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

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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, interacts in the replum with *REPLUMLESS* (*RPL*), an essential gene for replum development, and positively regulates the expression of this gene. Misexpression of *BP* in the gynoecium causes an increase in replum size, while the valve width is slightly reduced, and enhances the effect of mutations in *FRUITFULL* (*FUL*), 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 factors and replum factors, 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., 1999). 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 in valves 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 *ALCATRAZ* (*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).

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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 (Honma and Goto, 2001; Pelaz et al., 2001). Interestingly, *AS* 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 L*er* background; and transgenic lines *35S::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 at al., 2002) and the *BLR::GUS* line (Byrne et al., 2003), both in L*er* background, were provided by Robert Martienssen (Cold Spring Harbor Laboratory, NY). The *rpl-1* mutant (Roeder et al., 2003), in L*er* 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 L*er* 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 F_2 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 (GACGTT-TCAGTGTCGTACTGG) and KNAT2-1R (CAAGCCTCTTGGC-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 *35S::BP* construct and the *as1-1 bp-1* genotype was achieved by crossing twice to Col and *as1-1*, respectively, to obtain *35S::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 (H_o) 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-TCTTGTC) and AS1-8 (CAGGGGCGGTCTAATCTGC) and cloned into the pGEM-T vector (Promega). Two micrograms of *Nco*I-linearized plasmid were used to generate a DIG-labeled antisense riboprobe. A sense DIG-labeled riboprobe was generated after digestion with *Spe*I.

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 (AAGGGCTTGGCTCTTCGATC) 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 $\Delta C_T = C_T(RPL) - C_T(EF1\text{-}\alpha)$. Relative expression levels were calculated applying the formula $2^{-[\Delta CT\ (35S::BP)\ -\Delta CT\ (No\text{-}0)]}$.

RESULTS

Loss-of-function mutations in *AS* genes alter fruit morphology

Most hypotheses proposed to explain the origin of carpels argue that they have evolved from leaf-like organs, so that they are thought to have some kind of foliar attributes (Friedman et al., 2004; Dinneny et al., 2005; Balanzá et al., 2006). In an attempt to identify new functions involved in fruit patterning, we studied the possible role in this process of genes that determine important functions in leaf development. Two such candidates are *AS1* and *AS2*, the products of which physically interact to prevent the expression in leaves of the class I KNOX family genes *BP*, *KNAT2* and *KNAT6* (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Byrne et al., 2002; Xu et al., 2003).

We compared fruits of *as1-1* and Col, and found that the mutant displayed slightly altered siliques with a moderate bumpy appearance (Fig. 1A,B). A closer inspection of these fruits showed that they had developed enlarged repla (Fig. 1C,D). Cross sections revealed that mutant repla contained more cells than those of the wild type, as seen in the outer layer of the replum (Fig. 1E,F; Fig. 2; see Table S1 in the supplementary material). Unlike the case in the replum, the number of outer epidermal cells in mutant valves was smaller (Fig. 2; see Table S1 in the supplementary material), causing a reduction in valve width, which was the most likely reason for the bumpy aspect of the fruits. This phenotype was observed irrespective of the genetic background, as the *as1-104* mutant, homozygous for

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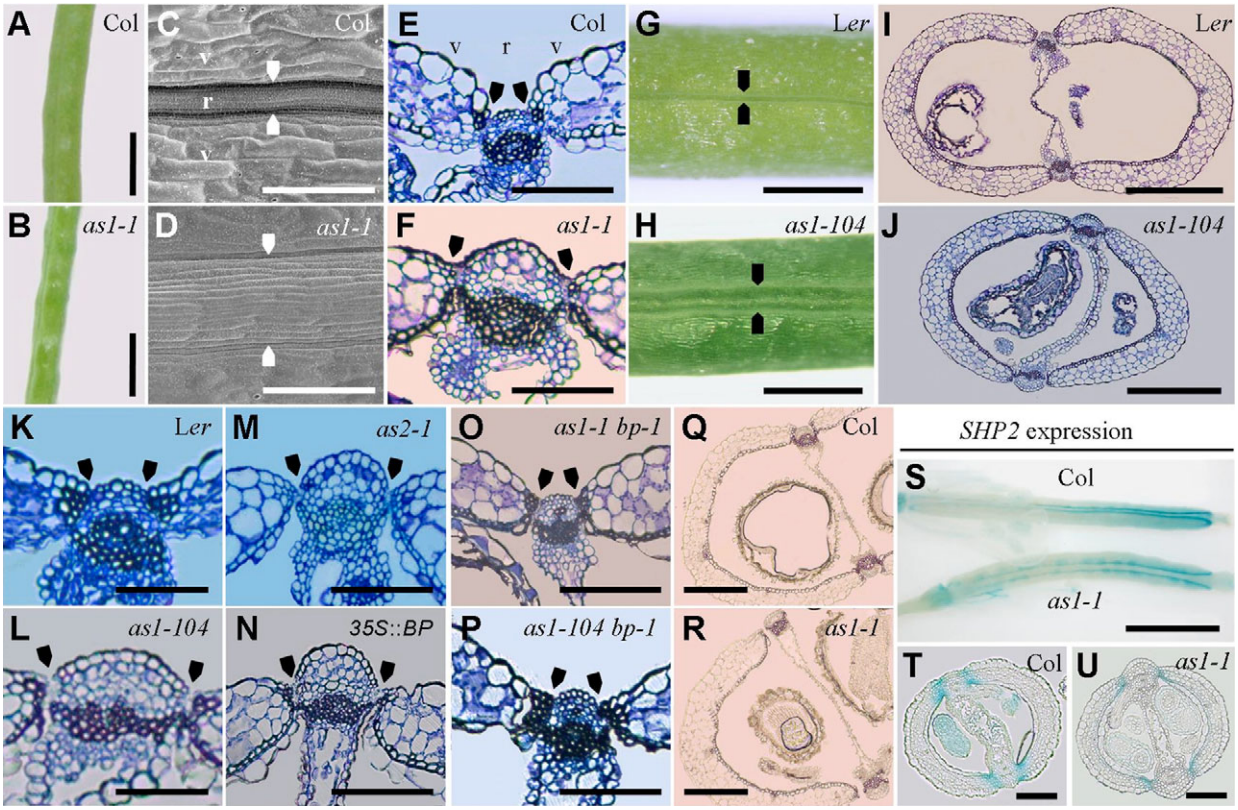

Fig. 1. Patterning defects in *as* mutants and *35S::BP Arabidopsis* plants. (**A**,**C**,**E**) Stage 17 fruits of the wild-type Col accession. (A) Bright-field stereomicroscope image; (C) SEM; (E) cross section. (**B**,**D**,**F**) Stage 17 fruits of the *as1-1* mutant, which produces bumpy fruits (B) and displays large repla both in SEM (D) and cross sections (F). (**G**,**I**,**K**) Bright-field stereomicroscope image (G) and cross sections (I,K) of stage 17 fruits of the L*er* accession. (**H**,**J**,**L**) Bright-field stereomicroscope image (H) and cross sections (J,L) of stage 17 fruits of *as1-104*. Notice the increased size of repla and the reduced size of valves in *as1-104* fruits (I-L). (**M**,**N**) Cross sections of stage 17 fruits of *as2-1* (M) and the *35S::BP* line (N), showing the large replum phenotype also seen in *as1-1* (D,F). (**O**,**P**) Cross sections of stage 17 fruits of the *as1-1 bp-1* (O) and *as1-104 bp-1* (P) double mutants that show strong recovery of the wild-type replum phenotype. (**Q**,**R**) Lignification pattern of stage 18 fruits of Col (Q) and *as1-1* (R) stained with phloroglucinol. (**S-U**) GUS expression in the valve margin driven by the *SHP2* promoter in Col (S,T) and *as1-1* (S,U) fruits at stage 15. Fruits in the pictures are in the *ER* background, with the exception of those in G-L and P, which are in the L*er* background. Arrowheads indicate the positions of valve margins. Scale bars: 2 mm in A,B,S; 1 mm in G,H; 500 μm in Q,R; 200 μm in I,J; 100 μm in C-F,K-P,T,U. r, replum; v, valve.

a null allele of *AS1* in L*er* background (see Fig. S1 in the supplementary material), also showed large repla and a reduction in valve width (Fig. 1G-L; Fig. 2; 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). Repla 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 repla 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 L*er* background. The alteration produced in fruits by both *as1* mutations was partially alleviated in the double mutants. Repla were narrow, practically reverting to the appearance of the wild type (Fig. 1O,P), and numbers of cells in both repla 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

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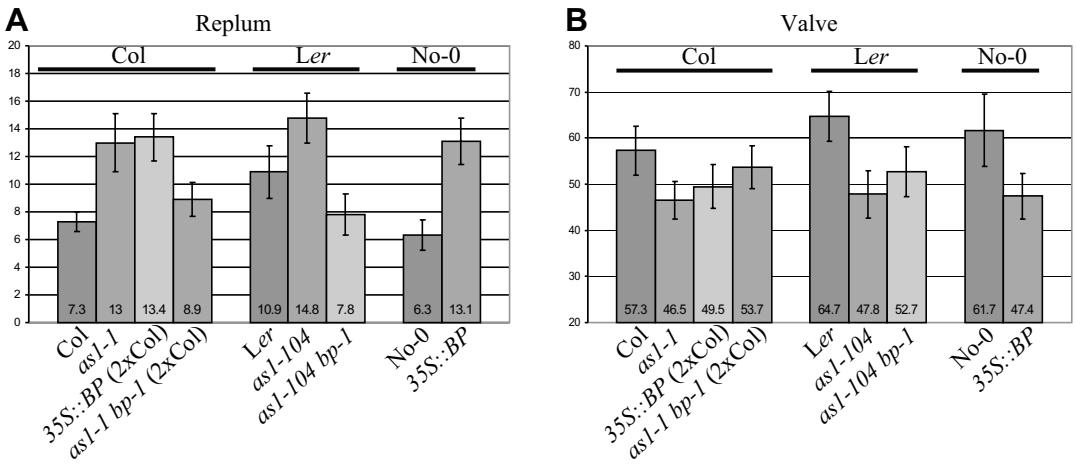

Fig. 2. Histograms indicating the number of outer epidermal cells in the valve and replum of several wild-type and mutant *Arabidopsis* lines. (**A**) Replum; (**B**) valve. Numbers inside the bars represent the mean number of cells, and lines on top represent standard deviations. Fruits from at least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

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.

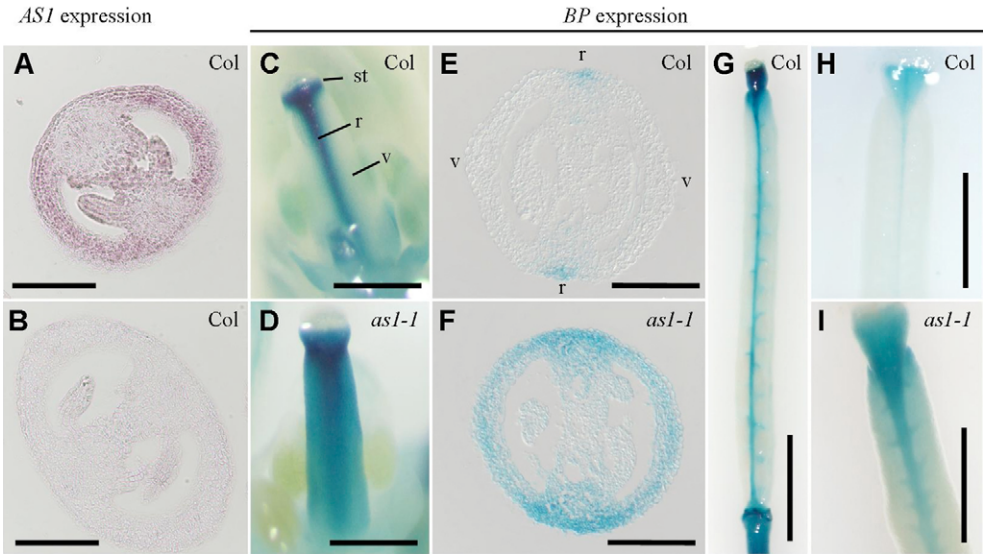

Fig. 3. *AS1* represses the expression of *BP* in the *Arabidopsis* gynoecium. (**A**) In situ localization of *AS1* mRNA in a cross section of a Col pistil (stage 10), showing strong expression in valves and lower levels in replum. (**B**) A control section of a Col pistil (stage 10) hybridized with 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-1* 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.

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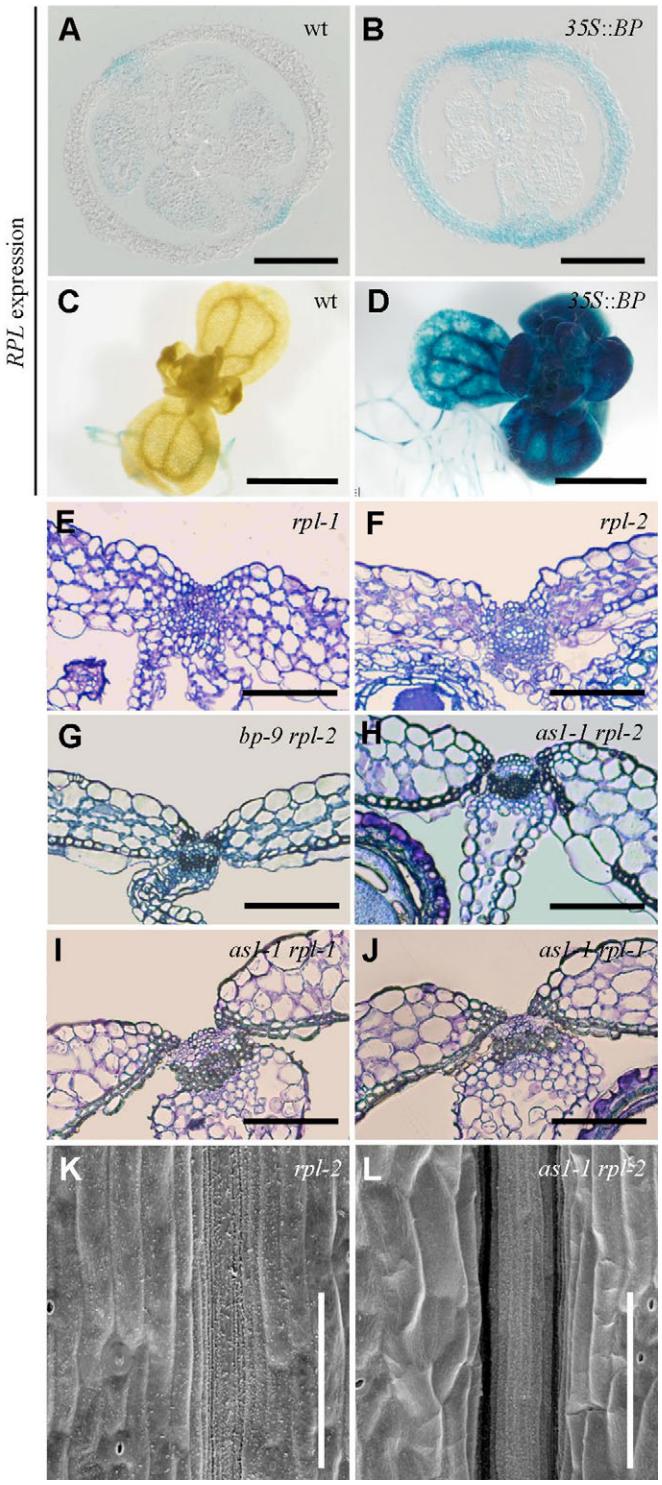

Fig. 4. Interactions of *RPL* with *BP* and *AS1*. (**A**,**B**) Cross sections of stage 13 gynoecia from wild-type and *35S::BP Arabidopsis* plants, showing GUS staining from the *BLR::GUS* transgene, a reporter of *RPL*. (A) GUS staining is restricted to the replum in the wild type. (B) GUS is detected throughout the ovary, including valves and valve margins, in *35S::BP* gynoecia. (**C**,**D**) GUS staining from the *BLR::GUS* transgene in rosettes. In the wild-type background (C), staining is detected in the meristem (hidden by the leaves in the picture), whereas the signal is ectopically seen in cotyledons and leaves of plants that overexpress *BP* (D). (**E**,**F**) Cross sections of stage 17 fruits from several mutants harboring *rpl* alleles. Fruits from *rpl-1* (E) and *rpl-2* (F) show narrow repla containing cells that adopt a valve margin identity. (**G**) The replumless phenotype is even stronger in the *bp-9 rpl-2* double mutant. (**H-J**) The *as1-1* allele rescues the replumless phenotype conferred by *rpl* alleles. The wild-type phenotype is observed in *as1-1 rpl-2* (H) and *as1-1 rpl-1* (I) fruits. (J) A moderate replumless phenotype is observed in an *as1-1 rpl-1* fruit. (**K**,**L**) SEMs of stage 17 fruits showing the replumless phenotype of *rpl-2* (K) and the wild-type phenotype of an *as1-1 rpl-2* fruit (L). The genetic background is *ER*, with the exception of E, I and J, in which *rpl-1* and *as1-1 rpl-1* are in the *er* background. Scale bars: 1 mm in C,D; 100 μm in A,B,E-L.

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 *BLR::GUS* line (Byrne at 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 F_1 individuals carrying the *BLR::GUS* construct in heterozygosis, those resulting from a cross between *BLR::GUS* and *35S::BP* plants (*35S::BP*/+;*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

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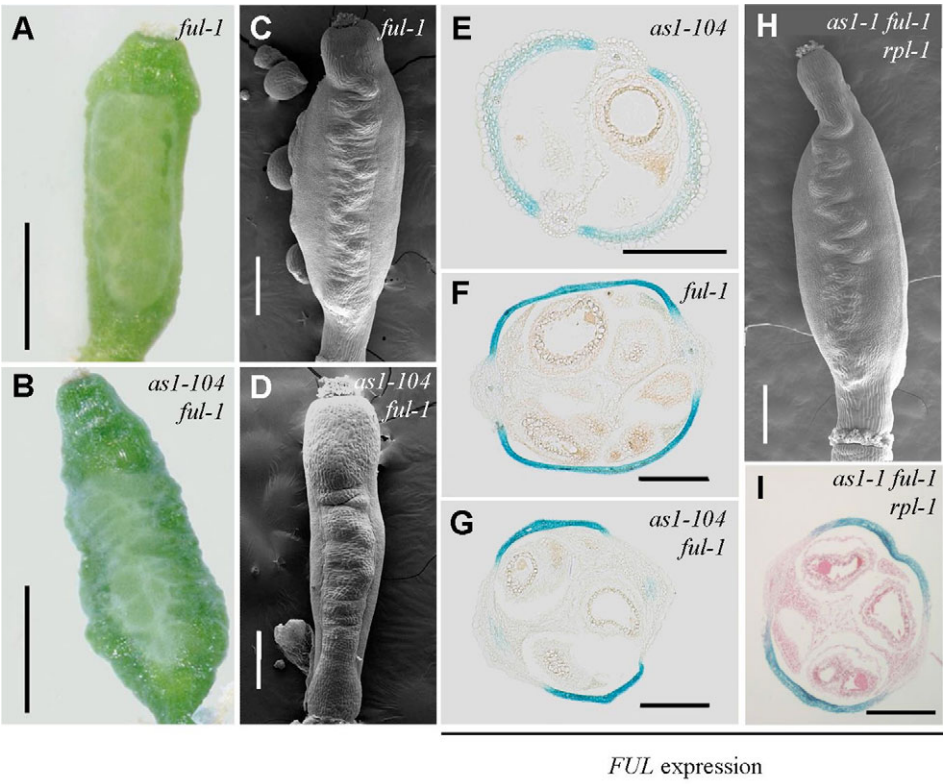

Fig. 5. Synergistic interaction between *ful-1* and *as1* alleles. (**A-D**) Bright-field stereomicroscope images (A,B) and SEMs (C,D) of stage 17 fruits. The *ful-1* mutant exhibits small valves and enlarged and creased repla (A,C). The *as1-104* allele enhances the mutant phenotype of *ful-1*, so that *as1-104 ful-1* double mutants show very small valves and very compressed and distorted repla (B,D). (**E-G**) GUS staining in cross sections of fruits containing the *ful-1* reporter. 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.

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,L), 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 (Fig. 5A,C), a phenotype that can also be interpreted in terms of a shift in the boundaries between valves and replum, giving rise to the small valves and large repla of *ful-1*. As predicted, the *as1-104 ful-1* double mutant displayed extremely small valves, and very large, rough and distorted repla, indicating a strong enhancement of the phenotypes of the two single mutants both in valves and replum (Fig. 5B,D), and favoring the hypothesis that *BP* overexpression affects the positioning of the borders between valves and replum. The same phenotype was also observed in *as1-1 ful-1*, *35S::BP ful-1* and *as2-1 ful-1* fruits (see Fig. S5 in the supplementary material).

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

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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,I). 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; Ori et al., 2000; Semiarti 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 at 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 at 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 at al., 2002). Thus, *BP* function in the replum of *bp* mutants could be assumed by *STM*, as their products share high homology (Byrne at 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

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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* (*FIL/JAG* 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 *FIL/JAG* activity in the valve would activate *FUL* expression, whereas the transcription of *SHP* genes would require only a weak *FIL/JAG* 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 (*FIL/JAG* 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 *FIL/JAG* 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 *FIL/JAG* 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 *FIL/JAG* 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 *FIL/JAG* activity to exert its role throughout the ovary.

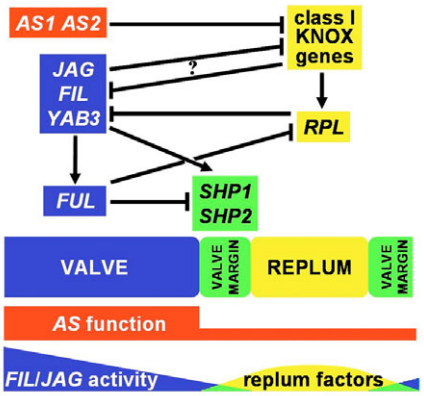

Fig. 6. A model for pattern formation along the mediolateral axis of the ovary in *Arabidopsis*. Three domains are specified by the opposing gradients of two antagonistic factors: valve factors (*FIL/JAG* activity; blue) and replum factors (class I KNOX genes; yellow). The *FIL/JAG* activity specifies valve formation, class I KNOX genes determine the replum, and the valve margin (green) is formed in the region in which both valve factors and replum factors are expressed. The *AS* function (red), carried out by *AS1* and *AS2*, represses class I KNOX genes, preventing the expression of these genes in valves and maintaining their expression below certain levels in the replum and valve margin.

This work provides further information on the connection between leaf and carpel development, through the establishment of the possible functions of *BP* and *AS1* 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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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/14/2663/DC1

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Table S1. *P*-values from Student's *t*-tests comparing the number of cells in valves and repla of several lines

Lines being compared		*P*-value	
		Valve	Replum
as1	Col	3.54×10^{-8}	1.03×10^{-6}
as1 bp (2xCol)	*as1*	1.19×10^{-4}	1.85×10^{-5}
as1 bp (2xCol)	Col	1.98×10^{-2}	8.86×10^{-6}
35S::BP (2xCol)	*as1-1*	0.13	0.57
35S::BP (2xCol)	Col	2.18×10^{-6}	1.37×10^{-10}
as1-104	Ler	5.61×10^{-11}	4.18×10^{-7}
as1-104	*as1-104 bp-1*	4.72×10^{-2}	3.17×10^{-6}
35S::BP (No-0)	No-0	5.88×10^{-9}	2.45×10^{-11}
wt (2xCol)*	*35S::BP* (2xCol)	5.03×10^{-8}	7.30×10^{-10}

Statistical significance was taken at $P<0.05$. A low *P*-value means that the two lines being compared are very likely to have different fruit phenotypes ($P<0.05$), and a high *P*-value indicates that both lines show a similar phenotype ($P>0.05$).
*Wild-type segregant of the second introgression of the *35S::BP* construct in Col.