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A novel role for the floral homeotic gene *APETALA2* during *Arabidopsis* fruit development

Juan José Ripoll, Adrienne H. K. Roeder*, Gary S. Ditta and Martin F. Yanofsky[‡]

SUMMARY

The majority of the *Arabidopsis* fruit comprises an ovary with three primary tissue types: the valves, the replum and the valve margins. The valves, which are derived from the ovary walls, are separated along their entire length by the replum. The valve margin, which consists of a separation layer and a lignified layer, forms as a narrow stripe of cells at the valve-replum boundaries. The valve margin identity genes are expressed at the valve-replum boundary and are negatively regulated by *FUL* and *RPL* in the valves and replum, respectively. In *ful rpl* double mutants, the valve margin identity genes become ectopically expressed, and, as a result, the entire outer surface of the ovary takes on valve margin identity. We carried out a genetic screen in this sensitized genetic background and identified a suppressor mutation that restored replum development. Surprisingly, we found that the corresponding suppressor gene was *AP2*, a gene that is well known for its role in floral organ identity, but whose role in *Arabidopsis* fruit development had not been previously described. We found that *AP2* acts to prevent replum overgrowth by negatively regulating *BP* and *RPL*, two genes that normally act to promote replum formation. We also determined that *AP2* acts to prevent overgrowth of the valve margin by repressing valve margin identity gene expression. We have incorporated *AP2* into the current genetic network controlling fruit development in *Arabidopsis*.

KEY WORDS: APETALA2, Fruit, Replum, Valve margin, Lignification, Growth

INTRODUCTION

Fruit have evolved a complex tissue organization that protects, nourishes and ultimately disperses the seeds at maturity (Ferrándiz et al., 1999; Ferrándiz, 2002; Balanzá et al., 2006; Roeder and Yanofsky, 2006; Martínez-Laborda and Vera, 2009). In *Arabidopsis thaliana*, the majority of the fruit consists of an ovary, and three distinct tissue types form along the mediolateral axis of the outer ovary (Fig. 1). Two laterally positioned valves (derived from the ovary walls) enclose and protect the seeds, and are separated from each other by a thin ridge of cells called the replum. A narrow stripe of small, rounded cells forms at the valve-replum boundary and is referred to as the valve margin. The valve margin is considered the ripening region in the *Arabidopsis* fruit and can be subdivided into a separation layer (SL) and a lignified layer (LL). When the fruit reaches its final length and is fully mature, it undergoes dehiscence. Fruit dehiscence involves enzymatic and mechanical processes at the valve margin that cause the valves to detach from the replum and release the seeds (Spence et al., 1996; Ferrándiz et al., 1999; Ferrándiz, 2002; Roeder and Yanofsky, 2006).

A suite of regulatory genes, collectively known as valve margin identity genes, is required in *Arabidopsis* for valve margin specification and to ensure seed dispersal (Liljegren et al., 2000; Rajani and Sundaresan, 2001; Liljegren et al., 2004). Although the MADS-box transcription factors SHATTERPROOF1 and SHATTERPROOF2 (SHP1, SHP2) (Liljegren et al., 2000) have been shown to also regulate other processes (Favaro et al., 2003; Pinyopich et al., 2003; Colombo et al., 2010), they are best known for their function in valve margin formation and dehiscence (Ferrándiz et al., 2000a; Liljegren et al., 2000). *SHP* genes work on top of the genetic hierarchy that regulates valve margin formation (Liljegren et al., 2000) and are positive regulators of *INDEHISCENT* (*IND*; Liljegren et al., 2004) and *ALCATRAZ* (*ALC*) (Rajani and Sundaresan, 2001), two genes that encode bHLH transcription factors also required for correct valve margin development. Whereas *IND* acts to specify the lignified layer identity, the combined actions of *IND* and *ALC* promote separation layer formation (Rajani and Sundaresan, 2001; Liljegren et al., 2004). Mutants defective in any of these genes fail to complete valve margin formation and generate indehiscent fruit with the seeds trapped inside (Rajani and Sundaresan, 2001; Liljegren et al., 2004).

The *FRUITFULL* (*FUL*) (Gu et al., 1998) MADS-box gene and the *REPLUMLESS* (also known as *BELLRINGER*, *PENNYWISE*, *LARSON* or *VAAMANA*) (Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004) (*RPL* hereafter) homeobox gene act within the valve and replum, respectively, to repress valve margin gene expression, thus ensuring that expression of the valve margin genes is limited to the valve-replum boundary (Roeder et al., 2003; Liljegren et al., 2004) (Fig. 7). Consistent with this idea is the observation that the valve margin genes are ectopically expressed in *rpl ful* double mutants, resulting in cells that would normally develop into valve and replum instead adopting a valve margin cell fate (Roeder et al., 2003; Liljegren et al., 2004).

In addition to *RPL* and *FUL*, another layer of regulation controls patterning along the mediolateral axis of the fruit. The class I KNOX (KNOTTED-LIKE HOMEOBOX) gene *BREVIPEDICELLUS* (*BP*) (Douglas et al., 2002; Venglat et al., 2002), also known as *KNAT1* (Chuck et al., 1996; Lincoln et al., 1994), has been proposed to promote replum formation, as

Division of Biological Sciences, Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA 92093-0116, USA.

*Present address: Department of Plant Biology, Weill Institute for Cell and Molecular biology, Cornell University, Ithaca, NY 14853, USA
[‡]Author for correspondence (marty@ucsd.edu)

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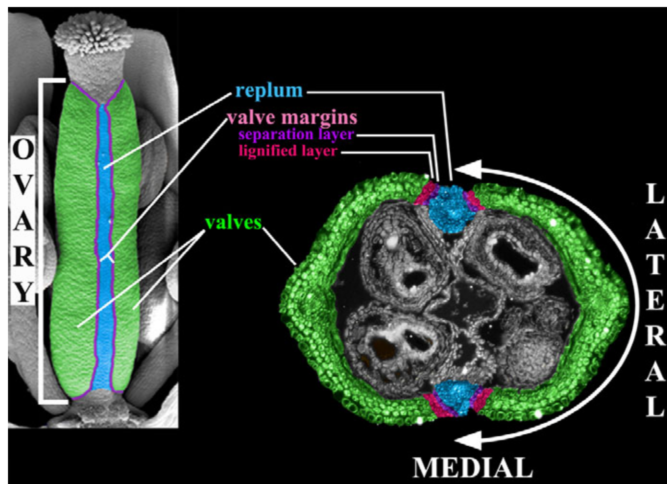

Fig. 1. Anatomy of the *Arabidopsis thaliana* fruit along the mediolateral axis. Scanning electron micrograph (left) of a pistil at stage 12 in which valves have been highlighted in green, replum in blue and valve margins in purple. Cross-section (right) of a stage 16 fruit in which the same color code has been used to delimit the territories. At the valve margin, the separation layer (SL) is highlighted in dark purple and the lignified layer (LL) in light purple.

35S::BP gain-of-function fruits have greatly enlarged repla, and *bp* mutations enhance the reduced replum phenotype of *rpl* mutants (Alonso-Cantabrana et al., 2007). *BP* is negatively regulated in fruits (Alonso-Cantabrana et al., 2007) and in leaves (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Byrne et al., 2002) through the combined activities of the MYB transcription factor *ASYMMETRIC LEAVES1* (*AS1*) (Byrne et al., 2000; Sun et al., 2002) and the *LATERAL ORGAN BOUNDARY* (*LOB*) domain protein *ASYMMETRIC LEAVES2* (*AS2*) (Iwakawa et al., 2002; Shuai et al., 2002; Guo et al., 2008). Mutations in either *AS1* or *AS2* result in fruit with enlarged repla and reduced valves (Alonso-Cantabrana et al., 2007).

To investigate replum formation in greater detail, we performed a suppressor screen on an EMS-mutagenized *rpl ful* population. Because the surface of *rpl ful* ovaries entirely comprises cells with valve margin identity (Fig. 2D-F), recovery of replum development can be easily scored. Surprisingly, one of the suppressors was found to be allelic to the flower organ identity mutant *apetala2* (*ap2*). The *AP2* gene is known to be post-transcriptionally regulated by microRNAs (Aukerman and Sakai, 2003; Chen, 2004). In addition to its role in flower organ identity (Bowman et al., 1989; Bowman et al., 1991; Drews et al., 1991; Jofuku et al., 1994; Kunst et al., 1989), *AP2* is known to regulate flowering time, shoot apical meristem maintenance and seed development (Jofuku et al., 1994; Jofuku et al., 2005; Mathieu et al., 2009; Ohto et al., 2005; Ohto et al., 2009; Würschum et al., 2006; Yant et al., 2010), although the role of *AP2* in fruit development has not been described in detail. During the preparation of this manuscript, two independent studies identified that *SlAP2a*, the true ortholog of *AP2* in tomato, controls fruit ripening via regulation of ethylene biosynthesis and signaling (Chung et al., 2010; Karlova et al., 2011). The role of AP2 in fruit is not only relevant to tomato and the data we present in this study incorporate *AP2* into the regulatory network that controls fruit patterning in *Arabidopsis* by demonstrating that *AP2* acts as a brake on valve margin and replum growth.

MATERIALS AND METHODS

Plant materials, genotyping and growth conditions

This work was performed in the *Arabidopsis thaliana* Columbia (Col) accession. The plant materials used in this study were: *as1-1* in Col (Redei, 1965); *bp-9* in Col (Mele et al., 2003); *bp-9 rpl-2* in Col (Smith and Hake, 2003); *rpl-2 ful-2* in Col (this work); *ap2-413 rpl-2 ful-2* in Col (this work); *ap2-413* in Col (this work); *ap2-7* in Col (Kunst et al., 1989); *ap2-12* in Col (Yant et al., 2010); *ap2-2* in Ler (Bowman et al., 1989); *35S::BP* in No-0 (3xCol) (Chuck et al., 1996); *ful-1* in Ler (3xCol) (Gu et al., 1998); *ful-2* in Col (Ferrándiz et al., 2000b); *shp1 shp2* in Col (Liljegren et al., 2000); *ind-2* in Ler (3xCol) (Liljegren et al., 2004); *BP::GUS* in Col (Ori et al., 2000); *RPL::GUS* in Col (Roeder et al., 2003); *SHP2::GUS* in Col (Savidge et al., 1995; Roeder et al., 2003); *GT140* (*IND::GUS*) in Ler (3xCol) (Ferrándiz et al., 2000a; Liljegren et al., 2004); *gAP2::YFP* in Ler (Wollmann et al., 2010). Transgenic or mutant strains previously generated or isolated in other accessions were backcrossed three times to Col before further experiments or crosses. Plants on soil or on MS plates were grown as described (Dinneny et al., 2004).

EMS mutagenesis of *rpl ful*

rpl-2 ful-2 seeds were treated overnight in 10 ml sterile water with 0.15% EMS (ethyl methanesulfonate) and 0.2% Tween 20. Seeds were washed 10 times in 0.2% Tween 20 and resuspended in 0.1% agar. After stratification (3 days at 4°C in darkness), seeds were sown on soil and grown to maturity. M2 seeds were germinated and adult plants were ultimately screened for restoration of replum formation. Seeds of the M2 mutants identified were saved and planted to rescreen the phenotype in the M3 generation.

Microscopy and histology

Plastic JB-4 thin sections were obtained and stained with Toluidine Blue (Ripoll et al., 2006). Staining of paraplast sections with Alcian Blue and Safranin O was performed as previously described (Roeder et al., 2003). GUS assays (Alonso-Cantabrana et al., 2007) and scanning electron microscopy (SEM) were performed as described (Roeder et al., 2003; Ripoll et al., 2006). Histological sections were analyzed using a Nikon E-600 microscope.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from fruits and treated with DNaseI, used for cDNA synthesis with an oligo(dT) primer and SuperscriptIII reverse transcriptase (Invitrogen). After 1/10 dilution of the cDNA, 1 μl was used as a template for the subsequent qPCR reactions. Relative changes in gene expression levels were determined using the $2^{\Delta\Delta\text{-CT}}$ method. RNA levels were normalized to the constitutively expressed gene *ACT2* (An et al., 1996) as previously reported (Ripoll et al., 2009). Each experiment was executed using three biological replicates. The standard deviation was calculated in Microsoft Excel. Primers used in this work can be found in supplementary material Table S1.

RESULTS

m413 suppresses *rpl ful* fruit phenotype

Previous studies have established that *RPL* and *FUL* negatively regulate the expression of valve margin identity genes in replum and valve, respectively (Ferrándiz et al., 2000a; Liljegren et al., 2000; Roeder et al., 2003; Liljegren et al., 2004). In *rpl ful* double mutants, epidermal ovary cells adopt valve margin identity, leading to a uniformly smooth surface and no apparent replum growth (Fig. 2D-F). Because even slight perturbations of this surface are easily visualized, we used the *rpl ful* mutant to undertake a genetic search for new loci involved in fruit patterning. Seeds of *rpl ful* were chemically mutagenized with EMS, and M2 plants screened for restoration of replum development. The replum rescue was confirmed in the M3 generation. Of particular interest was a mutation that suppressed the 'replumless' phenotype in *rpl ful* fruits; we called it *m413*. In *rpl ful m413* triple mutants (Fig. 2G,H), fruit showed a

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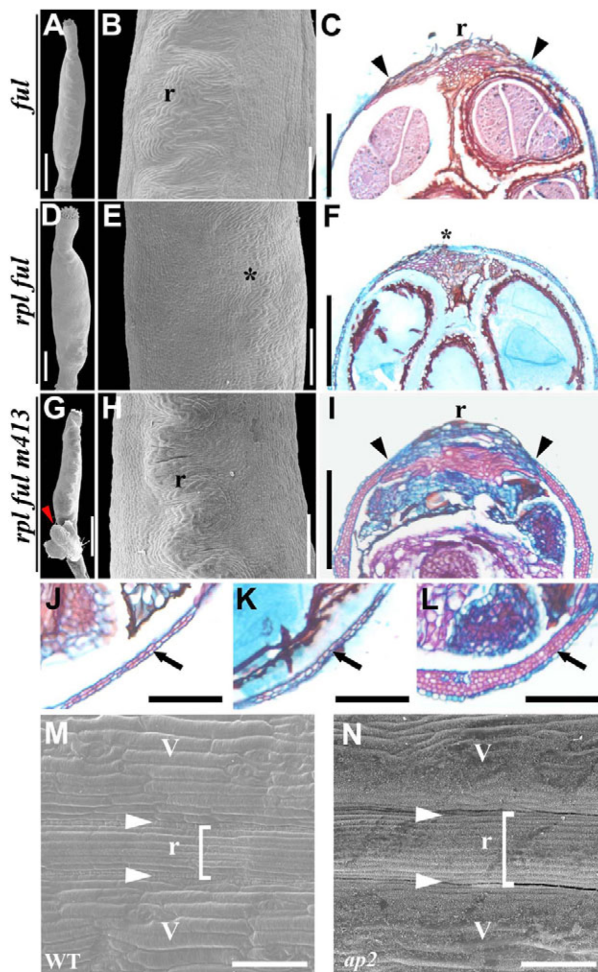

Fig. 2. *m413* mutation rescues replum development in *rpl ful*. (**A**,**B**,**D**,**E**,**G**,**H**) SEM micrographs of *ful* (A,B), *rpl ful* (D,E) and *rpl ful m413* (G,H). (B,E,H) Higher magnification views of the medial region of the fruits in A, D and G, respectively. In *ful*, the replum adopts a typical zigzag configuration (A,B). However, this structure is absent in *rpl ful* (E). Replum development is rescued in *rpl ful m413* background (G,H). The red arrowhead in G indicates the formation of carpeloid-sepals in *rpl ful m413*. (**C**,**F**,**I-L**) Cross-sections of *ful* (C,J), *rpl ful* (F,K) and *rpl ful m413* (I,L) fruits stained with Safranin O and Alcian Blue. Replum growth is observed only in *ful* (C) and *rpl ful m413* (I), but is largely absent (asterisks in F) in *rpl ful*. In *ful* (J) and *rpl ful* (K) valve cells, pink stain indicates ectopic lignification. More layers of lignified cells are observed in *rpl ful m413* valves (L). Arrows (J-L) indicate the ectopic layers of lignified tissue in valves. (**M**,**N**) SEM micrographs of wild-type (M) and *ap2* (N) fruits. In *ap2* mutants, the replum area is enlarged when compared with wild type. Arrowheads in M and N indicate the positions of the valve margins. r, replum; v, valves. Scale bars: 500 μm in A,D,G; 200 μm in B,C,E,F,H,I; 100 μm in J-L; 50 μm in M,N.

dramatic recovery of the zig-zag replum pattern seen in *ful* single mutants (Fig. 2A,B). Cross-sections further confirmed rescue of replum development in *rpl ful m413* (compare Fig. 2C,F,I).

A known consequence of the loss of *FUL* activity in *ful* and/or *ful rpl* mutants is the ectopic expression of valve margin genes in valves, which in turn results in ectopic valve cell lignification (Ferrándiz et al., 2000a). Interestingly, when compared with *ful* (Fig. 2J) or *rpl ful* (Fig. 2K) mutants, ectopic valve lignification was further enhanced in *rpl ful m413* mutant as consequence of an increase in the number of layers of lignified cells (Fig. 2L). Taken together, these observations suggest a role for *M413* in repressing lignification and replum growth.

m413 is allelic to the floral homeotic mutant *apetala2*

In addition to the fruit phenotypes described above, *rpl ful m413* flowers have a reduced number of stamens, no petals and carpeloid sepals (Fig. 2G). As this combination of traits is characteristic of *ap2* mutants (Bowman et al., 1989; Bowman et al., 1991; Jofuku et al., 1994), we performed an allelism test using the strong alleles *ap2-7* and *ap2-12* (Kunst et al., 1989; Yant et al., 2010) (supplementary material Fig. S1A,C). All of the F1 plants displayed *ap2*-mutant phenotypes, strongly suggesting that *m413* is a new allele of *ap2*. To further confirm this, the *AP2* locus (*At4g36920*) from the *m413* suppressor line was sequenced and a G-to-A change in the first exon was found that caused a premature stop codon truncating the protein before the first AP2 domain (supplementary material Fig. S1A). *rpl ful m413* was then outcrossed to Col to isolate the new *ap2* allele designated as *ap2-413* (supplementary material Fig. S1A). Floral defects observed in *ap2-413* single mutants were indistinguishable from those in strong *ap2* alleles (supplementary material Fig. S1C,D).

Although the *ap2* mutant has been characterized in detail (Bomblies et al., 1999; Bowman et al., 1989; Bowman et al., 1991; Drews et al., 1991; Jofuku et al., 1994; Okamuro et al., 1997; Chen, 2004; Ohto et al., 2005; Würschum et al., 2006; Wollmann et al., 2010; Yant et al., 2010), no prior studies have focused on defects during fruit development. The isolation of *ap2-413* in this work substantiates *AP2* as an important regulatory gene in fruit morphogenesis and motivated us to analyze *ap2* mutant gynoecia and fruit prior to dehiscence. We examined *AP2* expression using the *gAP2::YFP* translational reporter (Wollmann et al., 2010). Whereas no fluorescence was detected in carpel valves, YFP signal was detected in valve margins and replum tissues (supplementary material Fig. S1J,K), suggesting that AP2 may function during valve margin and replum development. The reporter activity gradually diminished as fruit growth proceeded (data not shown).

ap2 fruits have an enlarged replum

Because *ap2* mutations rescued replum formation in the *rpl ful* background, we next wanted to determine whether *ap2* single mutants have a replum phenotype. Close inspection of *ap2* fruits by SEM analysis revealed that the replum was conspicuously wider than in wild type (Fig. 2M,N), even at early stages of gynoecium development (data not shown). This enlarged-replum phenotype was observed in multiple, independent alleles, demonstrating that this is a general feature of *ap2* mutants (supplementary material Fig. S2J, Fig. S4A,B). Thin plastic cross-sections stained with Toluidine Blue showed the epidermal replum layer of *ap2* fruits to contain an average of 12 cells, substantially more than wild type, which contained an average of seven cells (Fig. 3A,B; supplementary material Fig. S2J). In addition, *ap2* replum epidermal cells appeared larger than those of wild type (Fig. 3A,B). Inner replum tissue in *Arabidopsis* accommodates a vascular bundle surrounded by a group of lignified cells. Although the inner replum of *ap2* mutants frequently contained more and larger lignified cells, this trait was variable (arrowhead in Fig. 3A,B).

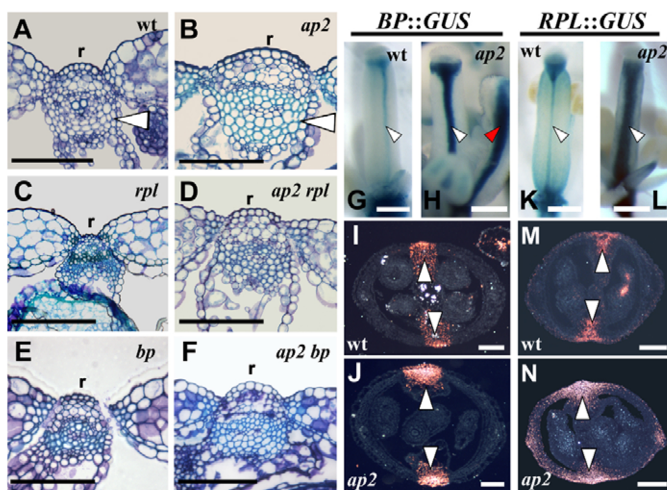

Fig. 3. ***AP2*** **regulates** ***BP*** **and** ***RPL*** **activities in the replum.** (**A-F**) Cross-sections of early stage 17 fruits from (A) wild-type, (B) *ap2*, (C) *rpl*, (D) *ap2 rpl*, (E) *bp* and (F) *ap2 bp.* An increase in replum epidermal cells causes the oversized replum present in *ap2* fruits (see supplementary material Figs S2, S3). *ap2* mutations rescue the *rpl*-replumless phenotype (D). In *bp* plants, no replum defects were reported (E). *bp* alleles largely alleviated the replum defects of *ap2* mutants (F). (**G**,**H**) Whole-mount staining for *BP::GUS* reporter in wild type (G) and *ap2* (H). (**I-N**) *BP::GUS* expression in cross-sections of stage 15 wild-type (I) and *ap2* (J) fruits. *RPL::GUS* staining in wild-type (I,M) and *ap2* (J,N) fruits. White arrowheads in A and B indicate the inner replum. In G-N, the white arrowheads indicate the replum. In H, the red arrowhead indicates the ectopic expression of *BP::GUS* in carpeloid sepals. Scale bars: 100 μm in A-F,I,J,M,N; 50 μm in G,H,K,L.

AP2 negatively regulates *RPL* and *BP* expression in the replum

The oversized replum that forms in *ap2* mutants identifies *AP2* as a repressor of replum growth. AP2 is one of the founding members of the large AP2/EREBP transcription factor family in which several members are known to act as transcriptional repressors in *Arabidopsis* (Dong and Liu, 2010; Fujimoto et al., 2000; Kirch et al., 2003; Nakano et al., 2006; Pandey et al., 2005; Song et al., 2005). In addition to the canonical AP2 domains (supplementary material Fig. S1A), the AP2 protein contains an EAR-motif near the C terminus (Kagale et al., 2010; Ohta et al., 2001) (supplementary material Fig. S1A), which is known to be involved in transcriptional repression (Ciftci-Yilmaz et al., 2007; Ikeda and Ohme-Takagi, 2009; Szemenyei et al., 2008; Dong and Liu, 2010; Pan et al., 2010; Pauwels et al., 2010). During floral patterning, it has been shown that AP2 mediates direct repression of *AG* (Drews et al., 1991; Bomblies et al., 1999; Yant et al., 2010).

As noted in the Introduction, replum development requires the activities of both *RPL* and *BP* (Roeder et al., 2003; Alonso-Cantabrana et al., 2007) (supplementary material Fig. S2). As a start towards determining whether *AP2* influences *BP* and *RPL* activities, we analyzed expression of *BP::GUS* (Ori et al., 2000) and *RPL::GUS* reporters (Roeder et al., 2003) in *ap2* fruits.

Both reporters showed a conspicuous increase in expression levels in *ap2* mutants, as well as an increase in the observed expression domains, suggesting that AP2 negatively regulates *RPL* and *BP* expression (*BP::GUS*, Fig. 3G-J; *RPL::GUS*, Fig. 3K-N). Consistent with this idea, we observed strong ectopic expression of both reporters in the first-whorl floral organs of *ap2* mutants (red arrowhead in Fig. 3H and data not shown). To substantiate this misregulation, we also performed quantitative reverse transcriptase-PCR (qRT-PCR) assays on these genes using gynoecia at stage 12 from both wild type and *ap2* (supplementary material Fig. S2K). To minimize differences in tissue composition between the samples, perianth organs and stamens of *ap2* and wild-type flowers were removed before total RNA preparation. Substantially higher levels of *BP* and *RPL* transcripts were observed in the *ap2* mutant than in the wild-type background (supplementary material Fig. S2K). Hence, these data demonstrate that, directly or indirectly, *AP2* negatively regulates *BP* and *RPL* expression in the replum.

rpl and *bp* mutations alleviate *ap2* replum defects

We have shown that the levels of *RPL* and *BP* expression are significantly elevated in *ap2* mutants. To determine whether this elevated expression is the cause of the enlarged replum of *ap2* mutants, we characterized *ap2 bp* and *ap2 rpl* double mutant fruit. We found that the oversized-replum phenotype of *ap2* mutants (12.4±0.9; Fig. 3B) was significantly mitigated by mutations in *rpl* (replum in *ap2 rpl* double mutants contained an average of 8.4±0.6 cells; Fig. 3D), although it remained slightly wider than that of wild type (7.2±0.8; Fig. 3A). Similarly, *ap2 bp* replum size (8.2±0.7 cells) was approximately the same as in *ap2 rpl* mutants (8.4±0.6) but again larger than wild type. In addition, cell size in the replum epidermis layer of *ap2 bp* (and also of *ap2 rpl*) fruits was restored to near that of wild type (supplementary material Fig. S2J). Taken together, these data suggest that *AP2* functions in the replum to negatively regulate *RPL* and *BP* expression, and that the oversized replum that forms in *ap2* mutants is caused by the elevated levels of expression of these genes.

ap2 mutations do not rescue replum development in *bp rpl* fruits

The reduced replum phenotype of *rpl* mutants is enhanced by mutations in *BP* such that replum growth is totally arrested in *bp rpl* double mutants (Fig. 4A) (Alonso-Cantabrana et al., 2007; Ragni et al., 2008). We have shown above that AP2 negatively regulates both *BP* and *RPL* expression and that the enlarged replum of *ap2* mutants is largely suppressed by *bp* and *rpl* mutations. We next wanted to determine whether *AP2* regulates replum size by primarily repressing both *RPL* and *BP* functions in fruit or, alternatively, whether *AP2* has other independent roles during replum formation. To address this issue we characterized the *ap2 bp rpl* triple mutant and found that the replum was completely absent, similar to *bp rpl* double mutants (Fig. 4A,B). These data demonstrate that the enlarged replum of *ap2* mutants is primarily due to the ectopic activities of *BP* and *RPL* and that the role for *AP2* in the replum is to prevent the overexpression of *BP* and *RPL*.

ind mutations do not rescue replum growth in *bp rpl*

It has been proposed that the negative regulation of valve margin genes by replum factors is required for the correct differentiation of replum (Roeder et al., 2003; Liljegren et al., 2004; Girin et al., 2010). To provide further evidence for the requirement of *BP* and *RPL* in replum formation, we characterized the *ind rpl* and *ind bp rpl* mutant fruit. In the Col background, the loss of replum formation that occurs in *rpl* mutants is restored in *ind rpl* double mutants (Fig. 3C, Fig. 4C). However, in the *ind bp rpl* triple mutant, replum formation was not rescued (Fig. 4D). These results reinforce the idea that specification and growth of replum tissue depends on both *BP* and *RPL* activities.

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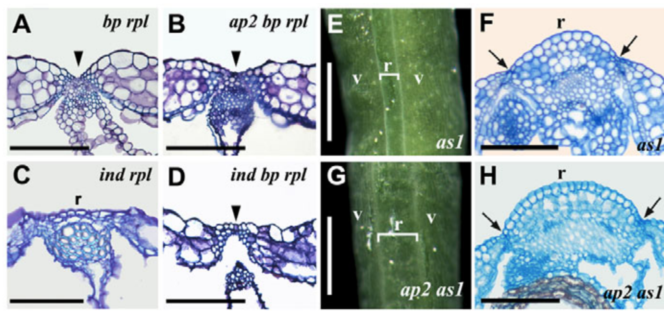

Fig. 4. *BP* and *RPL* activities promote replum formation, whereas *AP2* and *AS1* independently restrict its development. (**A**,**B**) No replum growth was observed in *bp rpl* (A) or ap2 bp rpl (B). (**C**,**D**) *ind* mutations rescue replum development in *ind rpl* (C) but not in ind *bp* rpl (D) fruits. (**E**,**G**) Images of the replum territory from stage 17 *as1* (E) and *ap2 as1* (G) fruits. (**F**,**H**) Stage 17 thin cross-sections of *as1* (F) and *ap2 as1* (H). Black arrowheads in A, B and D indicate medial tissues in the fruit. Arrows in F and H indicate the position of the valve margins. v, valve; r, replum. Scale bars: 100 μm in A-D,F,H; 500 μm in E,G.

AP2 and *AS1* interact during replum formation

As we have shown for *AP2*, the *ASYMMETRIC LEAVES1* (*AS1*) gene has previously been shown to be a negative regulator of *BP* expression in fruits (Alonso-Cantabrana et al., 2007), and, correspondingly, the size of the replum is significantly increased in *as1* mutants (compare Fig. 4E,F with Fig. 3A and supplementary material Fig. S2A). If *AP2* and *AS1* work independently to repress replum growth, an additive effect on replum size would be anticipated for an *ap2 as1* double mutant. Consistent with this hypothesis, we found that *ap2 as1* double mutants have an extremely enlarged replum (22.8±2.3 epidermal replum cells; Fig. 4H; supplementary material Fig. S2J), much larger than that observed for either *ap2* or *as1* single mutants (Fig. 3B and Fig. 4G, respectively), and dramatically larger than wild type (Fig. 3A). A similar genetic interaction was observed when *ap2 as2* fruits were examined (data not shown). Therefore, the *AS1* and *AS2* genes cooperate with *AP2* in *Arabidopsis* fruits to modulate replum development, most likely by independently regulating *BP* (and *RPL*) gene activities.

The lignified layer of the valve margin is enlarged in *ap2* mutants

We have shown that *AP2* is expressed in the replum where it functions to prevent replum overgrowth. Because *AP2* is also expressed in the valve margin (supplementary material Fig. S1K), we next looked to see whether *AP2* similarly negatively regulates growth of the valve margin. The LL layer of the valve margin is composed of several files of narrow cells that become lignified by stage 17 to facilitate fruit dehiscence (Fig. 5A; supplementary material Fig. S4A) (Ferrándiz et al., 1999; Ferrándiz, 2002; Liljegren et al., 2004; Dinneny and Yanofsky, 2005). In *ap2* mutants, we found that the LL was substantially larger than in wild-type fruit due to both an increase in the number and the size of the lignified cells in this region (Fig. 5A,B). These results are consistent with the phenotype seen in the original *rpl ful ap2-413* suppressor line in which more layers of ectopic lignified cells were also observed in the valve domain when compared with *rpl ful* (Fig. 2K) or *ful* (Fig. 2J). In contrast to the increased size of the LL layer, no obvious changes were found in the SL of *ap2* fruit. However, we observed that *ap2* fruit displayed, with some variability, a slight delay in dehiscence (not shown). This could be due to changes in cell wall properties that are not visible using the techniques employed.

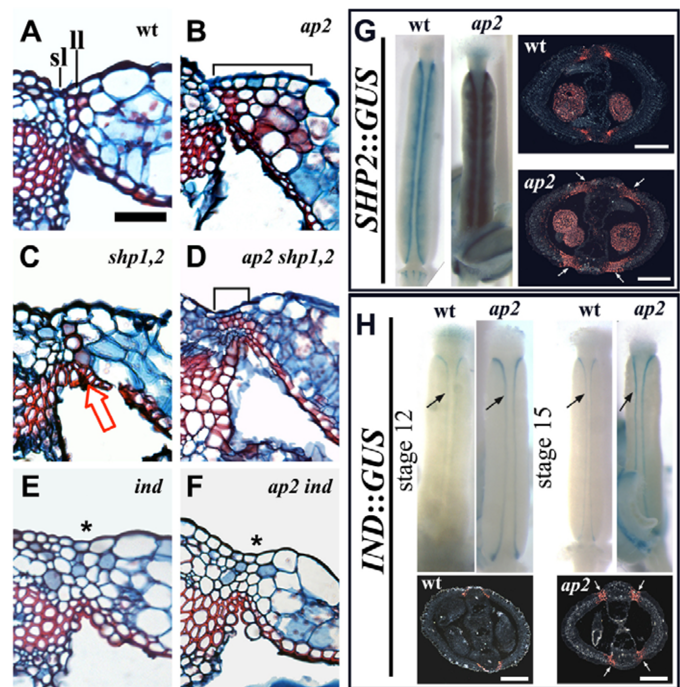

Fig. 5. *AP2* regulates lignification of the valve margin genes by controlling the expression of the valve margin identity genes. (**A-F**) Cross-sections of stage 17 fruits stained with Alcian Blue plus Safranin O dyes. (A) Wild type, (B) *ap2*, (C) *shp1 shp2*, (D) *ap2 shp1 shp2*, (E) *ind* and (F) *ap2 ind*. The dye mixture stains the separation layer (sl) light blue and the lignified layer (ll) in red. (B,D) Brackets delimit the ll region in *ap2* and *ap2 shp1 shp2* fruits, respectively. Arrow in C indicates the residual ll formation in *shp1 shp2* fruits. The abolishment of valve margin and, thus, lignification in *ind* (E) and *ap2 ind* (F) is indicated with an asterisk. (**G**) On the left, whole-mount histochemical activity of *SHP2::GUS* reporter in wild-type and *ap2* fruits. On the right, cross-sections of stage 15 fruits from wild type (top) and *ap2* (bottom). (**H**) Whole-mount staining for the *IND GUS*-reporter GT140 in wild-type (left) and *ap2* (right) stage 12 gynoecia and stage 15 fruits. Below, cross-sections of wild-type (left) and *ap2* (right) stage 15 fruits harboring GT140 reporter. Scale bars: 50 μm in A-F; 100 μm in G,H.

AP2 negatively regulates *SHP* expression

Because the size of the valve margin LL is significantly increased in *ap2* mutants (Fig. 5A,B), we next explored the possibility that the cause of this phenotype was an increase in expression of the valve margin identity genes. *SHP1* and *SHP2* are required for normal development of the LL and SL of the valve margin, and are expressed in thin stripes where the valve margins will later form (Fig. 5G) (Flanagan et al., 1996; Liljegren et al., 2000). In *ap2* mutants, the domain of *SHP2::GUS* expression was broader and expression levels appeared to increase when compared with wild type (Fig. 5G). These data suggest that AP2 negatively regulates *SHP* expression in the valve margin. This is perhaps not surprising, as it had previously been shown that AP2 also negatively regulates *SHP* expression in developing flowers (Savidge et al., 1995; Flanagan et al., 1996).

The expanded *SHP* expression domain that we observed correlates with an increased size for the LL in *ap2* fruits (Fig. 5B). Therefore, to determine to what extent misregulation of *SHP* expression might be responsible for the increased lignification in the valve margin of *ap2* mutants, we created *ap2 shp1 shp2* triple mutant plants. In this background, valve margin lignification was significantly reduced but remained considerably above the very low levels seen for *shp1 shp2* fruits (compare Fig. 5C with 5D). This indicates that although misregulation of *SHP* activity significantly contributes to the increased valve margin lignification seen in *ap2* fruit, additional factors are also involved.

AP2 negatively regulates *IND* expression

The bHLH transcription factor IND plays a major role in formation of the lignified layer and separation layer of the valve margin (Liljegren et al., 2004). Thus, we next used the *IND::GUS* reporter GT140 (Liljegren et al., 2000; Liljegren et al., 2004) to determine whether AP2 negatively regulates *IND* expression. In wild type, *IND::GUS* expression occurs at low levels within the valve margin region, beginning at around stage 12, just prior to fertilization (Liljegren et al., 2000; Liljegren et al., 2004; Sorefan et al., 2009) (Fig. 5H). In *ap2* mutants, we observed both higher levels of *IND::GUS* expression and a broader domain of expression (Fig. 5H). These data suggest that AP2 negatively regulates *IND* expression. To determine whether the increase in valve margin lignification that occurs in *ap2* mutants requires *IND*, we next examined lignification patterns in *ind* single (Fig. 5E) and *ind ap2* double (Fig. 5F) mutant fruit. In both cases, no lignification was observed, suggesting that the increased lignification that occurs in *ap2* mutants requires *IND* activity.

The loss of *AP2* does not affect valve development

The external surface of *ap2* mutant fruits was examined by SEM (supplementary material Fig. S3B), and in contrast to the increased size of the replum and valve margin domains, no change in cell size or cell number was detected in the valves (supplementary material Fig. S3A). Similarly, when the interior structure of *ap2* valves was examined by thin plastic cross-sections, no differences were observed in the mesocarp and endocarp layers (supplementary material Fig. S3C,D; J.J.R. and M.F.Y., unpublished). Last, the expression pattern for the valve marker gene *FUL* was unchanged in *ap2* when compared with wild-type fruits (supplementary material Fig. S3E,F). Thus, *ap2* mutants appear to be unaffected in valve development.

Mutations in *AP2* restore replum and valve margin in *35S::FUL* fruit

Our studies have shown that *AP2* prevents valve margin and replum overgrowth by negatively regulating the expression of valve margin and replum identity genes. Previous studies have shown that *FUL* is sufficient to negatively regulate valve margin and replum identity genes as constitutive misexpression of *FUL* (*35S::FUL*) leads to a conversion of the valve margins and replum into valves (Ferrándiz et al., 2000a). As a result, the entire surface of *35S::FUL* fruit consists of valve cells (Fig. 6B,D). To substantiate further a role for *AP2* in inhibiting valve margin and replum growth, we introduced the *ap2* mutation into *35S::FUL* fruit to see if valve margin and replum development would be restored. Analyses of SEM images shows an absence of valve margin and replum cells on the surface of *35S::FUL* fruit, whereas these tissues are evident on the surface of *ap2 35S::FUL* fruit (Fig. 6A-C). The restoration of replum and valve margin tissues is better visualized when cross-sections of *ap2 35S::FUL* fruits are compared with those of *35S::FUL* fruit (Fig. 6D,E).

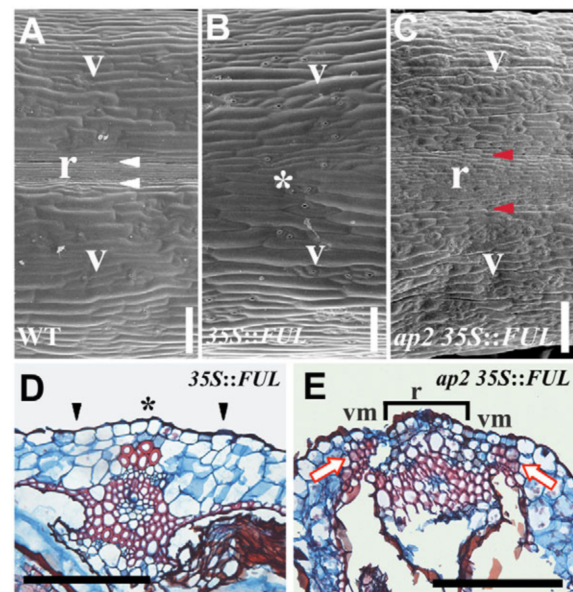

Fig. 6. Phenotypic characterization of *ap2 35S::FUL* backgrounds. (**A-C**) SEM pictures of the surface of stage 17 wild-type (A), *35S::FUL* (B) and *ap2 35S::FUL* (C) fruits. (**D**,**E**) Cross-sections of *35S::FUL* and *ap2 35S::FUL*, respectively, stained with Alcian Blue plus Safranin O, confirming the restoration of replum development and valve margin lignification. In A, the white arrowheads indicate the position of the valve margins. In B,D, asterisks indicate no replum formation. In C, red arrowheads indicate the valve margins. In D, the black arrowheads indicate the absence of valve margin tissue and lignification in *35S::FUL*. In E, the arrows mark the restoration of valve margin lignification *ap2 35S::FUL* siliques. r, replum; v, valves; vm, valve margin. Scale bars: 50 μm in A-C; 200 μm in D,E.

DISCUSSION

AP2 controls the development of replum and valve margin tissues

We used the *rpl ful* double mutant as a sensitized background to screen for new genes involved in fruit development. These studies allowed us to identify *AP2* as an important regulator of fruit patterning in *Arabidopsis*. Although *AP2* had been extensively studied with respect to its roles in flower, ovule and seed development (Bowman et al., 1991; Drews et al., 1991; Aukerman and Sakai, 2003; Chen, 2004; Ohto et al., 2009), little is known about the involvement of *AP2* in *Arabidopsis* fruit morphogenesis. Our studies show that *AP2* acts to prevent valve margin and replum overgrowth by negatively regulating the expression of valve margin and replum identity genes (Fig. 7).

AP2 negatively regulates *BP* and *RPL* to prevent replum overgrowth

We first identified AP2 as a negative regulator of replum growth because mutations in *AP2* restored replum growth in the *rpl* and *rpl ful* mutant backgrounds (Fig. 2). We subsequently found that *ap2* single mutants displayed a significant increase in the size of the replum when compared with wild type (Fig. 2M,N) owing to an increase in both the size and number of epidermal cells (Fig.

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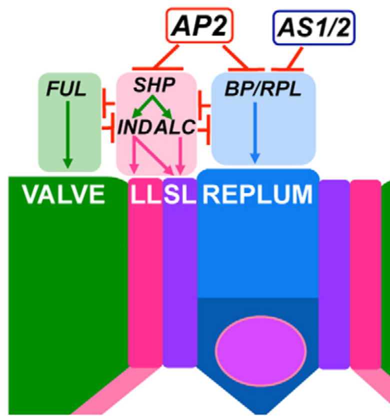

Fig. 7. Regulation of patterning along the mediolateral axis in the *Arabidopsis* fruit. *AP2* negatively regulates replum formation and valve margin lignification by repressing the replum genes (*BP* and *RPL*) and the valve margin genes (*SHP* and *IND*). *AS1* and *AS2* independently repress *BP* and *RPL*.

3A,B). This defect may be related to the observation that *ap2* mutant seeds are larger than wild type because of an increase in number and size of cells in the endosperm and seed coat (Jofuku et al., 1994; Jofuku et al., 2005; Ohto et al., 2005). Similarly, *ap2* embryonic cells are also larger than wild type, augmenting the final size of *ap2*-mutant embryos (Jofuku et al., 2005; Ohto et al., 2005; Ohto et al., 2009).

How does *AP2* normally act to prevent replum overgrowth? The answer comes from the analysis of *RPL* and *BP*, two genes that normally act to promote replum growth (Roeder et al., 2003; Alonso-Cantabrana et al., 2007) (this work). Mutations in either *bp* or *rpl* significantly reduced the replum-overgrowth phenotype of *ap2* mutants, and moreover, when both *rpl* and *bp* are inactivated, no replum formation occurs in *ap2* mutants (Fig. 4B). Analysis of reporter lines, as well as qRT-PCR data suggest that the levels of *RPL* and BP expression are significantly elevated in *ap2* mutants (Fig. 3G-N; supplementary material Fig. S2K). Taken together, our data indicate that the increased replum size of *ap2* mutants is caused by misexpression of *RPL* and *BP*, and that one of the functions of AP2 is to negatively regulate the expression of these two genes.

These data are consistent with the fact that AP2 functions as a negative transcriptional regulator in a number of developmental processes (Bomblies et al., 1999; Drews et al., 1991; Ohto et al., 2009; Yant et al., 2010). The AP2 protein, in addition to its two AP2 domains (Jofuku et al., 1994; Weigel, 1995), contains an EAR motif near the N-terminal region (supplementary material Fig. S1B) (Kagale et al., 2010), a motif that acts as a potent repression domain. The EAR motif is also involved in mediating protein-protein interactions with transcriptional co-repressors (Hiratsu et al., 2002; Hiratsu et al., 2004; Tiwari et al., 2004; Liu and Karmarkar, 2008; Szemenyei et al., 2008; Pauwels et al., 2010; Gallavotti et al., 2010).

Our data also showed that the enlarged-replum phenotype of *ap2* mutants is further enhanced by mutations in *AS1* (Fig. 4G,H). It is likely that this effect is caused by elevated levels of *BP* and *RPL* expression, because these two genes are known to also be upregulated in the replum of *as1* mutants (Alonso-Cantabrana et al., 2007). Taken together, it is likely that *AP2* and *AS1* act independently to prevent replum overgrowth by negatively regulating *RPL* and *BP* expression.

BP and *RPL* confer replum identity

The first gene shown to participate in replum formation was *RPL*. In *rpl* mutants, cells that would normally develop with a replum identity instead adopt a valve margin cell fate (Roeder et al., 2003). Correspondingly, the valve margin identity genes are ectopically expressed in the replum region of *rpl*, and mutations in the valve margin genes can suppress the replum defects of *rpl* fruits (Fig. 3C, Fig. 4C; supplementary material Fig. S2J). In fact, other mutations such as *fil, jag*, *as1*, *as2* and *ap2* can also restore replum formation to *rpl* mutants (Roeder et al., 2003; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007) (this work). Although these data substantiated a role for *RPL* in preventing replum cells from adopting an alternative cell fate, they did not address the issue of whether *RPL* also plays a direct role in promoting replum identity.

More recently, the *BP* gene was also found to promote replum formation. Although the replum is largely normal in *bp* single mutants, replum formation is completely abolished in *bp rpl* double mutants (Alonso-Cantabrana et al., 2007) (Fig. 4). Moreover, misexpression of *BP* causes a dramatic enlargement of replum tissue (supplementary material Fig. S2) (Alonso-Cantabrana et al., 2007). Importantly, whereas *fil*, *as1*, *as2*, *ap2* or mutations in valve margin identity genes restore replum tissue to almost wild-type size in *rpl* fruits, we have found that these mutations are not able to do so in *bp rpl* double mutants (Figs 3, 4; J.J.R., A.H.K.R., G.S.D. and M.F.Y., unpublished). These data support a direct role for *RPL* and *BP* in promoting replum identity in addition to their roles in preventing replum cell from adopting an alternative fate (Fig. 7).

It has been shown that heterodimer formation of class I KNOX- (including BP) and BELL- (including RPL) homeodomain transcription factors is required for their nuclear localization and target gene interaction (Bellaoui et al., 2001; Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004; Cole et al., 2006; Kanrar et al., 2006; Scofield et al., 2007; Rutjens et al., 2009; Hay and Tsiantis, 2010). The ability of closely related proteins to interact with and substitute for BP and RPL proteins may explain why *bp* or *rpl* single mutants fail to generate a completely replumless fruit, even though these related proteins are unable to promote replum formation in *bp rpl* double mutants (Chuck et al., 1996; Endrizzi et al., 1996; Long et al., 1996; Bhatt et al., 2004; Smith et al., 2004; Cole et al., 2006; Kanrar et al., 2006; Scofield et al., 2007; Rutjens et al., 2009) (our unpublished data).

AP2 represses valve margin lignification

In addition to controlling replum growth, we found that *AP2* also negatively regulates valve margin formation. Whereas the wild-type valve margin contains a narrow strip of cells that become lignified (Ferrándiz, 2002), *ap2* mutants have an increase in both the size and number of lignified cells (Fig. 5A,B). This increase is apparently caused by ectopic activity of the *SHP* and *IND* valve margin identity genes as *shp* mutations reduce, and *ind* mutations eliminate, valve margin lignification in the *ap2* mutant background (Fig. 5C-F). Consistent with this idea is the observation that the reporters for *SHP* and *IND* show increased levels of valve margin expression in *ap2* mutants relative to wild type (Fig. 5H,I).

Previous studies have shown that mutations in *FUL* lead to the ectopic expression of *SHP* and *IND*, causing valve cells to adopt a valve margin cell fate. Notably, *ful* mutant valve cells become ectopically lignified due to the misexpression of *IND* (Liljegren et al., 2004). Intriguingly, we found that this ectopic lignification of *rpl ful* (or *ful*) mutant valves was dramatically enhanced by mutations in *ap2* (Fig. 1L and not shown), perhaps because of elevated levels of *IND* expression. By contrast, it has previously

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been shown that misexpression of *FUL* (*35S::FUL*) is sufficient to convert valve margin and replum cells into valve cells, thus eliminating the normal lignification that occurs in the valve margin (Ferrándiz et al., 2000a) (Fig. 6D). We found that valve margin lignification was largely restored in *35S::FUL* fruit by mutations in the *AP2* gene (Fig. 6C,E). However, the restoration of valve margin was not complete as no separation layer was evident, and as a result, in *ap2 35S::FUL* fruit dehiscence did not occur or was incomplete (data not shown).

Based on our prior work and on data from other groups, a model to define patterning along the mediolateral axis of the gynoecium was proposed (Ferrándiz et al., 2000a; Liljegren et al., 2004; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007) (reviewed by Martínez-Laborda and Vera, 2009; Girin et al., 2009). This model proposes that formation of valve, valve margin and replum results from the interaction of opposite and antagonistic activities of replum and valve factors that act in a gradient manner. Accordingly, valve and replum identities are defined by high activities of valve factors and replum factors, respectively, and the valve margins develop where these activities are weakly expressed. Thus, misexpression of valve factors impairs (or represses) replum and valve margin identities (Ferrándiz et al., 2000a) and, conversely, misexpression of replum factors impairs valve identity (Dinneny et al., 2005; Alonso-Cantabrana et al., 2007) (J.J.R. and M.F.Y., unpublished). In agreement with this model, the loss of replum formation that occurs when *FUL* is misexpressed (*35S::FUL*) is overcome in *ap2 35S::FUL* plants, presumably because of the elevated levels of replum identity factors *BP* and *RPL* that occur in *ap2* mutants (Fig. 3). This further indicates that FUL and AP2 independently regulate replum identity genes.

Similarly, the loss of valve margin formation that occurs in *35S::FUL* plants is overcome in *ap2 35S::FUL* plants, most likely because of the elevated expression levels of valve margin identity genes such as *IND* and *SHP*. Previously, it has been showed that valve margin identity genes, such as *IND*, negatively regulate replum identity (Girin et al., 2010), and our data suggests this set of genes might be also negatively regulating valve factors (Fig. 7). The fact that *35S::SHP1* and *35S::SHP2* fruits resemble those of weak alleles of *ful*, or that misexpression of *IND* causes *ful*-like phenotypes (Liljegren et al., 2004; Girin et al., 2009) (J.J.R. and M.F.Y., unpublished) is also consistent with this idea.

Recently two independent studies reported that the *AP2* ortholog from tomato (*SLAP2a*) negatively regulates fruit maturation and ripening (Chung et al., 2010; Karlova et al., 2011). In this work, we have found that AP2 negatively regulates the formation of the valve margin, considered the ripening region in the *Arabidopsis* fruit (see Introduction) (Ferrándiz, 2002). The fact that AP2 plays related roles in both *Arabidopsis* and tomato suggests that AP2 is likely to be a major factor in controlling fruit patterning, growth and maturation in diverse plants species.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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DEVELOPMENT

Table S1. Oligonucleotides used in this work

Purpose	Name	Oligonucleotide sequence (5′-3′)	Gene/allele	Size (in base pairs)
RT-PCR	ACT2-F	CTCTTAACCGTAAAGCTAACAG	*ACT2*	
	ACT2-R	AGTGAGAATCTTCATGAGTGAG		
	oJJR156	AGGATTGTTGAGGATGTGAATGGG	*BP*	
	oJJR239	AACAGCACCACTCCTCAAAGAG		
	oRPLF	AAGGGCTTGGCTCTTCGATC	*RPL*	
	oRPLR	TCTGTATCTGTTGGATAAGGATGCA		
Genotyping	BP-X6	CACGATATTTAACCAACATGTCA	*bp-9*	W=432 M=405
	BP-X4	AATCATTGCTCATCCTCACTACTC		
	SPM1	CTTATTTCAGTAAGAGTGTGGGGTTTTGG		
	oAR41	CAAGCTTGCCATCTTCATGCTG	*rpl-2*	W=322 M=452
	oAR42	CTTTAAGTCCCTAGCTTTAAGATC		
	LBb1	AACCAGCGTGGACCGCTTGCTG		
	oJJR107	TTTAACCAAGGAAGAGTTCG	*ap2-12*	W=592 M=370
	oJJR108	GAGTAGTAGGATTCCCTGATG		
	oLBb1	AACCAGCGTGGACCGCTTGCTG		
	oJJR92	GATGCACTCGAAATCAGCCAATTTTAGAC	*shp1-1*	W=871 M=597
	oJJR93	GTGACGGAAGGAGGGTTGACG		
	oJJR94	GTGACGGAAGGAGGGTTGACG		
	oJJR95	GTCTACTGATGAGTTGTCACTAGG		
	oJJR96	GAGGATAGAGAACACTACGAATCGTC	*shp2-1*	W=1500 M=2800
	oJJR97	CAGGTCAAGTCAATAGATTCCCTAC		
	GT140-P	GCTAATGATCTTCTCACACAAGAAC	*ind-2*	dCAPS (AluI) W=100+30 M=130
	GT140-Q	ATCGCATCCATGTCTTCATCGTAC		

W, PCR product size for wild-type allele; M, PCR product size for mutant allele.