domain (Dinneny et al., 2005). A possible explanation is that a fall in FILJAG activity would produce an expansion of the expression of the counteracting replum genes, causing a shift in valve margin position. In 35S::FUL fruits, the ectopic expression of FUL would inhibit replum gene function, allowing FILJAG activity to exert its role throughout the ovary.

This work provides further information on the connection between leaf and carpel development, through the establishment of the possible functions of BP and ASI in fruit patterning. The pleiotropic behavior of these two genes is founded in their expression in several organs, the different morphologies of which could be explained by changes in the regulation of the genes, by different responses of their target genes and/or by the participation of other interacting genes. This same argument may be extended to the different contribution of one gene in two species. A recent work has demonstrated that a rice ortholog of RPL participates in seed shattering and that a punctual mutation in its regulatory sequence is involved in loss of seed shattering and domestication of this cereal (Konishi et al., 2006). Thus, although the dehiscence zone in the Arabidopsis fruit and the abscission layer at the base of the rice grain are structures that do not share the same botanical origin, both require RPL function for their formation. Understanding the contribution of specific genes in the formation of different structures will help to unravel the evolutionary relationships both between organs and between species.

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![Fig. 6. A model for pattern formation along the mediolateral axis of the ovary in Arabidopsis.](image)


