A genetic framework for fruit patterning in *Arabidopsis thaliana*

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Summary

In the model plant *Arabidopsis thaliana*, the establishment of organ polarity leads to the expression of *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*) on one side of an organ. One important question that has remained unanswered is how does this positional information lead to the correct spatial activation of genes controlling tissue identity? We provide the first functional link between polarity establishment and the regulation of tissue identity by showing that *FIL* and *YAB3* control the non-overlapping expression patterns of *FRUITFULL* (*FUL*) and *SHATTERPROOF* (*SHP*), genes necessary to form stripes of valve margin tissue that allow the fruit to shatter along two defined borders and disperse the seeds. *FIL* and *YAB3* activate *FUL* and *SHP* redundantly with *JAGGED* (*JAG*), a gene that also promotes growth in organs, indicating that several pathways converge to regulate these genes. These activities are negatively regulated by *REPLUMLESS* (*RPL*), which divides *FIL/JAG* activity, creating two distinct stripes of valve margin.

Key words: *Arabidopsis thaliana*, Development, Fruit, Patterning, Polarity, Dehiscence

Introduction

Remarkably, while multicellularity arose independently during the evolution of plants and animals, the processes that pattern organs in these two lineages are similar. In both lineages, organs such as limbs in animals and lateral organs in plants are patterned by two parallel systems: one system defines the type of organ that will develop, while the second system defines the coordinates of the organ (Engstrom et al., 2004; Logan, 2003). While the combination of factors that confer organ identity tend to be unique for individual organ types, the patterning system that defines the coordinates of the organ tends to be the same for all organs.

The development of the fruit in the model plant, *Arabidopsis*, provides an excellent system for dissecting the mechanisms that pattern an organ in plants because of the presence of distinctive morphological landmarks and the availability of reporter lines that mark specialized tissues (Dinneny and Yanofsky, 2005). The region of the fruit that encloses the seeds (ovary) is divided into three tissue zones: valve, replum and valve margin (Fig. 1A-D). The valves, or seed pod walls, protect the seeds during their development and detach after maturation to promote seed dispersal in a process known as dehiscence. The two valves are separated by a central ridge of replum tissue. At the valve/replum junction, a specialized stripe of tissue, termed the valve margin, forms that facilitates the detachment of the valves from the replum through the action of two different cell types. On the replum side of the valve margin, the separation layer forms, which permits the detachment of the valve from the replum through cell-cell separation (Fig. 1E) (Jenkins, 1999; Peterson, 1996). On the valve side of the margin, a layer of rigid lignified cells forms (Fig. 1E,F). The lignified layer of the valve margin is continuous with an adjacent layer of lignified cells present in the valves, termed the endocarp layer *b* (en*b*) (Fig. 1E-G). Together, these tissues are thought to provide spring-like tension that mechanically drives valve detachment (Spence, 1996).

Recent work has identified several genetic components that are important for specifying valve margin development and defining its borders (Fig. 7). The redundant MADS-box genes, *SHATTERPROOF 1/2* (*SHP*), are at the top of a cascade of transcription factor genes that specify valve margin identity (Liljegren et al., 2000). When *SHP* activity is eliminated, fruits are indehiscent and lack lignified layer and separation layer development. Acting both downstream and in parallel to *SHP*, the *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) basic helix-loop-helix-type transcription factor genes also specify valve margin identity with *IND* playing important roles in lignified and separation layer development, and *ALC* regulating separation layer development (Liljegren et al., 2004; Rajani and Sundaresan, 2001).

The expression of the valve margin identity genes is limited to the valve margin through negative regulation by *FRUITFULL* (*FUL*) in the valves and *REPLUMLESS* (*RPL*) in the replum. *FUL* encodes a MADS-domain transcription factor that is expressed in the valves (Gu et al., 1998). When *FUL* is mutated, the valve margin identity genes become ectopically expressed in the valves, imparting valve margin-like development, including the ectopic formation of lignified and separation layer-like cell types (Ferrándiz et al., 2000b). Similar to the role that *FUL* plays, *RPL* negatively regulates the expression of the valve margin identity genes in the replum

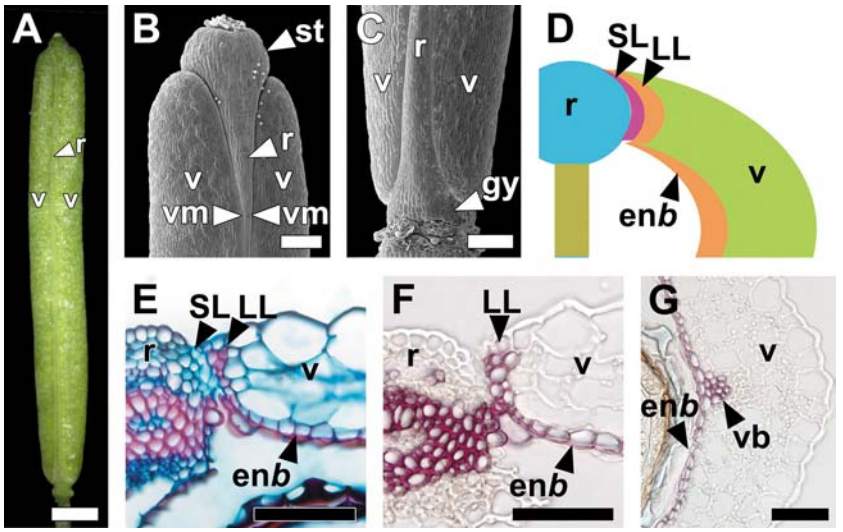

Fig. 1. Wild-type fruit development. (A) Bright-field image of a stage 17 fruit. (B,C) SEM of the apical region (B) and basal region (C) of a fruit. (D) Diagram of a cross section of a fruit with the various tissues indicated. (E) Safranin O- and Alcian Blue-stained cross section near the valve margin. The separation layer stains light blue and the lignified layer stains pink. (F,G) Phloroglucinol-stained cross sections of the valve margin (F) and valve (G). This stain is lignin specific and marks the lignified layer of the valve margin, the en*b* layer and cells of the vascular bundle. v, valve; r, replum; vm, valve margin; st, style; gy, gynophore; SL, separation layer; LL, lignified layer; vb, vascular bundle. Scale bars: 1 mm (A), 200 μm (B,C), 50 μm (E-G).

(Roeder et al., 2003). *RPL* encodes a BELL-family homeodomain transcription factor and is expressed in the replum. When *RPL* is mutated, ectopic *SHP* expression in the replum causes the valve margins to coalesce into a single stripe of tissue in place of the replum, rendering *rpl* fruit partially indehiscent. *SHP* expression is positively regulated by *AGAMOUS* (*AG*), a MADS-domain transcription factor gene that controls carpel identity (Savidge et al., 1995). However, *AG* probably acts redundantly with other factors, since *SHP* activity is still present in *ag apetala*2 mutants (Lee et al., 2005; Pinyopich et al., 2003).

A crucial aspect of fruit development that is not well understood is how the patterns of gene activities that control valve margin formation are initially established. Here, we show that the *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*) genes, which regulate the polarity of tissues in lateral organs (Eshed et al., 2004; Sawa et al., 1999; Siegfried et al., 1999), are required to promote the expression of *FUL* and *SHP* in the valves and valve margin, respectively. The unrelated gene, *JAGGED* (*JAG*), which promotes the growth of tissues in lateral organs (Dinneny et al., 2004; Ohno et al., 2004), acts redundantly with *FIL* and *YAB3* to promote the expression of *FUL* and *SHP*, with *jag fil yab3* triple mutants lacking *FUL* and *SHP* expression in the valves or valve margins. We also provide further insight into the mechanism by which *RPL* regulates valve margin development by showing that *RPL* promotes the formation of two stripes of valve margin by dividing *JAG*/*FIL* activities into two separate domains. Our work describes the first functional link between patterning systems in plants that define organ polarity and growth and those that control tissue identity, and provides a genetic framework for the patterning of the three tissue types composing the fruit.

Materials and methods

Plants

Landsberg *erecta* [Ler] or a wild-type segregant that is mutant for *erecta* when tissue is stained for GUS activity was the wild type used. *fil-8*, *yab3-2* (Kumaran et al., 2002), *jag-1*, *jag-5D* (Dinneny et al., 2004), *rpl-3* (Roeder et al., 2003), *shp1*, *shp2* (Liljegren et al., 2000), *ful-1* (Gu et al., 1998), *35S::FUL* (Ferrándiz et al., 2000b) and *SHP2::GUS* (Savidge, 1995) have been described before.

Genotyping

Genotyping of *jag-1* has been previously described (Dinneny et al., 2004). To genotype *fil-8* we used primers oJD174 and oJD175 to detect the wild-type *FIL* allele and oJD175 and oJD176 to detect the *fil-8* allele. To genotype *yab3-2*, we used primers oJD172 and oJD173 to detect the wild-type *YAB3* allele and oJD173 and oJD177 to detect the *yab3-2* allele. Genotyping of *ful-1* (Gu et al., 1998) and *rpl-3* (Roeder et al., 2003) has already been described. The *jag-5D*$^{+/-}$ *shp1,2* triple mutant was identified phenotypically.

The sequences of the oligonucleotide primers used were as follows:

oJD172, 5′-GAC TCC ATG TCG AGC ATG TCC ATG-3′;

oJD173, 5′-CTA GTA ATT CAT GTG AAA CCC TAG C-3′;

oJD174, 5′-GAC AAT GAA AGT AGT TTT AAT GAG-3′;

oJD175, 5′-CAG CTT TGA TAC GTT GGA TCT CCT CC-3′;

oJD176, 5′-ACC GTT ACG ACC GTT TTC ATC C-3′; and

oJD177, 5′-ACG GTC GGG AAA CTA GCT CTA C-3′.

In situ hybridization, microscopy and histology

In situ hybridization was performed as previously described (Dinneny et al., 2004). Tissue was prepared for scanning electron microscopy (SEM) as previously described (Dinneny et al., 2004). Late stage 17 fruit were fixed, sectioned (8 μm) and stained using Phloroglucinol (Liljegren et al., 2000) or Saffranin O and Alcian Blue (Roeder et al., 2003) as previously described.

GUS staining

To monitor *FUL* expression, the *ful-1* enhancer trap line was crossed to *fil-8 yab3-2* or *jag-1 fil-8 yab3-2*$^{+/-}$ mutants. F_2 segregants were genotyped for *jag-1*, *fil-8*, *yab3-2* and *ful-1* alleles and stained for GUS activity if plants were heterozygous for *ful-1*. The *yab3-2* allele is also a GUS enhancer trap line, however, no overlapping GUS staining was detected with that of *ful-1* in *fil yab3* and *jag fil yab3* mutant backgrounds during the stages of development described (data not shown). To monitor *SHP2* expression, the *SHP2::GUS* reporter line was crossed to *fil-8 yab3-2*, *jag-1 fil-8 yab3-2* and *jag-5D* mutant backgrounds. F_2 segregants were genotyped for *jag-1*, *fil-8*, *yab3-2* alleles and stained for GUS activity. Plants heterozygous for *jag-5D* were identified phenotypically. To monitor *AG* expression, the *KB9* reporter line was crossed to *fil-8 yab3-2* mutants (Busch et al., 1999). GUS staining was performed as previously described (Blázquez et al., 1997).

Results

The *fil yab3* mutant fruit has defects in dehiscence

To identify factors required for the establishment of fruit patterning we examined genes that are expressed early during gynoecium development. One candidate was the *YABBY* family

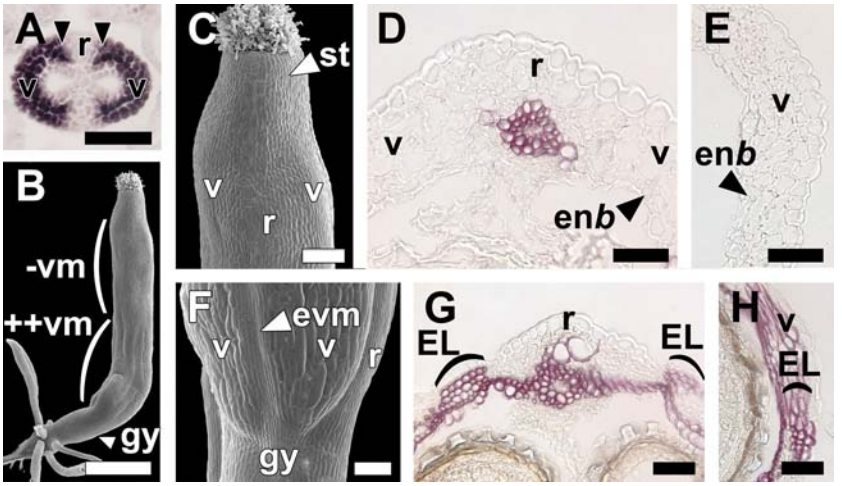

Fig. 2. The *fil yab3* double mutant has defects in valve margin patterning. (A) Expression of *FIL* detected by in situ hybridization in a stage-8 wild-type gynoecium. (B-H) The *fil yab3* fruits develop a complex arrangement of valve margin tissues in which the apical regions lack valve margin, while the basal regions develop ectopic valve margin. (B) SEM of a whole *fil yab3* fruit. Brackets mark regions that develop no visible valve margin (–vm) and that develop ectopic valve margin (++vm). The gynophore is also expanded in *fil yab3* mutants. (C) SEM of the apical region of a *fil yab3* fruit. Note the lack of creases that normally mark the location of the valve margin. (D,E) Phloroglucinol-stained cross sections of the apical region of a *fil yab3* fruit in the replum/valve margin region (D), or valve (E) region. The fruit lack lignin in cells normally found at the valve margin and en*b* layer. (F) SEM of the basal region of a *fil yab3* fruit. A large stripe of valve margin-like tissue (evm) is shown passing through the middle of a valve. (G,H) Phloroglucinol-stained cross sections of the basal region of a *fil yab3* fruit in the replum/valve margin (G) and valve (H) regions showing the expansion of lignified layer tissues into the valves. r, replum; v, valve; st, style; –vm, loss of valve margin; ++vm, ectopic valve margin; evm, ectopic valve margin; gy, gynophore; EL, ectopic lignification. Scale bar: 50 μm (A,D,E,G,H), 1 mm (B), 200 μm (C), 100 μm (F).

transcription factor gene, *FIL*, which is expressed strongly during floral stages 6-11 in the gynoecium, in the valves and in cells that will probably contribute to the formation of the valve margin (Fig. 2A) (Sawa et al., 1999; Siegfried et al., 1999). *YAB3*, the next closest homologue of *FIL* in *Arabidopsis*, is expressed in a similar manner (Siegfried et al., 1999). Previous studies have identified *FIL* and *YAB3* as downstream facilitators of the pathways that establish organ polarity (Eshed et al., 2004; Sawa et al., 1999; Siegfried et al., 1999). Lateral organs in plants develop bilateral symmetry with the half of the organ facing the shoot meristem, termed the adaxial side, while the half facing away from the shoot meristem is termed the abaxial side. All lateral organs develop distinct cell types that distinguish the adaxial and abaxial sides. In leaves, *FIL* expression is limited to the abaxial tissue layers, while in carpels, expression extends one cell layer adaxially to include the en*b* layer (Fig. 2A) (Siegfried et al., 1999). To determine whether *FIL* or *YAB3* genes play a role in patterning the tissues of the fruit, we examined mature fruit of *fil* and *yab3* single mutants but did not observe any major defects in dehiscence (data not shown). Since other work has shown that *FIL* and *YAB3* play redundant roles in leaf development, we examined fruit from *fil yab3* double mutants and found that they were largely indehiscent (Fig. S1A,B in supplementary material). The lack of dehiscence is most apparent in the apical regions of fruit, with some dehiscence occurring in the basal region (Fig. S1C in supplementary material). A closer examination of the *fil yab3* fruit, by scanning electron microscopy (SEM), revealed that, whereas the apical region of the fruit appeared to lack valve margins, the basal region appeared to develop regions with ectopic valve margin-like tissues (Fig. 2B, see below).

The apical region of *fil yab3* fruit lack valve margin development

Consistent with the reduction in dehiscence of *fil yab3* fruits, the apical region lacks valve margin development. Whereas wild-type fruit develop clear creases on either side of the replum, where the smaller valve margin cells develop (Fig. 1B), these creases are absent in *fil yab3* fruit (Fig. 2B,C). We took a closer look at the valve margin region of *fil yab3* fruits in cross section and stained for the presence lignified cells. In wild-type fruit, stripes of lignified cells develop on either side of the replum (Fig. 1F), however, in the apical region of *fil yab3* fruit these lignified cells are absent (Fig. 2D). Similar to our results for the lignified layer, we found that the apical region of *fil yab3* fruit lacked a separation layer at the valve margin region (Fig. 1E and S1D). Thus, the two cell types that compose the valve margin are absent in the apical region of *fil yab3* fruit.

The loss of valve margin development in *fil yab3* fruit suggests that *FIL* and *YAB3* have important roles in controlling the expression of the valve margin identity genes. To determine this relationship, we examined the activity of a *SHP2::GUS* reporter. Consistent with the loss of valve margin development in the apical region, reporter activity was absent from the valve margins of *fil yab3* mutants but was unaffected in regions where *FIL* and *YAB3* are not expressed (Fig. 3A-D). Thus, *FIL* and *YAB3* play important roles in promoting valve margin development, in part by promoting *SHP* expression at the valve margin.

The basal region of *fil yab3* fruit develops ectopic valve margin tissue

Paradoxically, *fil yab3* mutants develop ectopic valve margin-associated cell types in the basal region of the fruit. By SEM, ectopic valve margin can be seen as patches, or stripes, of small cells (Fig. 2B,F). In cross section these patches stain similarly to cells of the separation and lignified layers (Fig. 2G,H and Fig. S1E in supplementary material). Ectopic valve margin in *fil yab3* mutants can develop both as an expansion of the valve margin normally present on either side of replum and in stripes in the middle of the valves (Fig. 2F,G,H and Fig. S1E in supplementary material).

The development of the ectopic valve margin suggests that *SHP* expression might have expanded into the valves at the

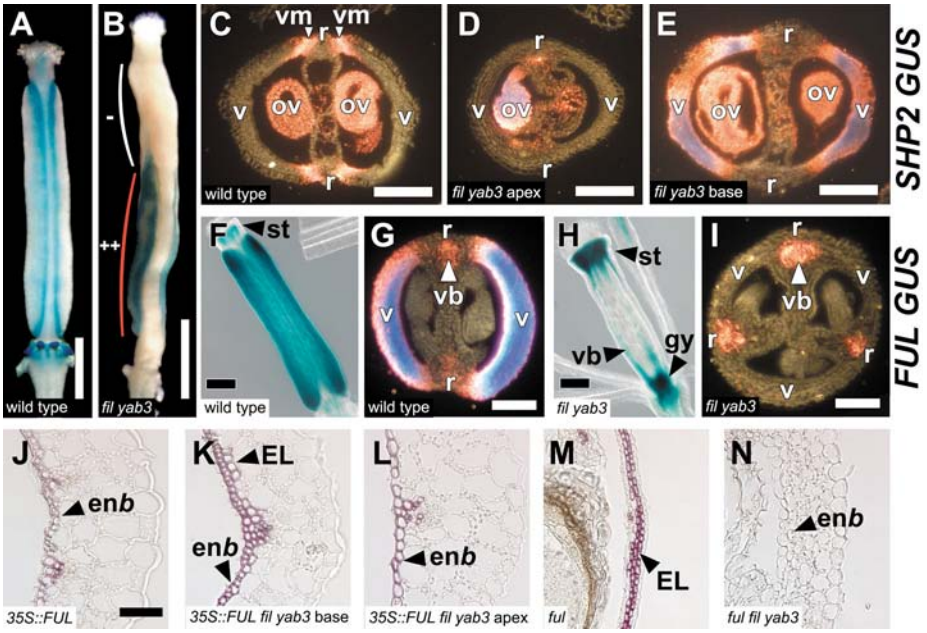

Fig. 3. *FIL* and *YAB3* are necessary for proper *SHP2* and *FUL* expression. *GUS* expression driven by the *SHP2* promoter (A-E) or the *ful-1* enhancer trap line (F-I). All plants stained for *FUL GUS* activity are heterozygous for *ful-1*. (A) In wild-type fruit, the *SHP2::GUS* reporter is active in the valve margins throughout the fruit at stage 14. (C) In cross section, reporter activity is visible in the ovules as well as the valve margins. (B,D,E) In *fil yab3* fruit, *SHP2* reporter activity is lost in the apical region of the fruit (B –,D) while ectopic reporter activity is present in the valves of the basal portion (B ++,E). (F,G) *FUL GUS* staining of a stage 12 wild-type gynoecium showing expression in the two valves as well as in the vasculature of the style (F) and replum (G). (H,I) In the *fil yab3* gynoecium, reporter activity is absent from the valves but remains in the vasculature. Note the presence of a supernumerary carpel in the *fil yab3* gynoecium (I). (J-N) Lignin staining of valve cells in mature fruit (stage ~17). (J) *35S::FUL* valves develop a lignified en*b* layer similar to wild type. (K) *35S::FUL* is able to suppress most of the ectopic lignification that occurs at the base of *fil yab3* mutants and is able to rescue en*b* layer lignification in the apical region (L). Note some ectopic lignification is still present in the basal region. (M) In *ful* mutants, valve mesophyll cells become ectopically lignified as a result of the expanded expression of valve margin identity genes. (N) In the apical region of *ful fil yab3* fruits, however, all cells of the valves are unlignified including the en*b* layer, suggesting that the valve margin identity genes are not active. v, valve; vm, valve margin; r, replum; ov, ovule; st, style; vb, vascular bundle; gy, gynophore; EL, ectopic lignification. Scale bar: 0.5 mm (A), 1 mm (B), 100 μm (C,D,E), 200 μm (F,H), 50 μm (G,I-N).

base of *fil yab3* fruit. We examined *SHP2::GUS* activity in *fil yab3* mutants and found that, in contrast to the apical region, reporter activity in the basal region was increased in the valves (Fig. 3A-E). The stark contrast in reporter activity between the apical and basal regions of *fil yab3* fruit is most dramatic in whole mounts (Fig. 3A,B).

FIL and *YAB3* are required for the expression of *FUL* in the valves

The development of ectopic patches of valve margin tissue at the base of *fil yab3* fruits is reminiscent of the *ful* mutant (Ferrándiz et al., 2000b). We used the *ful-1* enhancer trap line to examine *FUL* expression in *fil yab3* mutants and found that reporter activity was missing from valves in both the apical and basal regions (Fig. 3F-I). Occasionally a small patch of cells near the base of the valves showed reporter activity (data not shown). Reporter activity is present but elevated in the vasculature of the style, replum and gynophore where *FUL* is normally expressed, but *FIL* and *YAB3* are not. These data show that *FIL* and *YAB3* are important for promoting *FUL* expression in the valves and suggest that the ectopic expression of *SHP2* at the base of *fil yab3* fruits is an indirect effect, caused by the loss of *FUL* expression. This hypothesis is supported by the finding that constitutive expression of *FUL* can suppress most of the ectopic lignification that occurs at the base of *fil yab3* fruit (Fig. 2H, Fig. 3J,K).

FIL and *YAB3* are required for the maintenance of *SHP* expression at the valve margin and the activation of *FUL* expression in the valves

We examined the expression of *SHP2* and *FUL* during early stages of gynoecium development when *FIL* and *YAB3* are most strongly expressed. In wild type, the *SHP2::GUS* reporter is initially expressed in a broad domain during early gynoecium development in cells that will develop into the replum, septum and valve margins (Fig. S3 in supplementary material). Expression in the replum eventually fades, leaving *SHP2* expression at the valve margins and septum. In *fil yab3* mutants, *SHP2::GUS* expression initiates as in wild type, however, expression is quickly lost in the replum and presumptive valve margins (Fig. S2 supplementary material).

FUL expression, in wild-type gynoecia, initiates in the adaxial cell layers of the valves, and then expands into all cell layers by stage 10. In *fil yab3* mutants, however, expression is never detected in the valves. These data show that *FIL* and *YAB3* are required for expression of *SHP2* and *FUL* during early gynoecium development in addition to later stages in the fruit. The observation that *SHP2* and *FUL* expression is affected in tissues where *FIL* and *YAB3* are not expressed (i.e. replum for *SHP2* and adaxial epidermis for *FUL*) suggests that they can act cell non-autonomously.

The loss of en*b* lignification suggests that *FIL* and *YAB3* also regulate other valve margin identity genes

In *fil yab3* mutants, en*b* lignification is reduced in the apical region of the fruit (Fig. 1F,G and Fig. 2D,E). The development of the en*b* layer is redundantly specified by both *FUL* and the valve margin identity genes (Liljegren et al., 2004). Expression of at least one of these factors is sufficient for en*b* lignification: consistent with this scenario, a *35S::FUL* transgene restored en*b* lignification in the apical region of *fil yab3* mutants (Fig.

2D,E and Fig. 3J,L). Since a complete loss of en*b* lignification is otherwise only observed in the *ful shp1 shp2 ind alc* quintuple mutant, we conclude that besides *FUL* and *SHP*, the activity of *IND* and *ALC* is also compromised in *fil yab3* mutants. To test this possibility further, we examined the effect that loss of *FIL* and *YAB3* function had on the ectopic valve margin development seen in *ful* mutants (Fig. 3M). The apical phenotype of *ful fil yab3* fruit is very similar to *fil yab3* double mutants, with a clear absence of valve margin development and en*b* layer lignification, indicating that the *fil yab3* mutations are epistatic to *ful* (Fig. 2E and Fig. 3M,N) and necessary for the activity of the genes that confer ectopic valve margin identity to *ful* valves. Importantly, the base of *ful fil yab3* fruit still develop ectopic valve margin, however, the ectopic tissue appears to be composed only of cells with separation layer-like characteristics (not shown).

JAG acts redundantly with *FIL* and *YAB3* to regulate valve margin development

Our data show that *FIL* and *YAB3* promote the expression of both *SHP* and *FUL*. However, while *FIL* and *YAB3* are essential for *FUL* expression in the valves, *SHP* expression is only partially affected. One possible reason for this incomplete effect may be genetic redundancy of *FIL* and *YAB3* with another factor that also promotes *SHP* expression. We have tested whether *FIL* and *YAB3* act redundantly with other non-*YABBY* family members and in other work have found that *FIL* and *YAB3* function redundantly with an unrelated C_2H_2 zinc-finger transcription factor gene, *JAG*, to promote leaf blade growth (Fig. S3A,B in the supplementary material). To determine if this redundancy extends into the fruit we examined the effect of various combinations of *fil*, *yab3* and *jag* mutations.

Like *FIL* and *YAB3*, *JAG* is expressed during the early stages of gynoecium development in cells of the valves and presumptive valve margin (Fig. 4A) (Dinneny et al., 2004; Ohno et al., 2004). Unlike *FIL* and *YAB3*, however, *JAG* is expressed in all tissue layers of the gynoecium, between stage 6 and stage 8 (Fig. 4A), at which point *JAG* expression diminishes in the valves (Dinneny et al., 2004; Ohno et al., 2004). We have previously reported that *jag* mutants develop slightly bumpy fruit (Dinneny et al., 2004; Ohno et al., 2004). Upon closer inspection we noticed that the shoulders of the valves in the apical region of the fruit are somewhat sloped down (Fig. 4B). This phenotype is particularly apparent in the presence of the *erecta* mutation.

The combination of *jag* with *fil* results in the apparent expansion of the basal gynophore and apical style tissues into the ovary region (Fig. 4C). Furthermore, *jag fil* mutants develop a stripe of ectopic tissue that runs through the center

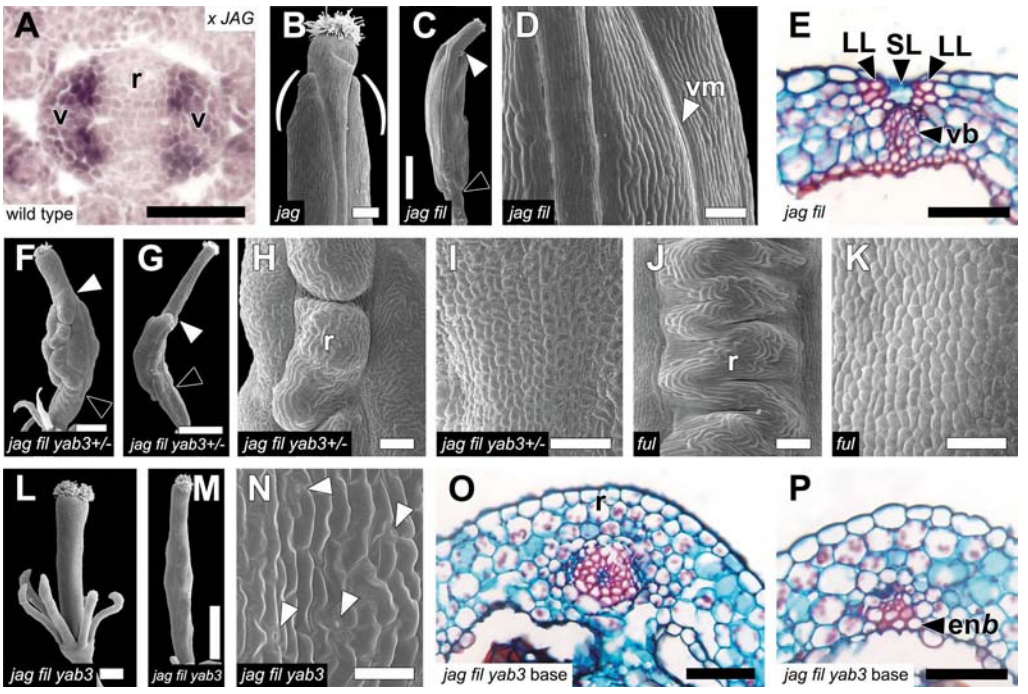

Fig. 4. *JAG* acts redundantly with *FIL* and *YAB3* to control valve margin patterning. (A) Expression of *JAG* detected by in situ hybridization in a stage 8 wild-type gynoecium. *JAG* is expressed in the valves and presumptive valve margin region and is expressed in all cell layers. (B) SEM of the apical region of a *jag* fruit. The shoulders of the valves are sloped downwards in *jag*. (C) SEM of a *jag fil* fruit. The style/ovary (white arrowhead) and gynophore/ovary (black arrowhead) boundaries have shifted, with the style and gynophore becoming enlarged. (D) *jag fil* fruit develop a stripe of valve margin tissue that runs down the middle of the valves. (E) In cross sections stained with Safranin O and Alcian Blue, separation layer and lignified layer cells are present overlying the vascular bundle. (F) SEM of *jag fil yab3*$^{+/-}$ fruit showing further expansion of style and gynophore into the ovary region. (G) SEM of *jag fil yab3*$^{+/-}$ *ER* fruit. The *erecta* mutation suppresses the growth of the style as it does in *ful* mutants (Ferrándiz et al., 2000a). (H) Close-up SEM of a *jag fil yab3*$^{+/-}$ replum. The normally narrow ridge of tissue has expanded in width and twists throughout the length of the fruit similar to the replum of *ful* mutants (J). (I) SEM of a patch of small cells that form in the valves of *jag fil yab3*$^{+/-}$ fruit. These cells appear very similar to the ectopic valve margin cells that replace the valves in *ful* mutants (K). (L) SEM of a *jag fil yab3* mutant flower. All the floral organs have patterning defects. Sepals, petals and stamens are replaced by filamentous organs with no apparent floral characteristics. (M) SEM of a fertilized *jag fil yab3* fruit. No valve margin development is present. (N) Epidermal cells of *jag fil yab3* valves are elongated with stomata interspersed (white arrowheads), similar to wild type. Occasionally, however, cells with an irregular shape can be seen. (O,P) Cross sections at the base of a *jag fil yab3* fruit stained with Safranin O and Alcian Blue. All cell types necessary for dehiscence, including the lignified layer, separation layer and en*b* layer are absent. Some en*b* lignification may be present internally to the vascular bundle. r, replum; v, valve; vm, valve margin; LL, lignified layer; SL, separation layer; vb, vascular bundle. Scale bar: 50 μm (A,E,I,K,N,O,P), 200 μm (B,L), 1 mm (C,G,M), 100 μm (D,H,J), 500 μm (F).

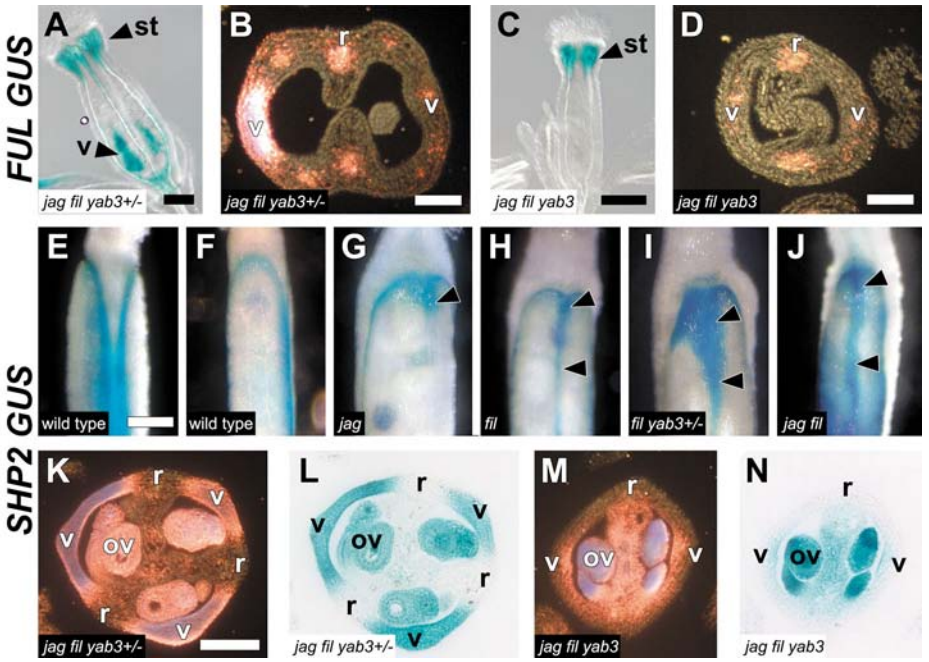

Fig. 5. *JAG* promotes *FUL* and *SHP* expression redundantly with *FIL* and *YAB3*. (A-D) *FUL GUS* reporter activity in the gynoecium using the *ful-1* enhancer trap line. (E-N) *SHP2::GUS* reporter activity in stage 15-16 fruit (E-J) and stage 12 gynoecia (K-N). (A,B) Whole mount (A) and cross section (B) of *FUL GUS* staining in a stage 12 *jag fil yab3*$^{+/-}$ gynoecium showing reporter activity restricted to small islands corresponding to the valves. (C,D) Whole mount (C) and cross section (D) of *FUL GUS* staining in a stage 12 *jag fil yab3* gynoecium. Reporter activity is completely absent from the valves. (E,F) Frontal (E) and side (F) views of *SHP2::GUS* expression in wild type. (G) In *jag* mutant fruit, a small wedge of ectopic reporter activity is detected in the apical region of the valves (black arrowhead). (H) In *fil* mutant fruit, a stripe of ectopic reporter activity can be seen extending through the center of the valve, overlying the vasculature. (I) In *fil yab3*$^{+/-}$ mutant fruit, the intensity of reporter activity in the valves is stronger than in *fil* single mutants. (J) In *jag fil* mutant fruit, strong reporter activity is present in a stripe in the valves with weaker staining elsewhere. (K,L) Cross section of a *jag fil yab3*$^{+/-}$ gynoecium showing strong ectopic reporter activity in the valves. Using dark-field microscopy, strong GUS staining appears purple, whereas weak staining appears pink/orange. (L) The same section as in K viewed under bright-field microscopy. (M,N) Cross section of a *jag fil yab3* gynoecium showing weak reporter activity in the valves (M). Using bright-field microscopy (N) to image the section in M, the loss of *SHP2::GUS* reporter activity in the valves and valve margins is even more apparent, compared to the strong GUS staining seen in the valve regions of *jag fil yab3*$^{+/-}$ gynoecia (L). v, valve; r, replum; ov, ovule and st, style. Scale bars: 200 μm (A,C); 100 μm (B,D); 0.5 mm (E-J); 100 μm (K-N).

of the valves (Fig. 4D). The cells in this region are narrow and can occasionally be seen separating from each other. Examination of cross sections of this stripe shows that it is composed of cells with separation and lignified layer characteristics, indicating that *jag fil* mutants develop an ectopic stripe of valve margin in the middle of the valves (Fig. 4E). Interestingly, this stripe of valve margin always develops overlying the main vascular bundle of the valves. The ectopic development of valve margin tissues also correlated with the expansion of *SHP2::GUS* activity into the valves of *jag fil* double mutants (Fig. 5F,J). Some ectopic reporter activity can be seen in the valves of *jag* and *fil* single mutants as well (Fig. 5F,G,H).

The ectopic development of valve margin cell types in the valves suggests that *FUL* activity might be compromised in *jag fil* mutants. Consistent with this hypothesis, we found that *jag fil* mutants develop a stripe of tissue in the valves in which *FUL* expression is not detected (Fig. S4A,C in supplementary material). We also examined the expression of *FUL* in *jag* single mutants and could also see a small cleft of *FUL*-negative tissue near the apical shoulder of the valves (Fig. S4A,B in supplementary material), suggesting that the downward sloping of the valves in the mature fruit may be caused by a loss of *FUL* expression.

The effect of the *yab3* mutation was next examined in the context of the *jag* and *fil* mutations. Loss of a single copy of *YAB3* resulted in a strong increase in the expansion of gynophore and style regions into the ovary of *jag fil yab3*$^{+/-}$ fruit (Fig. 4F). The increase in the size of the style was particularly apparent when the fruits were not mutant for *ERECTA* (Fig. 4G). The fruit of *jag fil yab3*$^{+/-}$ also developed a dramatic expansion of valve margin tissues, with large patches forming in and around the valves (Fig. 4I). The replum of *jag fil yab3*$^{+/-}$ fruit was also greatly expanded and twisted along the length of the fruit (Fig. 4H). These phenotypes are reminiscent of those observed in *ful* mutant fruit, which develop enlarged and twisted repla (Fig. 4J) along with ectopic valve margin tissue in the valves (Fig. 4K), although to a greater extent than *jag fil yab3*$^{+/-}$. Consistent with these phenotypes, the expression of the *SHP2::GUS* reporter further expands into the valve regions in *jag fil yab3*$^{+/-}$ fruit (Fig. 5K,L), with *FUL* expression diminishing to small islands (Fig. 5A,B). Together, these data indicate that *JAG* acts redundantly with *FIL* and *YAB3* to promote *FUL* expression in the valves. Importantly, *fil yab3*$^{+/-}$ mutants, which can develop stripes of ectopic valve margin in the valves, do not develop ectopic valve margin or ectopic *SHP2::GUS* activity to the extent of *jag fil yab3*$^{+/-}$ mutant fruit (Fig. 5I and data not shown).

Surprisingly, the loss of the second copy of *YAB3* in *jag fil yab3* triple mutants reverses the expansion of valve margin development seen in *jag fil* and *jag fil yab*$^{+/-}$ mutants. Fruits of *jag fil yab3* mutants do not develop valve margin-like cell types in either the apical or basal portions (Fig. 4M-P and data not shown). In the valve regions, the epidermis is composed of elongated cells with interspersed stomata, similar to wild-type valve cells (Fig. 4N). Some valve cells have an irregular shape. The expression of *FUL* is completely absent from the valve regions of *jag fil yab3* mutant gynoecia (Fig. 5C,D), similar to *fil yab3* mutants. Contrary to what was observed in *jag fil yab3*$^{+/-}$ fruit, however, *SHP2::GUS* activity is strongly reduced

in both the valves and valve margins (Fig. 5M,N). A low level of *SHP2* expression occurs only in the vicinity of the vascular bundles (Fig. 5M,N). These data indicate that *JAG* not only acts redundantly with *FIL* and *YAB3* to promote *FUL* expression, but also to promote *SHP* expression. Furthermore, the strong reduction of *SHP* expression in *jag fil yab3* mutants demonstrates that genetic redundancy with *JAG* is a cause of the remaining *SHP* expression seen in *fil yab3* mutants. The other floral organs of *jag fil yab3* mutants are also severely affected and develop little, if any floral characteristics, suggesting that *JAG, FIL* and *YAB3* promote the patterning of other floral organs as well (Fig. 4L).

Ectopic *JAG* activity in the replum promotes valve margin development

The data presented thus far show that *JAG*, *FIL* and *YAB3* act redundantly to promote the expression of *FUL* in the valves and *SHP* in the valve margins. Presumably, the domains of *FUL* and *SHP* expression are limited to the valves and valve margins, respectively, because the expression domains of *JAG, FIL* and *YAB3* are limited to these tissues. To test this hypothesis, we wanted to determine if ectopic expression of *JAG*, *FIL* or *YAB3* could promote the ectopic development of valve or valve margin-like tissues. We used the dominant activation-tagged allele, *jag-5D*, in which *JAG* expression is expanded into the replum (Fig. S5A in supplementary material) (Dinneny et al., 2004). When we examined the fruits of *jag-5D* mutants we found that the outer replum, which normally develops in between two stripes of valve margin, was apparently missing (Fig. 6A,B). Cross sections of the fruit showed that the replum had been replaced with tissues characteristic of the separation and lignified layers of the valve margin. In strongly affected regions of *jag-5D* fruit, the normally separate lignified layers fused, forming a 'lignified bridge' that joined the two valves (Fig. 6B). Consistent with this observation, fruit of *jag-5D* mutants are partially indehiscent (data not shown).

The ectopic development of valve margin tissues in *jag-5D* mutants suggested that the presence of *JAG* activity in the replum might be able to promote the expansion of *SHP* expression into this region. We therefore examined *SHP2::GUS* activity in *jag-5D* mutants, and found that reporter activity had expanded into the replum (Fig. 3C and Fig. 6C). To test whether expanded *SHP* expression was the cause of the ectopic valve margin development, we removed *SHP* activity by constructing a *jag-5D shp1 shp2* triple mutant and found that replum development was rescued (Fig. 6A,B,D,E). Thus, *JAG* is sufficient to drive the ectopic expression of *SHP* in the replum, converting this region into valve margin.

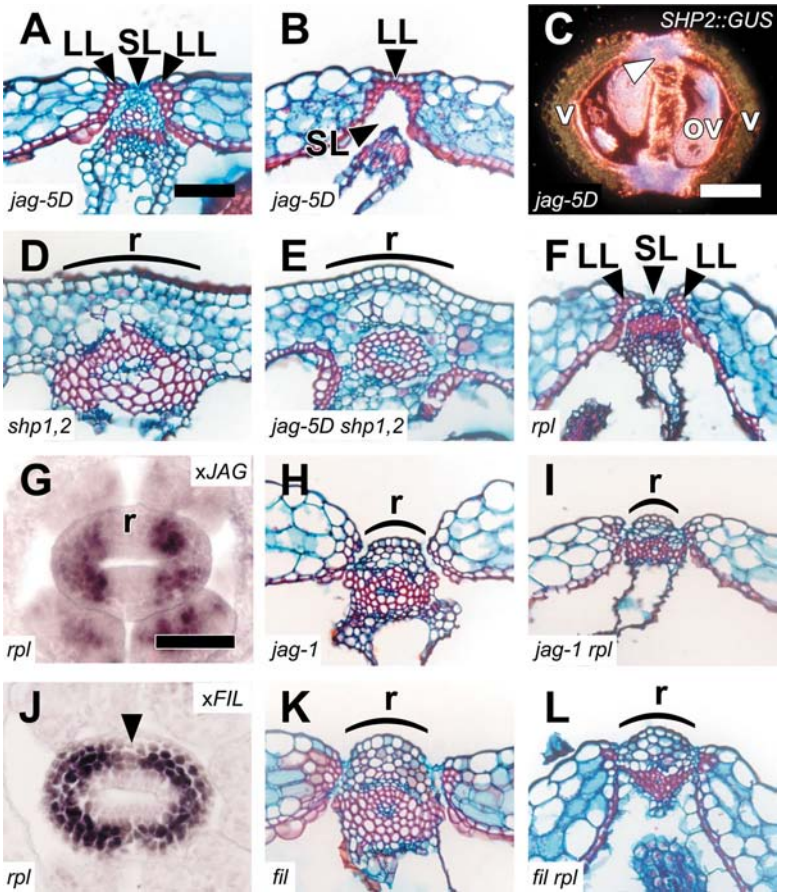

Fig. 6. *RPL* promotes the formation of two valve margins by negatively regulating *JAG* and *FIL* activity. The loss-of-function *JAG* allele *jag-1*, and the activation-tagged allele *jag-5D*. (A,B,D-F,H,I,K,L) Cross sections of stage 17 fruit from various mutants stained with Alcian Blue and Saffranin O. (A) *jag-5D* fruit. The replum is replaced by lignified layer and separation layer-like cell types. (B) Example of a strongly affected *jag-5D* fruit showing the fusion of the lignified layer to form a 'lignified bridge'. Note that the two valves have detached from the rest of the fruit as a single unit. (C) *SHP2::GUS* reporter activity in a stage 13 *jag-5D* fruit expands into the replum region. Note that the *SHP2::GUS* reporter is not activated as highly in the vascular bundle where *FUL* is expressed (white arrowhead). (D) *shp1,2* fruit lack separation and lignified layer development. The cross section is taken near the base of the fruit where the *shp* mutant phenotype is strongest. (E) *jag-5D shp1,2* fruit near the base. The ectopic development of valve margin tissues is suppressed, indicating that *SHP* is necessary for the replumless phenotype of *jag-5D* mutants. (F) *rpl* fruit have patterning defects similar to *jag-5D*. (G) In situ hybridization of *JAG* in a stage 8 *rpl* mutant gynoecium. Expression of *JAG* is not detectable in the replum region. (H) *jag-1* fruit are similar to wild type. (I) *jag-1 rpl* fruit showing a rescue of replum development. Note that replum rescue is variable and this is a strong example. (J) Expression of *FIL* in a stage 8 *rpl* gynoecium expands into the replum region (black arrowhead). (K) *fil* mutant fruit. The replum tends to be larger than in wild type. (L) *fil rpl* fruit display a consistent and strong rescue of replum development. LL, lignified layer; SL, separation layer; r, replum; v, valve; o, ovule. Scale bars: 50 μm (in A for A,B,D-F,H,I,K,L; in C; and in G for G,J).

RPL promotes the formation of two stripes of valve margin by repressing *JAG*/*FIL* activity in the replum

The conversion of the replum into valve margin in *jag-5D* is very similar to the effect that the *rpl* mutation has on replum development (Fig. 6F) (Roeder et al., 2003). As in *jag-5D*, *rpl* mutants develop ectopic separation and lignified layer tissues in the replum, which is caused by the expansion of *SHP* expression into the replum. Likewise, loss of *SHP* function in a *rpl* mutant background rescues replum development. These similarities suggest the basis of the *rpl* mutant phenotype may

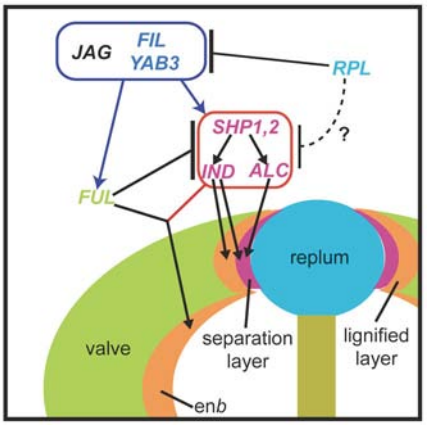

Fig. 7. Model for fruit patterning in *Arabidopsis*. *JAG, FIL* and *YAB3* function redundantly to promote the expression of the valve margin identity genes and *FUL*. These activities are negatively regulated by *RPL* in the replum.

be ectopic *JAG* expression in the replum. To test this, we examined *JAG* expression in *rpl* mutants by in situ hybridization, however, we could not detect *JAG* transcript in the replum (Fig. 6G). Nevertheless, we examined the effect of removing *JAG* activity from a *rpl* mutant background by constructing *jag rpl* double mutants and found that replum development was partially rescued (Fig. 6F,H,I). The rescue of replum development is somewhat variable, however (data not shown). Thus, while expression of *JAG* can not be detected in the replum of *rpl* mutants, genetic evidence suggests some *JAG* activity may be present.

Since *JAG* and *FIL* play similar roles in patterning the fruit, we wanted to determine if *FIL* was also regulated by *RPL* in the replum. We examined *FIL* expression by in situ hybridization in *rpl* mutants and found a clear expansion of *FIL* expression into the replum region (Fig. 2A, Fig. 6J). This expansion was particularly apparent during floral stages 7-9. To determine if this ectopic *FIL* expression was a cause of the *rpl* phenotype, we constructed *fil rpl* double mutants. The loss of *FIL* function had a strong effect in rescuing the *rpl* phenotype with *fil rpl* fruit consistently developing large repla (Fig. 6F,K,L).

We also examined the expression of *FIL* in *jag-5D* mutants to see if the replumless phenotype could be caused by ectopic *FIL* expression in the replum. No effect on *FIL* expression was apparent in *jag-5D* gynoecia, however, indicating that *JAG* is not sufficient to activate *FIL* expression in these tissues (Fig. S5B in supplementary material). Together, our data suggest that the effect of *RPL* on *SHP* is indirect, and mediated by repression of *FIL* and *JAG*, two activators of *SHP* expression. This hypothesis is consistent with in situ hybridization experiments that show *RPL* expression in the replum during the same stages that *FIL* expression expands into the replum region of *rpl* mutants. Our data, however, do not exclude the possibility that *RPL* may repress *SHP* expression through a parallel pathway as well.

Discussion

The initiation of lateral organ development at the shoot apex sets off a series of patterning events that result in the formation of a mature organ with a unique set of tissues. In plants, one of the first events to take place in organ development is the establishment of polarity, which helps to determine the arrangement of tissues in the organ. In this work, we have determined that *FIL* and *YAB3* play an important role translating this polarity information into the regulation of genes necessary for fruit patterning. *FIL* and *YAB3* are expressed early during the initial stages of gynoecium development in cells of the valve and presumptive valve margin and promote the expression of *FUL* and *SHP* in these tissues, respectively. *FIL* and *YAB3* regulate *FUL* and *SHP* expression in cooperation with another unrelated gene, *JAG*. The involvement of *JAG*, which was originally identified by its role in promoting growth during organ development, suggests that multiple developmental pathways may converge to regulate *FUL* and *SHP* expression. We have also shown that *JAG* and *FIL* activity is negatively regulated by *RPL* in the replum, dividing *JAG/FIL* activity into two separate domains corresponding to the two valves of the fruit. Together, these data provide a mechanistic basis for the patterning of the valves, valve margin and replum and the activation of *FUL* and *SHP*, which control the identity of tissues in these regions.

The relationship between organ polarity and fruit patterning

Plant organogenesis is regulated by a genetic system that divides organs into abaxial and adaxial halves. Members of a miRNA-regulated clade of HD-ZIP family transcription factors, including *PHABULOSA*, *PHAVOLUTA* and *REVOLUTA*, control the identity of the adaxial domain, the half of the organ that is proximal to the shoot meristem (Emery et al., 2003; McConnell and Barton, 1998; McConnell et al., 2001). On the other side, the GARP-type transcription factor genes, *KANADI 1* and *2* control the identity of the abaxial domain (Eshed et al., 1999; Eshed et al., 2001; Eshed et al., 2004; Kerstetter et al., 2001). Not only do these factors impose adaxial or abaxial development on their respective sides, but they also restrict the spread of the opposing identity. *FIL* and *YAB3* also function with *KAN* genes to control abaxial identity, however, they do not appear to be involved in the initial establishment of organ polarity. Instead it has been proposed that *FIL* and *YAB3* function downstream of *KAN* to facilitate abaxial identity and to promote the growth of the leaf blade (Eshed et al., 2004). Thus, *FIL* and *YAB3* may function downstream of polarity establishment to begin carrying out an abaxial-specific developmental program.

Although it is known that the establishment of organ polarity controls the distribution of cell types in an organ, it is not yet known what the functional relationship is between the specification of adaxial/abaxial identity and the development of specialized cell types that constitute these regions. By showing that *FIL* and *YAB3* promote the expression of the *FUL* and *SHP* genes, our work provides the first functional link between the establishment of organ polarity and the regulation of genes that control tissue identity. Because the *FIL* and *YAB3* genes are expressed in all lateral organs, it is likely that they control tissue identity in the fruit by interacting with other factors, such as those encoded by the floral homeotic genes. Thus, *FIL* and *YAB3* would provide positional information that is common to all organs, with the floral homeotic genes

contributing identity information specific to individual organs. Importantly, we have found that the expression of the floral homeotic gene, *AG*, which regulates the identity of the carpels, is unaffected in *fil yab3* mutants (Fig. S6 in supplementary material). This indicates that *FIL/YAB* and *AG* represent independent pathways regulating *FUL* and *SHP* expression.

The *fil yab3* double mutants and *jag fil yab3* triple mutants also have defects in patterning all other floral organs (Siegfried et al., 1999). The defects seen in these other organs do not resemble the patterning defects of mutants, such as *phb-1D* or *kan1 kan2*, that have been classified as having ectopic adaxial development (Eshed et al., 2001; McConnell and Barton, 1998). It will be interesting to examine these floral organs more carefully to determine the exact nature of the patterning defects and to see if specific floral organ cell types are missing, as they are in the fruit.

Activation of *FUL* in the valves and *SHP* in the valve margin

While *FIL, YAB3* and *JAG* are expressed in both the valves and presumptive valve margin, *FUL* and *SHP* are expressed in mutually exclusive domains in these tissues. How is it then that *FIL*, *YAB3* and *JAG* pattern the expression of both sets of genes? Our loss-of-function genetic results show that *FUL* expression is most severely affected by reductions in *FIL/YAB3/JAG* activity whereas *SHP* expression is more robust. For example, *FUL* expression is strongly affected in *fil yab3* mutants, whereas *SHP* expression is lost in only part of the fruit. These data suggest that the activation of *FUL* and *SHP* expression may require different levels of *FIL*, *YAB3* and *JAG* activity. In this light, it is intriguing that *SHP* is expressed at the periphery of the *FIL/YAB3/JAG* expression domain where *FIL/YAB3/JAG* activity may be weakest; possibly pointing to their proteins having properties of a morphogen that controls different downstream targets in a concentration-dependent manner. It will be necessary to develop tools to quantitatively detect FIL, YAB3 and JAG proteins, in order to enable a close comparison of their distribution with *FUL* and *SHP* expression during fruit development.

Mechanistic insight into the role of *RPL* in fruit development

We have gained further insight into how fruit patterning is established by showing that *JAG/FIL* activity is negatively regulated by *RPL* in the replum. In *rpl* mutants, the two valve margins coalesce into a single stripe of tissue with valve margin characteristics. This expanded valve margin development correlates with the presence of ectopic *FIL* expression in these medial tissues. Removal of either *JAG* or *FIL* activity from a *rpl* mutant rescues replum development, demonstrating that ectopic *JAG/FIL* activity in the replum is the cause of the *rpl* phenotype. Thus, *RPL* effectively divides *JAG/FIL* activity into two domains, creating two separate valve margins that enable each valve to separate independently from the fruit.

The spatial regulation of *JAG/FIL* activity in the fruit identifies an interesting parallel to lateral organ and meristem patterning at the shoot apex. In the shoot, antagonism between the meristem and lateral organ primordia ensures that the meristem is not consumed during the process of organogenesis and prevents organ primordia from fusing together. In the fruit, *RPL* [a.k.a. *BELLRINGER* (Byrne et al., 2003), *PENNYWISE* (Smith and Hake, 2003) and *VAAMANA* (Bhatt et al., 2004)] determines the limits of valve margin development by inhibiting *JAG/FIL* activity. *BELLRINGER* also plays important roles in meristem development and acts redundantly with *SHOOTMERISTEMLESS* to prevent the fusion of the cotyledons (seed leaves) at their base (Byrne et al., 2003). Thus, the mechanism that prevents the margins of the valves from fusing may be derived from the process by which organ fusion is suppressed in the seedling shoot. It will therefore be interesting to determine whether ectopic *FIL* or *JAG* activity is responsible for any of the shoot defects seen in *rpl* mutants.

Fruits as modified leaves

In 1790, Goethe proposed that floral organs represented modified vegetative leaves. It would take nearly 200 years for plant biologists to develop the technologies to test this intriguing hypothesis. The formation of the ABC model of floral organ development led to an understanding of the molecular basis of floral organ identity (Coen and Meyerowitz, 1991). These discoveries demonstrated that the expression of a handful of genes was responsible for transforming simple leaves arising on flowers into sepals, petals, stamens and carpels. These principles came full circle when it was shown that the constitutive expression of floral homeotic genes and their co-factors could convert vegetative leaves outside of flowers into individual floral organs (Honma and Goto, 2001; Pelaz et al., 2001). The observation that these ectopic floral organs formed all of the appropriate tissue types in the correct spatial arrangement, despite the constitutive expression of the homeotic genes, suggests that these genes do not directly control the arrangement of tissues. Furthermore, since leaves can be converted into floral organs, and vice versa, the positional information that controls the arrangement of tissues must be the same for every organ. Our work showing that *FIL*, *YAB3* and *JAG*, which are expressed in all organs, are important for the development of fruit-specific tissues and for the development of all floral organs, suggests that they are important for this positional information. Thus, not only do floral organs share a common ancestry with leaves, but the mechanisms that pattern the arrangement of tissues are also common. It will be interesting to determine how the *FIL/YAB3* and *JAG* pathways were co-opted to pattern the wide array of organ types found in plants.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/21/4687/DC1

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