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

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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 fruit, and is referred to as the valve margin. The valve margin is 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; 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).

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., 2000; 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; 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 HOMEBOX) gene BREVIPEDECELLUS (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

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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: asl-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) (Chuch et al., 1996); ful-1 in Ler (3xCol) (Gu et al., 1998); ful-2 in Col (Ferrándiz et al., 2000b); shp1 shp2 in Col (Liijegren et al., 2000); ind-2 in Ler (3xCol) (Liijegren et al., 2004); BP::GUS in Col (Neg et al., 2000); RPL::GUS in Col (Roeder et al., 2003); SHP2::GUS in Col (Savidge et al., 1995; Roeder et al., 2003); GT40 (IND::GUS) in Ler (3xCol) (Ferrándiz et al., 2000a; Liijegren et al., 2004); gAP2::YPFP 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 from 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 Alican 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 2ΔΔCT 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; Liijegren et al., 2000; Roeder et al., 2003; Liijegren 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
growth is observed only in m413 (I,L) fruits stained with Safranin O and Alcian Blue. Replum layers of lignified tissue in valves. (observed in absent (asterisks in F) in rpl ful m413 C). The red arrowhead in G indicates the formation of carpeloid-sepals in (E). Replum development is rescued in zigzag configuration (A,B). However, this structure is absent in m413 A,D,G; 200 μC,F,I). rescue of replum development in single mutants (Fig. 2A,B). Cross-sections further confirmed 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 apetalal2

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; Okamura et al., 1997; Chen, 2004; Ohno 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).
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; Bombles 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. S2I). 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.
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 AS1 (H). (A) No replum growth was observed in bp rpl (A) or ap2 bp rpl (B). 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; Dimmeny 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.

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).

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.

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 (S) light blue and the lignified layer (L) 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.
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).

**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. 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.
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 (Bombilies 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; Dimmery 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 (Bellouai 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), in the valve margin gene mutants (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; Dimmery 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.

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
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 shown 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 plant species.

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Competing interests statement
The authors declare no competing financial interests.

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