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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Transcriptional regulation of *FRUITFULL*, a MADS-box gene involved in *Arabidopsis* fruit development

A Thesis submitted in partial satisfaction of the requirements
for the degree Masters of Science

in

Biology

by

Ann Luu Nguyen

Committee in charge:

Professor Martin F. Yanofsky, Chair
Professor Nigel Crawford
Professor Robert Schmidt

2008

The Thesis of Ann Luu Nguyen is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2008

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ABSTRACT OF THE THESIS

Transcriptional regulation of *FRUITFULL,*
a MADS-box gene involved in *Arabidopsis* fruit development

by

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Masters of Science in Biology

University of California, San Diego, 2008

Professor Martin F. Yanofsky, Chair

The MADS-box gene *FRUITFULL (FUL)* plays a critical role in *Arabidopsis thaliana* fruit development after fertilization, particularly in the valves. While research has uncovered a number of genes that function upstream of *FUL*, there is little information on how *FUL* is directly regulated. To address this, the *FUL* promoter was studied in order to isolate the cis-acting regulatory regions that are required for *FUL* transcription. A 3.9 kb long promoter upstream the *FUL* genomic sequence was

demonstrated to direct the expression of the ß-glucuronidase (GUS) reporter gene in a pattern that mimicked the *FUL* expression pattern previously reported in mRNA *in situ* hybridization studies and *ful-1* DsE enhancer trap lines. In a “promoter-bashing” study, we identified a 682 bp long region within this 3.9 kb promoter that contained at least one cis-element required for transcriptional activation of *FUL* in the valves. Site-directed mutagenesis revealed that a CArG box in this aforementioned region was required for the repression of ectopic transcription in the ovules but was not required for expression in the valve cells. Further studies will precisely identify the cis-element(s) in this regulatory region that positively regulate *FUL* transcription in the valves. Additionally, our data showed that the 3’ region of the 3.9 kb promoter was required for *FUL*-like expression in the inflorescence stem.

INTRODUCTION

The structure of wild-type *Arabidopsis* fruit

The unique pathways of flower and fruit development by Angiosperms have been crucial to their evolutionary success. The fruit, which is arguably the most complex plant organ, nourishes and protects the developing seeds, and, at maturity, mediates seed dispersal.

Arabidopsis thaliana, a model plant, is typical of more than 3,000 other Brassicacea species. Its fruit consists of a dehiscent seed pod called a silique that derives from the gynoecium, the female structure of the flower (Bowman et al., 1999; Ferrandiz et al., 1999). The fruit morphology that *Arabidopsis* displays is similar to that of certain families (including legumes), and its physiological and biochemical characteristics are comparable to many others (Barendse et al., 1986). Mature seeds are released when the fruit "shatters" (or dehisces), easily falling to pieces under mechanical pressure (Dinneny and Yanofsky, 2005). The *Arabidopsis* fruit is derived from the gynoecium, which develops as a tubular structure derived from two fused carpel primordia emerging at the center of the floral meristem.

Different regions are visible in gynoecium along the longitudinal axis (Bowman et al., 1999; Dinneny and Yanofsky, 2005). In the apical region is the stigma specialized in pollen reception and germination, followed by a short style with a core of transmitting tract tissue. The medial region corresponds to the ovary. The septum internally divides the ovary into two cavities to accommodate and protect the ovules, and, after fertilization, the seeds (Figure 1B; Bowman et al., 1999). The ovules develop from the placenta on

either ends of the septum and are attached to both it and the replum through the funiculus. At the basal end is the gynophore, a short solid cylinder that connects the ovary to the flower (Figure 1A).

The diagram in figure 1B represents a cross-section of the *Arabidopsis* ovary, showing its bilateral symmetry (Ferrandiz et al., 1999; Roeder and Yanofsky, 2006). The *Arabidopsis* ovary can be divided in three major regions: the valves, the valve margins and the replum (Figure 1B; Bowman et al., 1999).

The valves are located at the lateral parts of the ovary and we can distinguish three different regions from outer to inner. The abaxial (outer) epidermal layer or exocarp consists of long rectangular and stomata. The mesocarp is below formed by chloroplast-containing cells and the vasculature of the valves runs through this region. The endocarp is conformed by two layers, the adaxial epidermal layer named the en*a* layer and a lignified layer, or en*b* layer (Bowman et al., 1999).

The replum regions refer to the abaxial region of the septum, with the replums dividing the fruit into halves (Figure 1B). Each replum is located in the medial regions of the fruit and contains one of the medial vascular bundles. After dehiscence (fruit opening) the replum remains attached to the fruit.

The valve margins are the zones where the fruit opens and located at the valve-replum boundaries (Figure 1B). The valve margins are subdivided into two distinct regions, the separation layer (or dehiscence zone) and the lignified cell layer (Roeder and Yanofsky 2006).

Fruit fertilization and dehiscence

Upon successful fertilization, a series of signals are triggered, causing the gynoecium to undergo final differentiation into a mature fruit (Spence et al, 1996). After fertilization, the valve and replum epidermal cells divide and expand in primarily longitudinal direction, and the valve stomata differentiate in the outer epidermis (Roeder and Yanofsky, 2006). Secondary vascular strands that begin developing from the lateral vasculature continue differentiating and branching throughout the mesophyll layers of the valve to form a reticulate pattern (Alvarez and Smyth, 2002). The en*b* layer expands and lignin is deposited into the cell walls. The en*a* layer expands then breaks down as the fruit matures and is gone by stage 17b (Spence et al., 1996). Cell expansion and division at the valve margin is limited compared to the valve and replum, causing it to form an increasingly prominent indentation at the valve-replum junction as the silique expands (Vivian-Smith and Koltunow, 1999; Roeder and Yanofsky, 2006).

At stage 17b, hydrolytic enzymes are recruited by the separation layer in the valve margin to breakdown the middle lamella between cell walls, leaving the valves free to detach from replum (Roeder and Yanofsky, 2006). The tissue facing the valve is one to two cells wide and becomes lignified. It has been proposed that the lignified cells of the valve's en*b* layer is continuous with the lignified layer of the valve margin, forming a spring-loaded tension mechanism that causes dehiscence pulling valves away from the replum to release seeds (Liljegren et al., 2000).

Genes involved in fruit patterning.

While the required signals produced after fertilization to cause final differentiation into the mature fruit have not been studied in depth, genes controlling post-fertilization and fruit morphogenesis have been studied in detail (Dinneny and Yanofsky, 2005). Several studies have identified regulatory genes involved in fruit formation, leading to a genetic model that explains how this organ is patterned (Figure 3; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007). The model explains how the valve margin forms as narrows stripes at the valve-replum boundary through the control of both replum and valve gene activities (Figure 3).

The role of *FUL* in fruit development

The MADS-box domain transcription factor *FRUITFULL (FUL)* was the first of these genes discovered to mediate fruit development after fertilization (Gu et al., 1998; Dinneny and Yanofsky, 2005). *FUL* is most related to fellow MADS-box genes *APETALA1 (AP1)* and *CAULIFLOWER (CAL),* and the non-flowering *ap1 cal ful* mutant indicates that all three genes redundantly regulate flower meristem identity (Ferrandiz et al., 2000a). Though *FUL* plays a role in floral meristem identity, gynoecium development of *fruitfull (ful)* mutants is largely unaffected through stage 11, with the exception of the en*a* layer (Ferrandiz et al., 1999).

FUL is important to development of the normal silique, playing a prominent role in regulating post-fertilization development in all cell layers of the valves. Mutants with the strong *ful-1* allele, resulting from an DsE enhancer trap insertion in the 5' untranslated region of the *FUL* gene, have a severely reduced valve length that results in

compacted and smaller seeds, and a widened and winkled replum; occasionally, seed-crowding in the shortened siliques will cause valves to rupture before seeds mature (Figure 2; Gu et al., 1998). *ful* mutants also have an elongated style (Ferrandiz et al., 2000a). Stomata fail to differentiate in the abaxial valve epidermis, the lateral and medial vascular bundles of the valve poorly differentiate, and valve cell growth is reduced in *ful* (Figure 2A and 2B; Gu et al., 1998). Overexpression of *FUL* by the cauliflower mosaic virus (CaMV) 35S promoter results in ectopic valve cell development in the valve margin and replum, and indehiscent fruit (Ferrandiz et al., 2000b).

Although it was originally proposed that *FUL* positively regulated valve cell differentiation, the discovery of genes that specified valve margin identity showed that *FUL* did not promote valve cell identity, but instead inhibited ectopic expression of *SHATTERPROOF1* and *SHATTERPROOF2* (*SHP1,2*), *ALCATRAZ (ALC)* and *INDEHISCENT (IND)* (Dinneny and Yanofsky, 2005; Figure 3). *SHP1 and SHP2* are redundantly functioning MADS-box genes that promote valve margin identity (Ferrandiz et al., 2000b). *shp1,2* mutants are indehiscent and have reduced margin definition, and *SHP* expression is inhibited in the valves margins by ectopic *FUL* expression (Ferrandiz et al., 2000b; Liljegren et al., 2000). Expression of both *ALC*, a basic helix-loop-helix (bHLH) transcription factor, and *IND,* an atypical bHLH protein, are positively regulated by *SHP* in the valve margin (Rajani and Sundaresan, 2001; Liljegren et al., 2004). *IND* is required for development of both the separation and lignifed layers of the valve margin, while *ALC* is required for cell differentiation in the separation layer.

Removal of *SHP1,2* activity from *ful* mutants only partially rescues defects in the valves (Liljegren et al., 2000). However the combination of *alc, ind* and *shp1,2*

mutations in the *ful* mutant background rescues valve development and most defects. This demonstrates that *FUL* function represses valve margin gene expression in the valve and does not confer valve cell identity (Liljegren et al., 2004). However, *FUL* and the valve margin identity genes redundantly cooperate to promote lignification of the en*b* layer (Dinneny and Yanofsky, 2005).

FILAMENTOUS FLOWER (*FIL*)*, YABBY3* (*YAB3*), and *JAGGED* (*JAG*) genes positively regulate expression of *FUL* and *SHP* in the valves and valve margin respectively, and establish a functional link between the genes that regulate tissue differentiation in fruit development and those that regulate polarity (Dinneny et al., 2005). *FIL* and *YAB3* belong to the *YABBY* family, genes regulating tissue polarity in lateral organs of plants, while *JAG* encodes a C2H2 zinc-finger transcription factor that regulates tissue growth in lateral organs by limiting cell division inhibition in the distal regions of organs (Siegfried et al., 1999; Dinneny et al., 2004). *FIL* and *YAB3* act redundantly in the gynoecium and are required for FUL expression in the valves and positive regulation of *SHP*. Ectopic valve margin tissue is occasionally seen in the basal portion of *fil yab3* valves due to *JAG* activity, which also promotes *SHP* expression. *JAG* also plays a role in regulating *FUL* expression in the valves as there are *FUL*-negative clefts present in the apical shoulders of *jag* valves. *FUL* and *SHP* are expressed in mutually exclusive domains in the gynoecium (the valves and valve margins respectively); the fact that they are both regulated by *JAG, FIL*, and *YAB3* indicate that *FUL* and *SHP* may be activated by these genes in a concentration-dependent manner (Dinneny et al., 2005).

REPLUMLESS (RPL) is a BELL1 subfamily homeodomain transcription factor that regulates replum development by the repressing valve margin identity genes, like FUL does in the valves (Roeder et al., 2003). In the absence of *RPL,* ectopic *JAG/FIL/YAB3* activity promotes expression of *SHP* and *IND* in the replum, resulting in ectopic formation of valve margin tissue in this territory (Roeder et al., 2003; Dinneny et al., 2005; Liljegren et al., 2004).

Genes regulating of *RPL* expression have been recently discovered (Figure 3). *BREVIPEDICELLUS (BP*) transcription factor, a member of the class I *KNOTTED1*-like homeobox genes (*KNOX*) associated with meristem development, is expressed in high levels in the replum and style and is sufficient to activate *RPL* expression (Alonso-Cantabrana et al., 2007). The expression pattern of *BP* is controlled by *ASYMMETRIC LEAVES1* (*AS1*) expressed in the valves (Alonso-Cantabrana et al., 2007). *AS1* encodes a myb transcription factor that interacts with *ASYMMETRIC LEAVES2 (AS2),* a plant-specific transcription factor containing a *LATERAL ORGAN BOUNDARIES* domain, to directly inhibit expression of class I *KNOX* genes like BP (Guo et al., 2008). In the absence of *AS1* function, the replum is wider, valve width is reduced, and fruits are bumpy, presumably due to misexpression of *BP* in the valves (Alonso-Cantabrana et al., 2007).

Previous research establishes how *FUL* is essential to fruit patterning in *Arabidopsis* (Gu et al., 1998; Ferrandiz et al., 2000b; Liljegren et al., 2004; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007). While how *FUL* epistatically influences tissue differentiation in the fruit after fertilization has been studied in-depth, the transcription factors necessary for direct regulation of *FUL* expression are poorly understood.

However, isolation of the minimal active *FUL* promoter can lay the groundwork for future study of how transcription factors interact with the promoter and help in the discovery of established or novel DNA motifs required for interaction with said transcription factors.

An approximately 3.9 kilobase (kb) genomic fragment from upstream the *FUL* start codon was used as the putative *FUL* promoter. The promoter was used to drive a β-glucuronidase (GUS) reporter construct containing a minimal CaMV 35S promoter and analyzed for *FUL*-like expression in the Columbia background of *Arabidopsis thaliana*. Deletions as well as site-directed mutagenesis were used to characterize the promoter fragment and establish which regions of the promoter were required for wild-type expression of *FUL* in the valves and inflorescence stem.

MATERIALS AND METHODS

Cloning details

The 3.9 kb *FUL* promoter fragment was previously isolated and inserted into the cloning vector pBin19 to create the pBin-FUL construct. The promoter fragment was then cloned out of pBin-FUL using the restriction enzyme EcoRI and inserted into the bluescript SK+ plasmid vector (Stratagene) to create pAN1.

Reporters were in the pDW294 background, which contains a minimal CaMV 35S promoter used to drive transcription of GUS (Hong et al., 2003). The pAN4 reporter construct was made by cloning the *FUL* promoter from pAN1 into pDW294 via BamHI. pANR4 contained the BamHI promoter insertion in reverse orientation.

pAN5 was created by reducing the pAN1 construct to 4.8 kb with SpeI to create pAN3; a 1.2 kb XbaI-BamHI promoter fragment from pAN3 was cloned into the pDW294 to create pAN5. pAN6 and pAN22 were created by either inserting 500 or 700 base pair (bp) long promoter fragments, respectively, from pAN1 into pAN5 via XbaI.

All other reporters with truncated promoters were synthesized using PCR to isolate fragments of the *FUL* promoter from pAN1, with BamHI sites added to both the 5' and 3' ends. The PCR product was subcloned into a pCR2.1 TOPO vector (Invitrogen), and promoter fragments were then shuttled into the pDW294 using BamHI; see Table 2 for oligonucleotides used to isolate promoter fragments and Table 1 for the sequence of those oligonucleotides. Orientation of insertions was checked using restriction enzyme sites present in the *FUL* promoter and the vector.

Plants and plant transformation

The floral dip method was used to generate transgenic lines in the Columbia (Col) background, and plants expressing reporter constructs were selected on kanamycin (Weigel and Glazebrook, 2002).

GUS staining, histology, and microscopy

GUS staining was performed as previously described with the exception that tissues were vacuum infiltrated for 20 minutes with staining solution (0.2% Triton-X-100, 50 mM sodium phosphate buffer pH 7.2, 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, 1 mM X-gluc) at room temperature (Sessions et al., 1999). Standard light microscopy was performed on whole-mounts. Tissue samples from strong GUS expressing transgenic lines were taken through an ethanol series into Histoclear (National Diagnostics) and embedded in Paraplast Plus, before using a microtome to generate 8 μm tissue sections. Slides were prepared and viewed as described (Roeder et al., 2003).

Site-directed mutagenesis

The *FUL* promoter CArG-box located 2512 bp upstream the *FUL* ATG was mutagenized in the pAN1 construct using PCR with Pfu DNA polymerase and the QuikChange Kit (Stratagene). The CArG-box sequence was specifically mutated from 5'-CCAATTTTGG-3' to 5'-GGAATTTTCC-3' using the oAN29 and oAN30 primers, which overlapped the mutagenized site, to make pAN23 (Table 1). DpnI was used to digest the parental DNA templates and the PCR product was transformed into

Escherichia coli. pAN23 was verified by sequencing. The site-mutated *FUL* promoter was then cloned from pAN23 into pDW294 using BamHI to create pAN26.

Promoter analysis

Known motifs of plant cis-acting elements that could potentially mediate *FUL* transcription were identified in the *FUL* promoter by scanning the sequence against the online Plant Cis-acting Regulatory DNA Elements database (http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999).

RESULTS

Reporter GUS activity by the pAN4 promoter resembles wild-type *FUL* expression

A promoter fragment upstream the ATG translation initiation site for *FUL* was previously isolated in the construct pBinFUL by a graduate student in Dr. Martin Yanofsky's lab. The 3873 bp *FUL* promoter fragment 66 bp, upstream the ATG (-3938, -66; 5', 3' promoter coordinates refer to the *FUL* ATG as +1) from pBinFUL was then inserted in front of a minimal 35S promoter and used to drive GUS expression; this construct was denoted pAN4 and transformed into the Columbia (Col-0) ecotype of *Arabidopsis thaliana* (Figure 4A).

Transcriptional activity of pAN4 generally agreed with data from *FUL* mRNA *in situ* hybridizations assays and GUS activity reported in heterozygous *ful-1* plants that contain a DsE enhancer trap previously described in published literature (Mandel and Yanofsky, 1995; Gu et al., 1998). The GUS expression was detected in the inflorescence meristems, and throughout the developing carpel primordia before valve differentiation. GUS reporter activity was high in the floral meristem after stage 2 in comparison to stage 1 and 2 (Figures 5 and 6). At stage 8 the expression was restricted to the adaxial layers of ovary walls; residual expression was found in the replum vascular bundle (Figure 5E). GUS activity then extended to all layers of the valve by stage 10 and restricted to the medial vascular bundle in the replum (Figure 5F). GUS activity was not reported in the medial ovary, which includes septum tissue and developing ovules (Figures 5A, 5F and 5G).

Whole-mounts and longitudinal tissue sections of the gynoecia of pAN4 transformants showed that GUS was preferentially expressed in the apical and basal regions of the valves from stage 12 up to stage 17 and correlates to previously published data (Figures 5A-5D; Gu et al., 1999; Dinneny et al., 2005). As reported for *ful-1*/+ plants, pAN4 transgenic plants showed GUS activity in the nectaries, gynophore, style and the vasculature of the sepals (Figures 5 and 6A). The expression was also detected in the inflorescence stem in high levels throughout the tissues, but with the highest levels in the vascular bundles and epidermal tissues (Figures 6A, 6D, and 12A).

Transcriptional activity by the *FUL* promoter fragment in the pAN4 transgenic line was considered wild-type for *FUL,* and the pAN4 promoter was used as the starting point for *FUL* promoter analysis. A series of constructs using progressively larger 5' or 3' deletions of the pAN4 promoter fragment to drive reporter activity were used to identify the minimal *FUL* promoter (Figure 4B). An initial approach using site direct mutagenesis was performed to analyze the importance of DNA motifs. The study was focused on the transcriptional activity in the valves and inflorescence stem.

The 5' region of the pAN4 promoter is required for *FUL* valve-specific expression

Reporter activity in the valves of transformants expressing the 5' deleted *FUL* promoter in pAN37 or promoters with progressively larger 5' deletions in pAN 38 and pAN39 resembled wild-type transcriptional activity of pAN4 (Figure 4B). The pAN39 promoter is 2.9 kb (-2952,-66) and is the smallest 5' deleted *FUL* promoter fragment capable of driving pAN4-like reporter activity in the valves. The pAN39 transformants

showed a GUS pattern similar to that of pAN4 plants with the exception of expression in the abaxial region of the replum (Figures 7B and 8G).

Reporter activity by the 5' deleted *FUL* promoter of pAN22 was not valve-specific (Figure 4). High activity was detected throughout the replum and the style (Figure 7C). The GUS expression was low in the valves, losing the typical apical-basal gradient observed in pAN4 gynoecia (Figure 7A and C). Interestingly, GUS activity in the gynoecia of pAN6 and pAN5 transformants, which have reporter constructs with smaller 5' deleted promoter fragments than pAN22, is similar to pAN22 transformants (data not shown).

GUS expression driven by 3' deleted promoter fragments of pAN53, pAN54, and pAN58 (Figure 4B) was similar to reporter activity of pAN4 in the valves of transformants (Figure 7D). In addition, GUS activity was detected in the style of pAN53, pAN54, or pAN58 reporter constructs and none of these lines had GUS activity in the medial vascular bundle of the replum (Figures 7D and 8I; data not shown for pAN53 and pAN54). The pAN58 *FUL* promoter is approximately 1.7 kb long, and is the smallest 3' deleted promoter fragment able to drive pAN4-like GUS expression in the valves. Although, on the other hand, we did not detect signal in the carpel primordia until the valve were completely differentiated (stage 7 and stage 8).

The 3' deleted promoter of the pAN59 (Figure 4B) transgenic line reports in the valves, but not in a pAN4-like fashion (Figure 7E). GUS activity did not appear until late stage 8, where it was visible in the adaxial layers (Figure 8E). As the gynoecium developed, the reporter activity extended throughout the inner layers of the valve but not the endocarp*a* layer or outer epidermis (Figure 8J). At stage 12, GUS activity

preferentially stained the secondary vasculature that generates a reticulate network in the mesophyll layers and the borders of the valve (Figure 7E).

With these results, we can say that the 682 bp *FUL* promoter region, overlapping the 5' deleted promoter in pAN39 and the 3' deleted promoter in pAN58 likely contains either one or more cis-acting regulatory elements necessary for *FUL* wild-type transcription in the valves (Figure 9). Several motifs of putative cis-elements were unique (occurring three or less times) in the pAN4 *FUL* promoter region and shared by pAN58 and pAN39, and were identified using a database of nucleotide sequence motifs found in plants. Several good candidates for cis-elements regulating *FUL* expression are shown in Table 3. A perfect CArG-box, with the 5'-CCAATTTTGG-3' sequence, located 2512 bp upstream the *FUL* ATG, was selected for site-directed mutagenesis and further study.

The 5'-CC(A/T)$_6$GG-3' sequence will bind most plant MADS-box proteins, and the interaction between transcription factors and CArG-boxes have been studied extensively (Ferrandiz et al, 1999; Riechmann et al., 1996; Folter and Angenent, 2006). The CArG-box located in the *FUL* promoter region overlapping pAN58 and pAN39 (or the 5' CArG-box) is one of two perfect CArG-boxes located within 500 bp of each other, with the other CArG-box (or 3' CArG-box) found 2038 bp upstream the *FUL* ATG. The 5' CArG-box of the pAN4 *FUL* promoter was mutated by converting the 5'-CC(A/T)$_6$GG-3' core motif to 5'-GG(A/T)$_6$CC-3', which has been shown to eliminate the binding activity of MADS-box transcription factors (Figure 10A; Savidge et al., 1995). The site-mutated *FUL* promoter fragment was used to drive GUS transcription by the pAN26 reporter construct. GUS activity was pAN4-like in most tissues of the inflorescence including the valves, indicating that the 5' CArG box is not required for

FUL transcription in the valves (Figure 10). GUS was weakly expressed in the adaxial replum. Interestingly, GUS was expressed through stage 12 in the developing ovule primordia and ovules of the gynoecia. Reporter activity was largely gone from the ovules by stage 14. This ectopic expression was also detected in the developing ovules of pAN22, pAN6, and pAN5 transformants. In these lines, the GUS reporter genes was driven by a 5' deleted *FUL* promoters that do not contain the aforementioned 5' CArG box (Figure 8H; data not shown for pAN6 and pAN5).

The 3' region of the pAN4 promoter is required for inflorescence stem transcription

Reporter activity occurs throughout the tissues of the inflorescence stem in pAN4 transformants, with GUS expressed in high levels in the vascular bundles (including the xylem and phloem) and the outer tissues (epidermis, cortex, and endodermis). GUS is expressed in relatively weak levels in the pith (center of the inflorescence stem). The pAN53 reporter harbors the largest 3' deleted *FUL* promoter (1.4 kb long deletion, Figure 4B). The GUS expression level is reduced in the inflorescence stems of pAN53 transformants and they lack transcriptional activity in the vascular bundles (Figures 11D and 12D). Transcriptional activity by pAN54 (Figure 4B), which has the next largest 3' deleted promoter, is completely absent from the inflorescence stem (Figure 12E).

All available constructs with reporter expression driven by 5' deleted *FUL* promoters share the same pAN4-like pattern of expression in the inflorescence stems of transformants – where GUS is expressed in all inflorescence stem tissues, with higher levels of activity in the vascular bundles and outer tissues (Figures 11 and 12). Though, on average, the independent GUS-expressing transformant lines (T1) of pAN22, pAN6,

and pAN5 had relatively higher levels of reporter activity in the inflorescence stem compared to analyzed T1 lines of pAN4 (Figure 13). In addition, the 2.1 kb promoter fragment in pAN22, the 1.7 kb promoter fragment in pAN6 and the 1.2 kb promoter fragment in pAN5 were still capable of activating transcription in the inflorescence meristem and floral meristem (data not shown).

Since the deletion in the pAN53 promoter includes the pAN5 promoter fragment, it is highly probable that pAN5 contains one or more cis-elements required to promote transcription in the vascular bundles of the inflorescence stem. The pAN5 promoter was analyzed for potential cis-elements that positively regulate *FUL* transcription in the inflorescence stem by searching the sequence against a database of motifs found in cis-elements; a selection of promising motifs that were both unique in the pAN4 promoter (found three or less times) and found in the pAN5 promoter fragment are given in Table 4.

It appeared that specific reporter activity in the sepal vasculature of pAN22 and pAN6 transformants was lower than wild-type/pAN4, and that ectopic activity occurred in other sepal tissues; this complements the expression patterns of all 3' deleted promoter fragments analyzed by this reporter series, which were capable of specifically activating transcription in the sepal vasculature (Figure 11; data not shown for pAN58 and pAN59).

Reversed *FUL* promoter verifies transcriptional data for 5' and 3' regions.

The *FUL* promoter fragment in pAN4 was inserted in reverse orientation following the same procedure as used for the other transgenic lines. This reporter was called pANR4. GUS activity by pANR4 in the valves was similar to pAN4, with

expression levels highest in the apical and basal regions of the valve. Reporter expression was also visible in the nectaries, medial tissues of the style, and vasculature of the sepals. GUS activity was weak or absent in the inflorescence stem, gynophore, and medial vascular bundle of the replum in T1 lines. The expression patterns obtained in pANR4 lines indicate that the elements in the 5' region of the *FUL* promoter that positively regulate transcription in the valves are position and orientation independent, while elements in the 3' region of the *FUL* promoter (which positively regulate expression in the inflorescence stem) are either position- and/or orientation-dependent (Figure 14).

DISCUSSION

The proper regulation of *FUL* expression is crucial to *Arabidopsis* fruit development after fertilization. While research has uncovered a number of genes that function upstream of *FUL*, there is little information on how *FUL* is directly regulated. Here, the *FUL* promoter was studied in order to isolate cis-acting elements required to regulate *FUL* activity. Using fragments of the promoter region to drive reporter expression in a "promoter-bashing" study, this research identified that a 682 bp region, located 2271 bp upstream the *FUL* start codon, contained at least one cis-element required for transcriptional activation of *FUL* in the valves. Additionally, study of generated reporter lines revealed that the 1.2 kb promoter region directly upstream the *FUL* start codon contained cis-elements required for *FUL* activity in the inflorescence stem.

pAN4 promoter mimics *FUL* expression

A 3.9 kb genomic fragment isolated 66 bp upstream the *FUL* start codon was used as the starting point for promoter analysis. The reporter activity by this 3.9 kb promoter mimicked the *FUL* expression pattern previously reported in mRNA *in situ* hybridization studies and the *ful-1* DsE enhancer trap lines (with the DsE enhancer trap element inserted into the 5' untranslated region of *FUL*; Mandel and Yanofsky, 1995; Gu et al., 1998; Dinneny and Yanofsky, 2005). Reporter activity by the 3.9 kb promoter (in pAN4 transgenic lines) was detected in the inflorescence meristem, style, inflorescence stem,

nectaries, gynophore, and vasculature of the sepals. GUS was also expressed throughout the tissues of the carpel primordia prior to stage 7, and was later restricted to the valves.

GUS was expressed at distinctly higher levels in the apical and basal regions of the valves compared to the middle region, which is not evident in DsE enhancer trap lines (Figures 2D and 5). However, GUS activity by this promoter was still observed in all cell layers of the valve, even in the middle region. This difference between GUS activity by this promoter and GUS activity in the enhancer trap lines may be because the expression gradient of *FUL* is more visible in the Col-0 background. The Landsberg *erecta* ecotype used in enhancer trap lines produce smaller, more compact fruits compared to the Col-0 ecotype used in this study, which lack the *erecta* mutation. Another explanation may be that transcriptional activation by the *FUL* promoter is influenced by its position in the *Arabidopsis* genome, resulting in minor differences in reporter activity by this promoter compared to the expression pattern reported in enhancer trap lines. Further studies either crossing the *ful-1* enhancer trap into the Col-0 background or transforming pAN4 into the Ler ecotype will verify if this observed expression pattern is due to the different ecotypes.

682 bp region contains cis-elements required to regulate valve expression

This study primarily evaluated the promoter region affecting transcriptional activation in the valves, since that tissue carries significant phenotypic defects in *ful* mutants. The promoter was subject to various deletion mutations and these mutated promoters were used to generate reporter lines. A 682 bp region, between positions -2952 and -2271, was identified to contain at least one cis-element required to regulate *FUL* transcription in the valves (Figure 9). Data from the reversed promoter in pANR4

indicates that these cis-elements are orientation- and position-independent (Figure 13). Several putative signal sites in the promoter were selected as candidates that may mediate *FUL* signal response in the valves.

5' CArG box represses ectopic activity in developing ovules

A CArG box located in the 682 bp region was chosen for site-directed mutagenesis in the promoter. The CArG box signal site, containing a 5'-CC(A/T)$_6$GG-3' core motif, is involved in the binding of MADS box transcription factors and is linked to MADS factor regulation of other MADS factors (Folter and Angenent, 2006). For instance, previously reported data indicates that *AGAMOUS* binds a CArG box to directly regulate *SHP2* expression (Flanagan et al., 1996). The core motif was altered to 5'-GG(A/T)$_6$CC-3' in the 3.9 kb promoter and the mutated promoter was inserted into a reporter construct. Reporter activity in pAN26 transgenic lines remained in all cell layers of the valves and maintained a basal-apical expression gradient, indicating that the CArG box was not necessary for transcriptional activity in all cells of the valves. This does not exclude the possibility that this motif co-regulates some aspect of *FUL* activity in the valves.

Interestingly, data indicated that the CArG motif represses ectopic GUS activity in the ovules through stage 12. Possible candidates may be MADS factors that bind this region to repress *FUL* activity and would include *SHP1* or *SHP2*, which are expressed in mutually exclusive domains to *FUL* and are active in the ovule primordia (Pinyopich et al., 2003). Other candidates include *AGAMOUS* and *SEEDSTICK*, which redundantly promote ovule development along with *SHP1,2* (Pinyopich et al., 2003)..

Two CArG boxes are capable of cooperating to bind ternary protein complexes, even in cases involving autoregulation (Folter and Angenent, 2006). The CArG box mutagenized in pAN26 is located at position -2512 and was one of two perfect CArG boxes identified in the putative *FUL* promoter; an additional CArG box, located 2038 bp upstream the *FUL* start codon, was found. While deletion data indicates that the 3' CArG box is not required for wild-type activity in the valves, expression activity is altered when both CArG boxes are not present in the deleted promoters. It is possible that the 3' CArG box works in conjunction with the 5' CArG box to regulate some other aspect of *FUL* activity in the valves (or other tissues), and further study should be done in order to address this point. However, activity by the truncated promoter lacking both CArG boxes (pAN59) – which expresses some aspects of *FUL* transcription in the valve mesophyll layers – indicates that there is at least one additional cis-acting element mediating expression in the valves.

Future isolation of signal motifs promoting transcription in the valves

Further studies of other unique motifs in the 682 bp region should be performed in order to identify the cis-element(s) that mediate(s) valve activity (Table 3). There are three additional putative cis-elements motifs that are unique in this region and are excellent candidates for future "promoter-bashing" experiments using site-directed mutagenesis or deletion (Table 3). One of them is the palindromic CATATG sequence (2893 bp upstream the *FUL* start codon), which is the SAUR motif (Small Auxin-Up RNA motif) consensus sequence involved in early auxin response (Xu et al., 1997). Auxin is a class of major plant hormones, and plays an essential role in coordinating

many growth and behavioral processes in the plant life cycle. In terms of fruit development, auxins are linked to apical-basal tissue specification as well as vasculature development. In addition to the SAUR motif, there are several putative auxin response elements (AuxRE, TGTCTC) located upstream and downstream of the 682 bp region critical for valve expression (see Table 4) that may work in conjunction with the SAUR element to regulate *FUL* expression (Ulsamov et al., 1999). Additional study of the 5' promoter region required for transcriptional activation of *FUL* should test if the 682 bp region is sufficient to drive *FUL*-like reporter expression in the valves.

This research does not exclude the possibility that there are additional cis-elements upstream the identified 682 bp region capable of promoting aspects of transcriptional activation in the valves of pAN59 transformants. For instance, a *WUS*-box motif (TTAATGG) is located in reverse-orientation at position -3168 (Higo et al., 1999). The *WUS*-box is linked with direct regulation of MADS box factors by *WUSCHEL (WUS)* and is associated with the maintenance of stem cell populations in both the floral and inflorescence meristem (Lohmann et al., 2001).

Another putative cis-element upstream the 682 bp region is a motif with the ACAAAGAA sequence (designated "XYLAT" in the PLACE database) located in reverse orientation at position 3772, and is linked to regulation of secondary xylem development in *Arabidopsis* (Higo et al., 1999; Ko et al., 2006). The pAN59 construct contains this motif, and the resulting plants show high reporter activity in the vasculature of the style and in the reticulate network of the valves, which may correspond to the vascular network that begins thickening at stage 12 in the mesophyll layers of the valve. Thus, this motif may promote *FUL* expression in the vascular tissue.

3’ promoter region is required for *FUL* transcription in the inflorescence stem

While this study primarily focused on expression in the valves of developing fruit, “promoter-bashing” revealed that the 3’ region of the promoter is required for expression in the inflorescence stem. The smallest 5’ truncated promoter, located between positions -1255 and -66, used in pAN5 lines is sufficient to drive expression in the inflorescence stem, with particularly strong expression in the vascular bundles and outer layers. However, reporter lines driven by the promoter with a 3’ deletion mutation between positions -1473 and -66 (pAN53) lacked expression in most inflorescence stem tissues, including the vascular bundles. The larger 3’ deletion mutation (with a 1.8 kb deletion) in pAN54 caused loss of all transcriptional activity in the inflorescence stem. Loss of GUS expression in the inflorescence stem by the reverse-oriented promoter in pANR4 transgenic lines indicated that cis-elements mediating transcriptional activation of the reporter in this area were position- and/or orientation-dependent.

The 1190 bp promoter in pAN5 was sufficient to drive expression in the inflorescence meristem, corresponding to previously reported data using a 2.3 kb *FUL* promoter fragment to drive a *GUS* reporter (Mandel and Yanofsky, 1995). pAN5 also directed GUS activity in the floral meristem. pAN58, a reporter containing a 3’ deletion, was incapable of directing GUS expression in either the floral or inflorescence meristem. This evidence suggests that the 3’ promoter is responsible for activating *FUL* during early floral organ development.

In the absence of the 5’ promoter region required for valve-specific activity, reporter activity by the promoter regions in pAN5 generally mimicked expression pattern

by the 3.9 kb promoter in pAN4 transformants, with the GUS expressed in tissues of the presumptive carpel primordia until the valves begin to differentiate. By stage 8, defects in expression patterning in the gynoecium were already noticeable, with high levels of ectopic expression in the replum, ectopic activity in the presumptive tissues of the ovule primordia, and lowered activity in the valves.

A number of promising motifs of putative cis-elements found in the 1190 bp 3' region were associated with regulating expression in either ovules/seeds and meristematic tissues (Table 4). In addition, a number of motifs associated with auxin-response elements were located in the 3' promoter region, and may be associated with activity in the inflorescence stem. Further dissection of the pAN5 promoter fragment will be able to narrow down the exact region containing cis-elements required for expression in the inflorescence stem and other tissues.

Determining the minimal *FUL* promoter

Rescue studies using fragments of the 3.9 kb promoter to direct expression of *FUL* cDNA in the *ful* background will clarify the minimal *FUL* promoter region and the region required for valve development. It is uncertain if the 5' region of the promoter requires transcriptional activity by the 3' region to mediate valve-specific expression, particularly after successful fertilization. The rescue by the minimal promoter region required for *FUL*-like reporter expression in the valves (pAN58 in this reporter series) should be compared against rescue using the pAN39 promoter, which contains both the 5' promoter region required for valve-specific expression and the 3' promoter region required for inflorescence stem and floral meristem expression. If the 5' promoter region

is unable to rescue valve development, the 3' promoter region may regulate some aspect of valve development or set up conditions in the floral meristem required for differentiation of the valves in later stages.

The characterization of the *FUL* promoter performed here will provide the basis for more directed studies on the *FUL* activity and the upstream regulating elements. Since regions were identified to carry cis-elements required to either promote expression in the valves or inflorescence stem, or repress ectopic activity in developing fruits, isolation of particular promoter(s) will allow the expression of target gene(s) in a specific tissues where *FUL* is present. Ultimately, further analysis using different approaches (chromatin immuno-preciptation, gel retardation, et cetera) will identify the transcription factors that bind to the *FUL* promoter to regulate *FUL* activity.

APPENDIX 1: FIGURES AND FIGURE LEGENDS

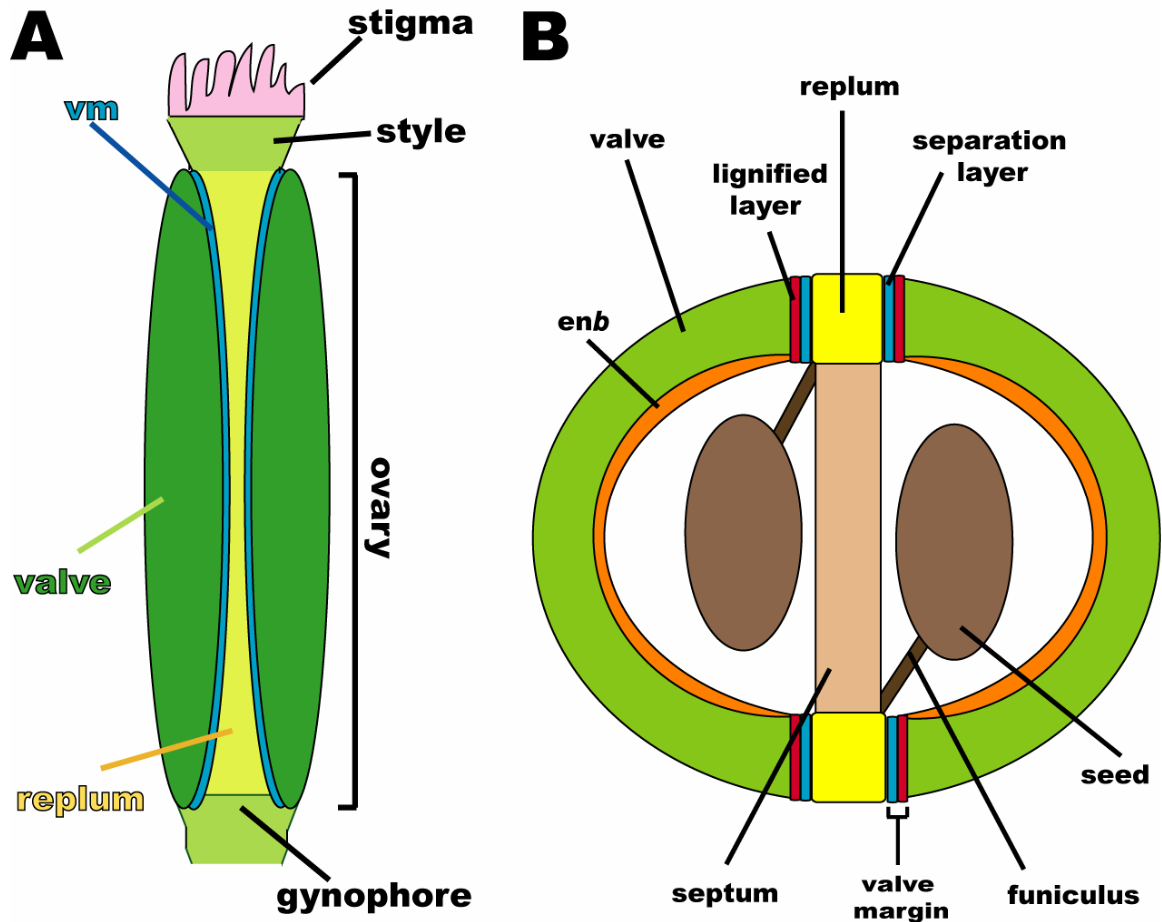


Figure 1. Organization of *Arabidopsis thaliana* fruit tissues.
(A) Model of a typical stage 12 *Arabidopsis* gynoecium. **(B)** Diagram of a lateral cross-section of a mature fruit. Lignified cells in the en*b* layer are thought to be continuous with cells in the lignified layer, driving dehiscence. The valve margin is composed of both the lignified layer by the valve side and separation layer by the replum side.

Valve margin (vm).

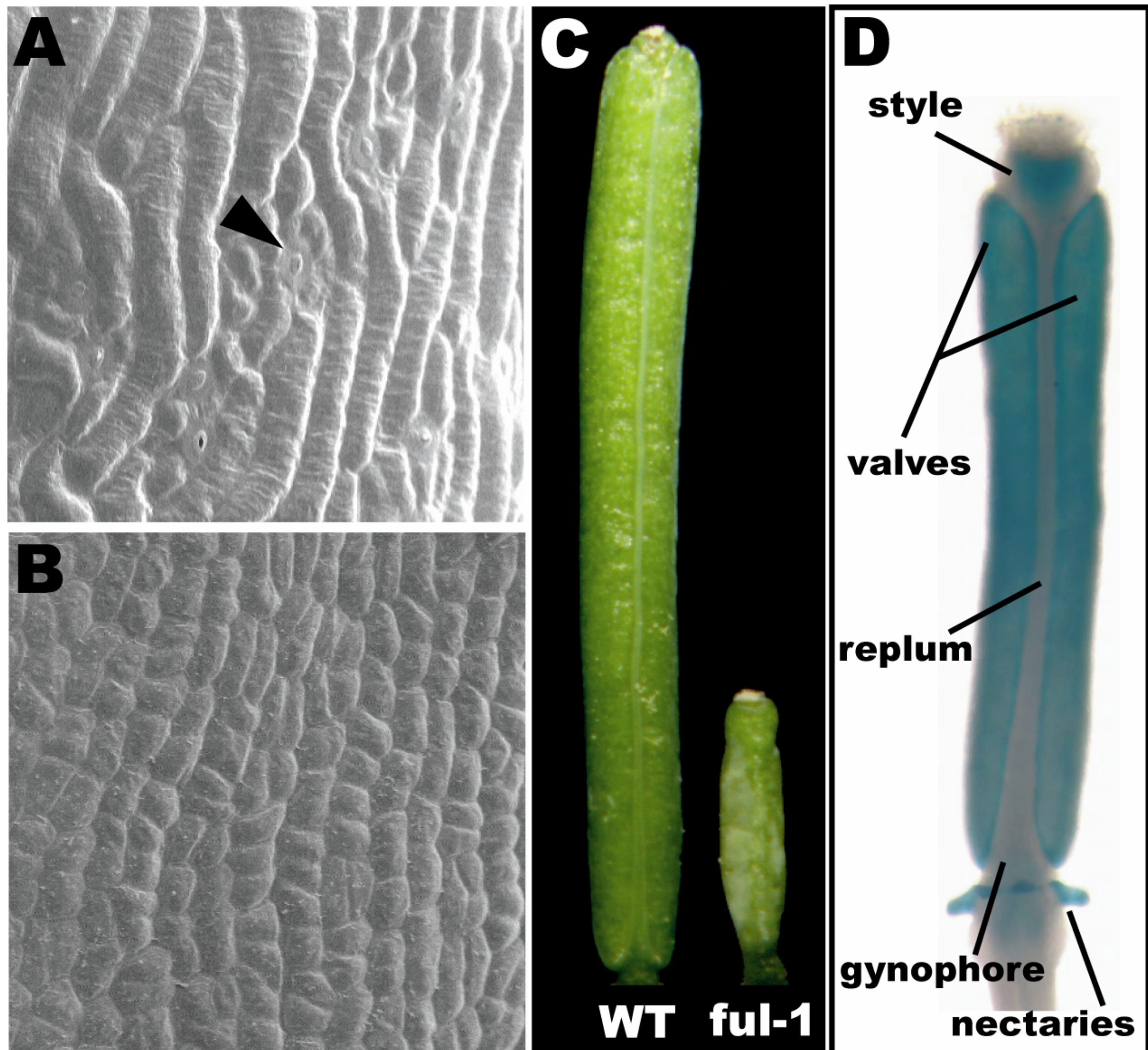


Figure 2. ***FUL*** **expression plays a crucial role in valve development.**
(A) Scanning electron micrograph (SEM) of the valve cells of a wild-type fruit. Arrow indicates presence of a stoma in the outer epidermis of the valve. **(B)** SEM of valve cells in a *ful-1* mutant. Valve cells are significantly smaller and stomata do not differentiate in the valves of *ful-1*. **(C)** Comparison between wild-type (WT) and *ful-1* fruits in the Lansberg *erecta* (Ler) ecotype. Wild-type fruits are approximately four times the length of *ful-1* fruits. **(D)** Gus expression (blue staining) of the *FUL* enhancer trap in heterozygous (ful-1/+) stage 16 fruit. *FUL* is strongly expressed in the valves, nectaries, and vasculature of the style.

(A-D) pictures provided by and printed with permission of Dr. Martin F. Yanofsky.

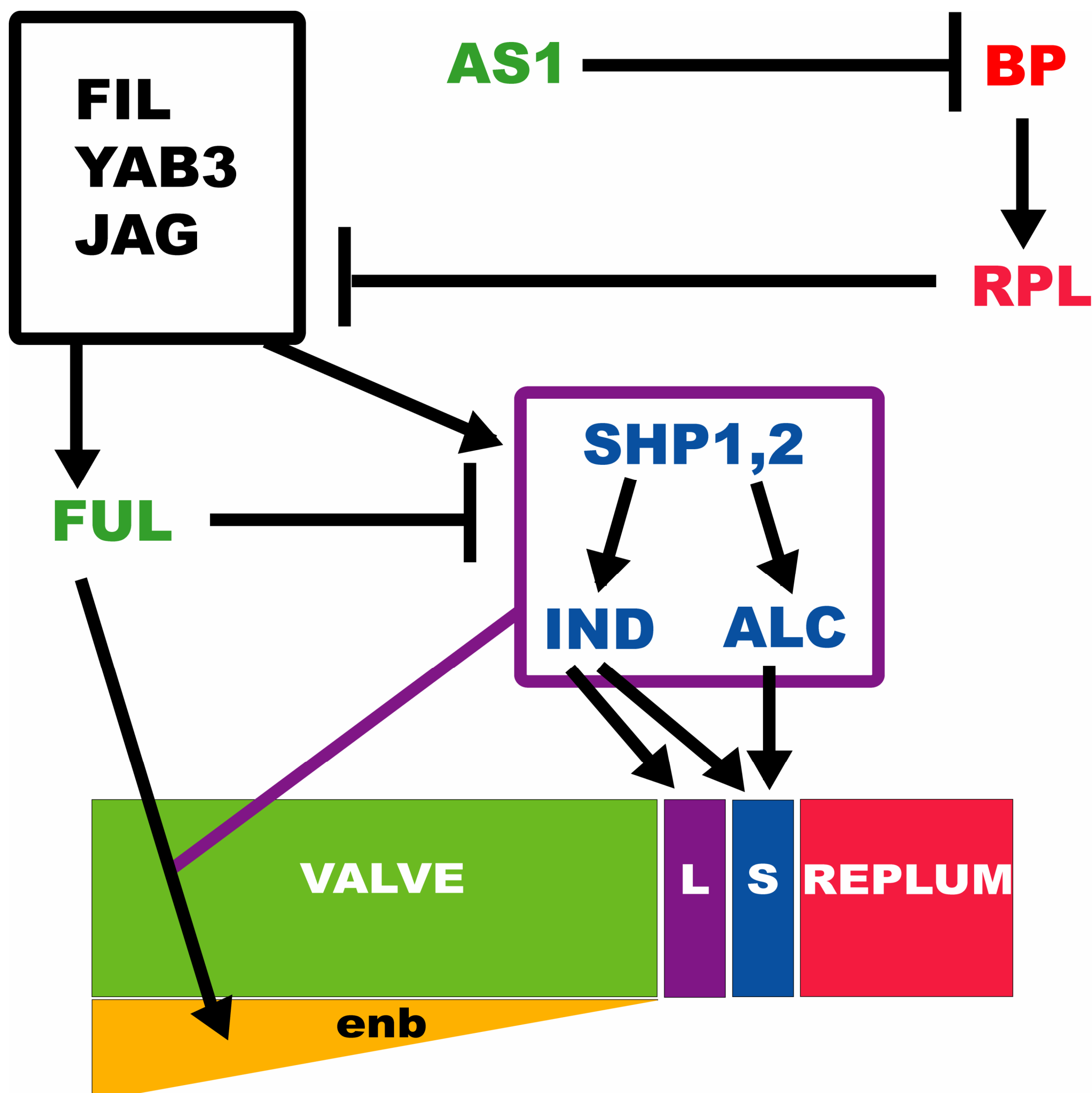


Figure 3. A model for *Arabidopsis* fruit patterning along the mediolateral axis. Valve margin identity is specified by the *SHATTERPROOF* (*SHP1* and *SHP2*), *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) genes; *FRUITFULL* (*FUL*) is expressed in the valves and limits the expression of the valve margin genes to this region. The *REPLUMLESS* (*RPL*) gene carries out a similar role in the replum by the negative regulation of the *FILAMENTOUS FLOWER* (*FIL*), *YABBY3* (*YAB3*) and *JAGGED* (*JAG*) genes. The *FIL/YAB3/JAG* action activates the expression of *FUL* and *SHP* in the valves and valve margin respectively. On the other hand, the activities of *FUL* and the valve margin genes redundantly cooperate to lignify the en*b* cell layer. Replum formation is driven by *RPL*, whose expression is promoted by *BREVIPEDICELLUS* (*BP*). *BP*, in turn, is negatively regulated by *ASYMMETRIC LEAVES1* (*AS1*), expressed in the valves (Dinneny et al., 2005; Alonso-Cantabrana et al., 2007).

Lignified layer (L), separation layer (S).

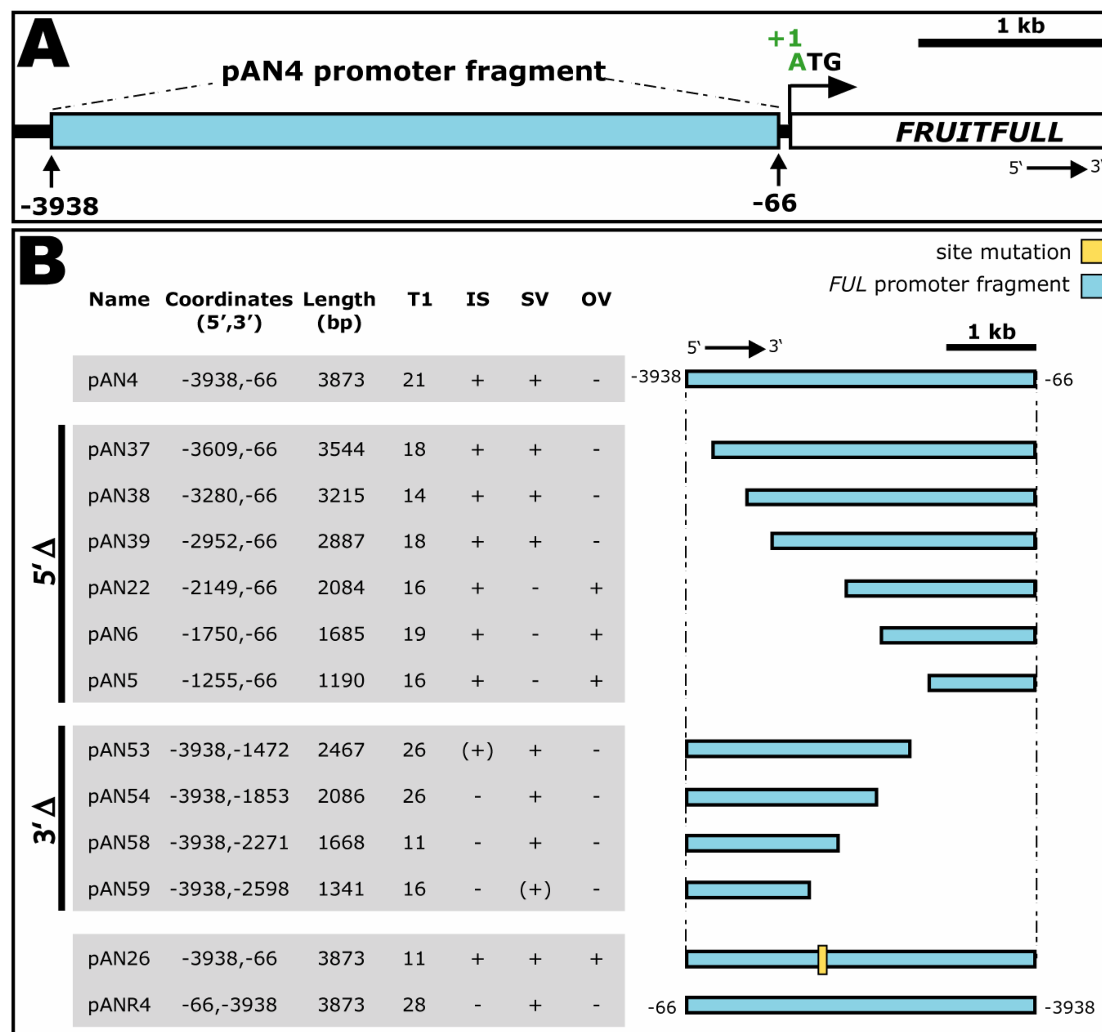


Figure 4. *FUL* reporter constructs generated.
(A) A diagram showing where the pAN4 promoter fragment lies relative to the *FUL* coding region. The pAN4 promoter is derived from the 3873 bp genomic region found 66 bp upstream the *FUL* ATG (the "A" is considered +1). **(B)** A schematic representation of the GUS reporter construct series made. The table on the left indicates the name of the construct, the coordinates of the genomic region relative to the *FUL* ATG start codon, the total length of that *FUL* promoter fragment, and the number of independent GUS-expressing transformant lines that were studied (T1). Additionally, the table indicates if GUS was expressed by *Arabidopsis thaliana* (Col-0 type) transformants in the inflorescence stem (IS) and ovules (OV), and if GUS expression was valve-specific (SV) [-, negative; +, positive; (+), positive but not pAN4-like]. The right side shows a diagram representing the promoter fragment of each construct, relative to pAN4. 5' and 3' deletion constructs are marked as 5'Δ and 3'Δ, respectively. The pAN26 construct harbors a site mutation of the 5' CArG motif (at position -2512). pANR4 uses the reverse-oriented pAN4 *FUL* promoter.

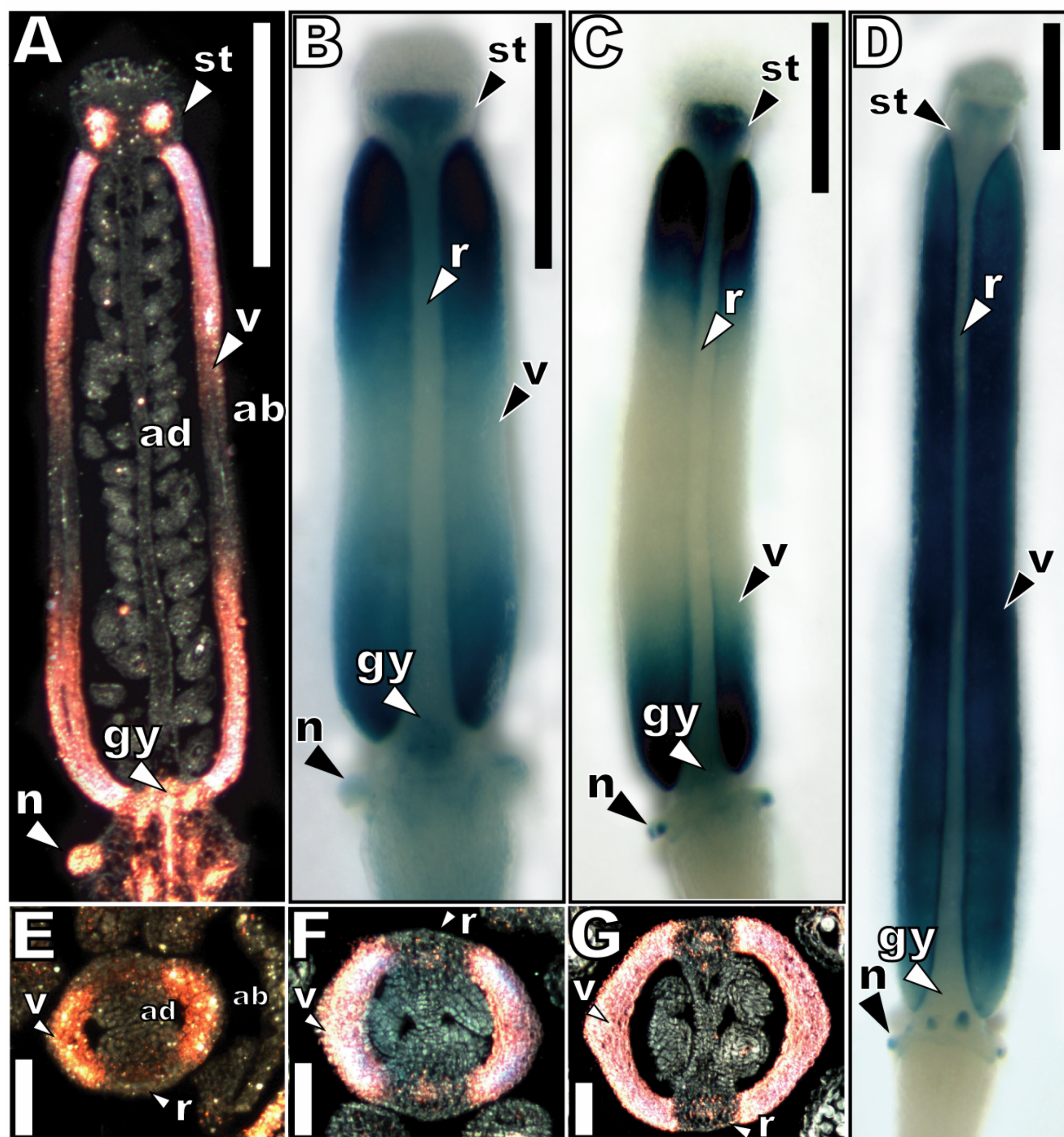


Figure 5. Analysis of pAN4 GUS activity in the *Arabidopsis* gynoecium.
(A) Longitudinal section of a gynoecium of a pAN4 transformant at stage 13. GUS staining appears orange or blue in dark field. **(B-D)** GUS activity by pAN4 transformants is shown for **(B)** stage 12, **(C)** stage 15, and **(D)** stage 17b gynoecia. Note that GUS activity in the valves preferentially expresses in the apical and basal portions of the valve at stage 12, but extends throughout by stage 17b. **(E-G)** Sections of the gynoecia of pAN4 transformants at **(E)** stage 8, **(F)** stage 10, and **(G)** stage 12 are shown. GUS is expressed in the adaxial layers of the developing valves at stage 7 and 8, but later expands to all the tissues of the valve by stage 10.

Style (st), valve (v), gynophore (gy), nectaries (n), replum (r), adaxial (ad), abaxial (ab). Scale bars are 250 μm for (A) and (B), 500 μm for (C) and (D), 50 μm for (E-G).

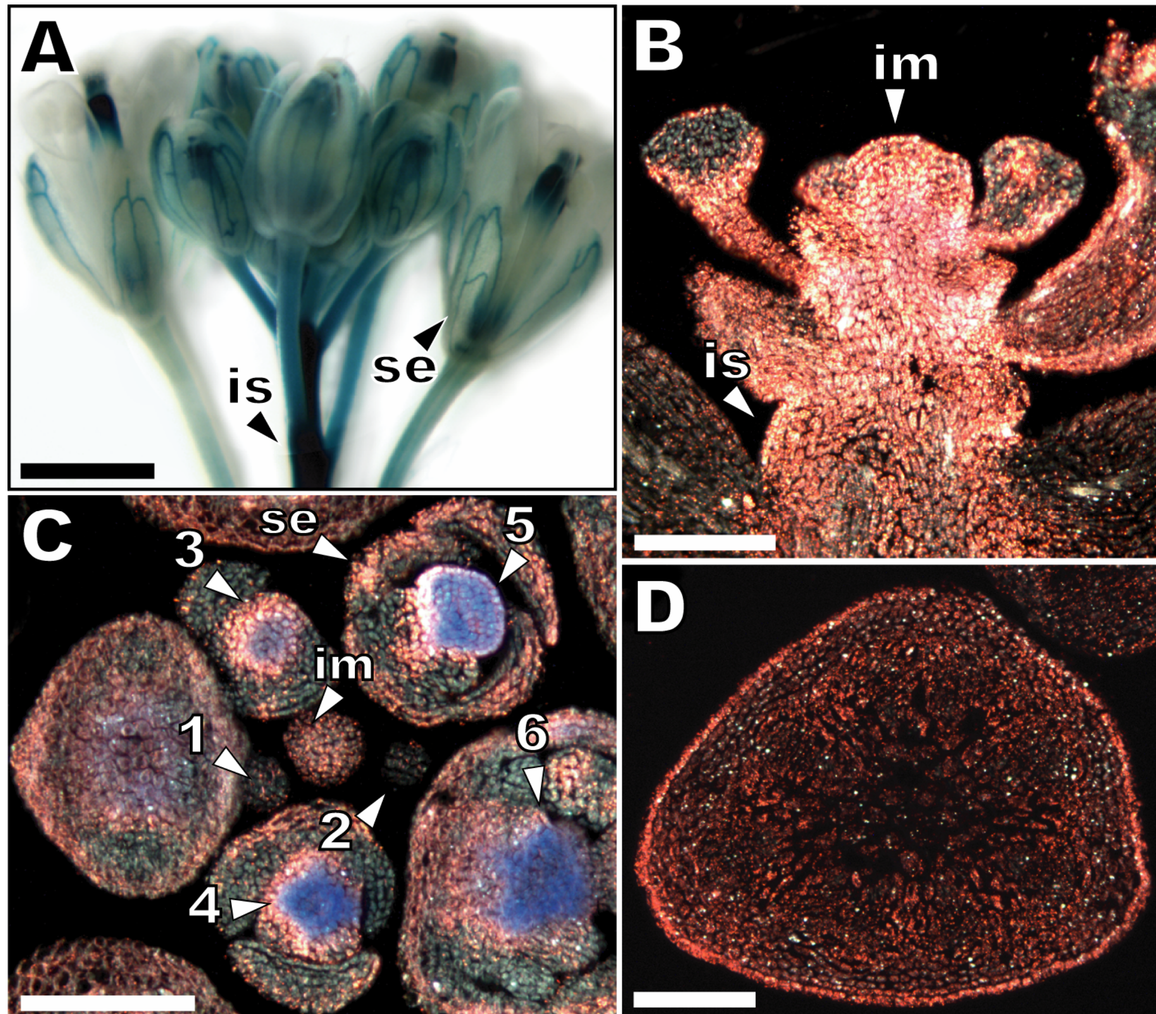


Figure 6. Analysis of pAN4 GUS activity in the *Arabidopsis* inflorescence.
(A) Whole-mount GUS staining of a pAN4 transformant plant. GUS activity is strongly expressed in the inflorescence stem and vasculature of the sepals in addition to the developing fruits as previously shown (Gu et al., 1998). **(B)** Longitudinal section of a pAN4 inflorescence. **(C)** Cross-section of a pAN4 inflorescence. The stages of the developing carpel primordia are indicated. **(D)** Cross-section of the pAN4 inflorescence stem.

Inflorescence stem (is), sepal (se), inflorescence meristem (im). Scale bars is 1 mm for (A), 100 µm for (B-D)

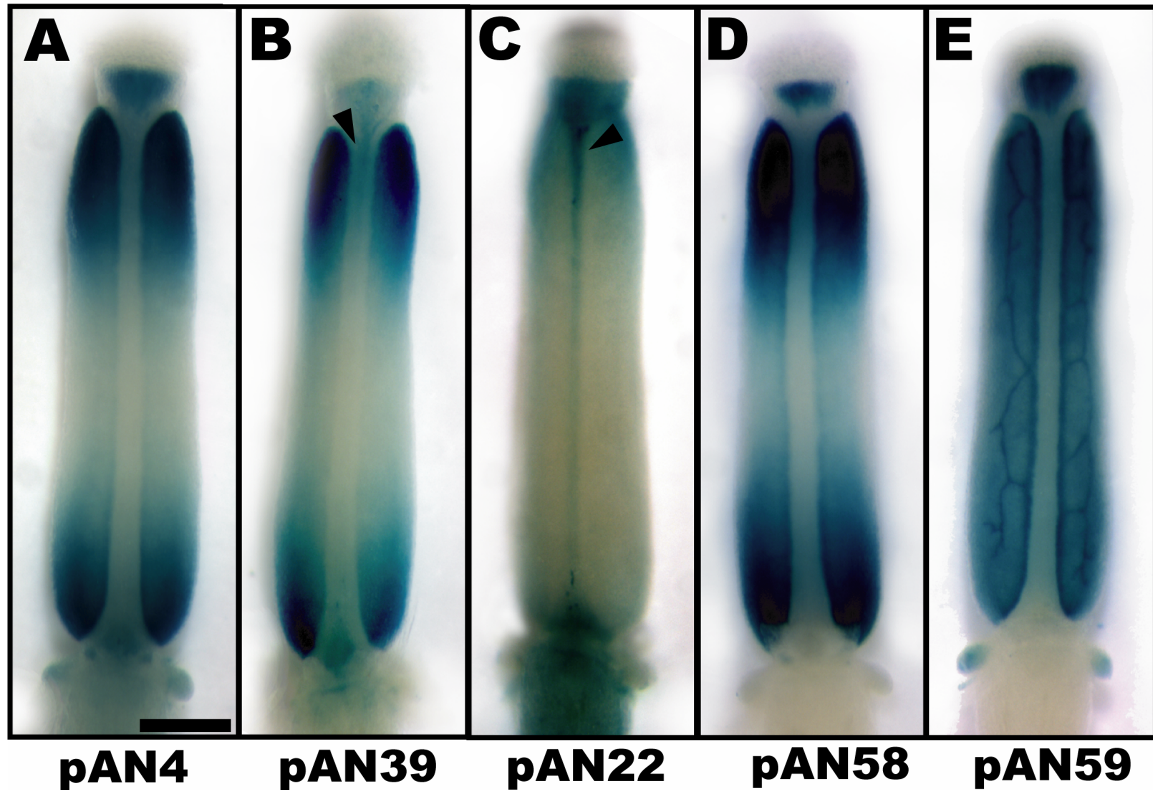
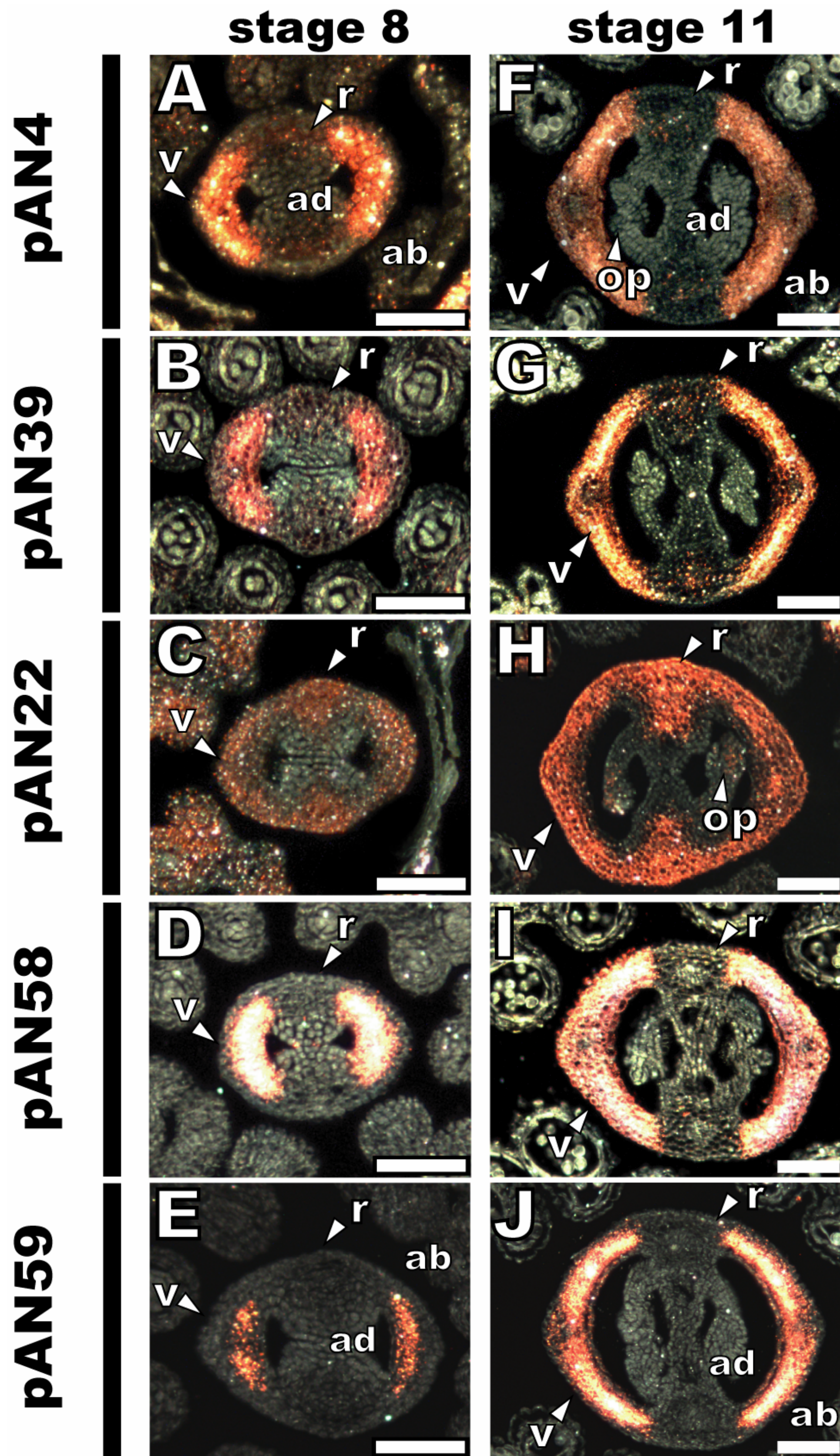


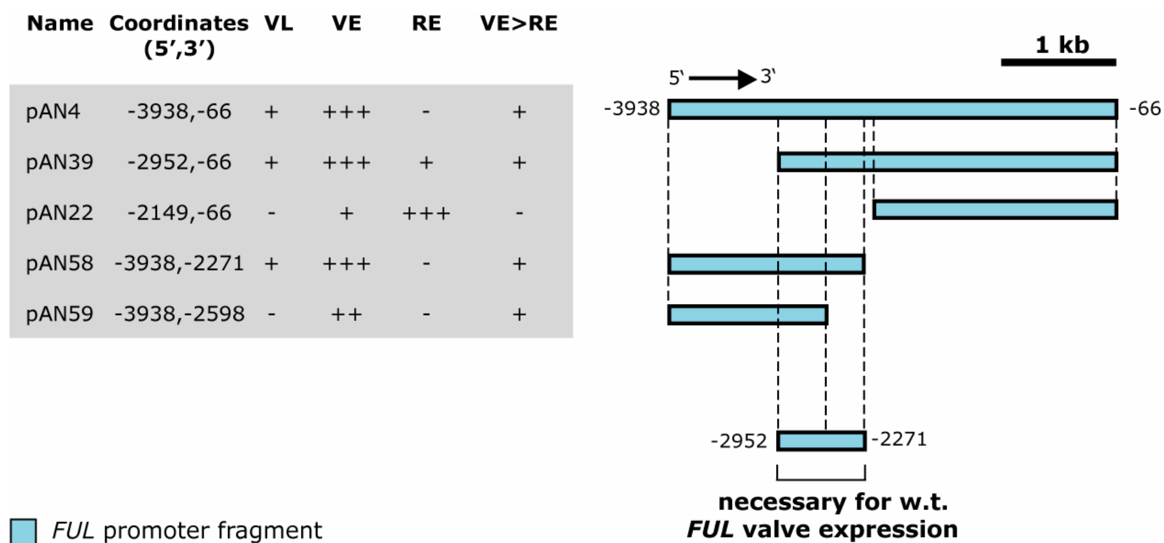
Figure 7. GUS expression pattern of the deletion constructs in the *Arabidopsis* gynoecium.
(A-E) Stage 13 gynoecia of T2 reporter lines. **(A)** GUS activity driven by the pAN4 promoter is considered the wild-type expression pattern. **(B)** In pAN39 gynoecia, the GUS expression pattern is similar to pAN4. **(C)** In pAN22, the style expression still remains but the valve expression levels are dramatically reduced. Typical valve-specificity is gone, and GUS is clearly expressed in the replum (black arrowhead).
(D) The 3' deletion in pAN58 shows a similar expression pattern as observed for pAN4.
(E) GUS activity is valve-specific in pAN59 although not in a pAN4-like way. In this transgenic line, GUS is preferentially expressed in a reticulated pattern probably corresponding to the vascular tissue in the valves. Interestingly, the GUS signal becomes stronger at the valve-replum boundaries, where the valve margins are located.
Arrowheads indicate ectopic GUS activity in the abaxial replum.

Scale bar in (A) is 250 µm and applies to (B-E).

Figure 8. ***FUL*** **promoter activity has a valve-specific transcription.**
Cross-sections of **(A-E)** stage 8 and **(F-J)** stage 11 gynoecia of T2 plants, respectively. **(A)** and **(F)** show GUS activity of pAN4 wild-type promoter in stage 8 and stage 11 fruits respectively. In pAN4 the signal is strongly detected in the developing valves. At **(B)** stage 8 and **(G)** stage 11, the pAN39 construct yields a similar expression pattern as shown for pAN4. The **(C)** and **(H)** panels show GUS activity by pAN22, where GUS is ectopically expressed in the ovule primordia and at high levels in the replum. pAN58 at **(D)** stage 8 and **(I)** stage 11 show similar expression patterns as shown for pAN4. At **(E)** stage 8, the pAN59 valve expression level is lower than pAN4 and the territory is reduced and restricted to the adaxial region. Later, at **(J)** stage 11, the GUS is detected only in the mesocarp layer, and is absent from the inner and outer epidermal layers.

Valve (v), replum (r), adaxial (ad), abaxial (ab), ovule primordium (op). Scale bar in (A) is 50 µm and applies to (B-E). Scale bar in (F) is 50 µm and applies to (G-J).


stage 8
stage 11
pAN4
pAN39
pAN22
pAN58
pAN59
A
B
C
D
E
F
G
H
I
J
r
v
ad
ab
op



Name	Coordinates (5′,3′)	VL	VE	RE	VE>RE
pAN4	-3938,-66	+	+++	-	+
pAN39	-2952,-66	+	+++	+	+
pAN22	-2149,-66	-	+	+++	-
pAN58	-3938,-2271	+	+++	-	+
pAN59	-3938,-2598	-	++	-	+

Figure 9. 5' *FUL* promoter region positively regulates valve-specific expression
On the table to the left are the names of the constructs and the genomic coordinates relative to the *FUL* gene start codon. The next columns represent the GUS expression in *all* valve layers (VL) [+, yes; -, no], the relative expression levels of the valves (VE) and abaxial replum (RE) based on whole-mount observations [+++, strong; ++, moderate; +, weak; (+), trace; -, absent], and if the GUS expression was valve-specific (VE>RE) [+, yes; -, no] for each construct. On the right, a diagram shows the *FUL* promoter fragments for each construct relative to the pAN4 wild-type promoter. The promoter region determined necessary for *FUL* valve-specific expression is located between 2952 and 2271 bp upstream the *FUL* start codon.

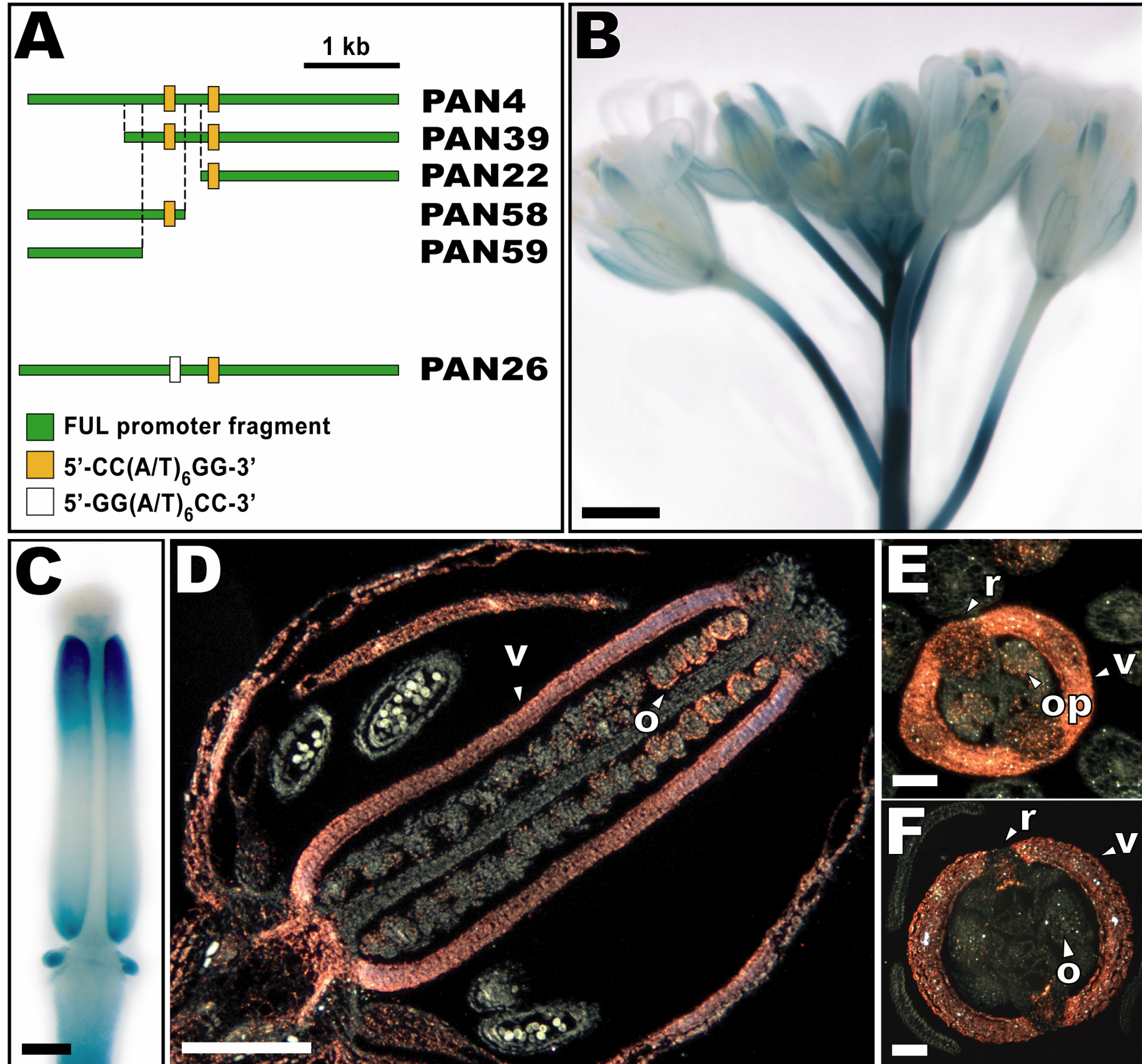


Figure 10. 5' CArG box inhibits GUS ectopic expression in the ovule primordia. **(A)** Diagram of pAN26 promoter relative to pAN4 promoter and other promoter deletions. The pAN4 promoter has 2 CArG boxes located at -2512 and -2038 bp. The 5' CArG box (-2512) is mutated in pAN26. **(B)** Inflorescence of a pAN26 transformant. GUS activity is expressed in the developing fruit, inflorescence stem, and vascular tissues of the sepal. **(C)** Stage 12 gynoecium of a pAN26 transformant. **(D)** Longitudinal section of a stage 12 gynoecium from the pAN26 transformant line. Compared to pAN4, ectopic GUS activity occurs in the ovules of pAN26 transformants. **(E)** Cross-section of a stage 11 gynoecium expressing pAN26. While GUS expression is valve-specific, GUS is ectopically expressed in the ovule primordia and abaxial replum compared to pAN4 transformants. **(F)** Cross-section of a stage 14 gynoecium expressing the pAN26 GUS reporter.

Valve (v), ovule (o), replum (r), ovule primordium (op). Scale bars are 1mm for (B), 200 µm for (C) and (D), and 50 µm for (E) and (F).

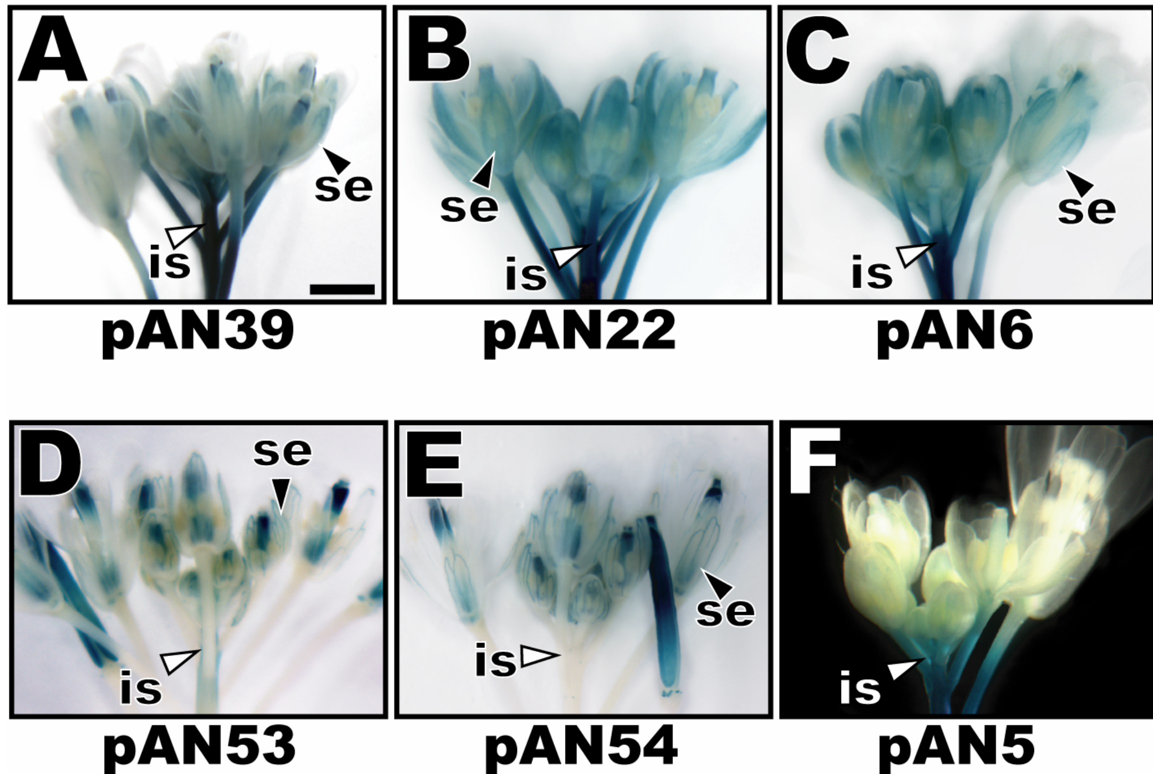
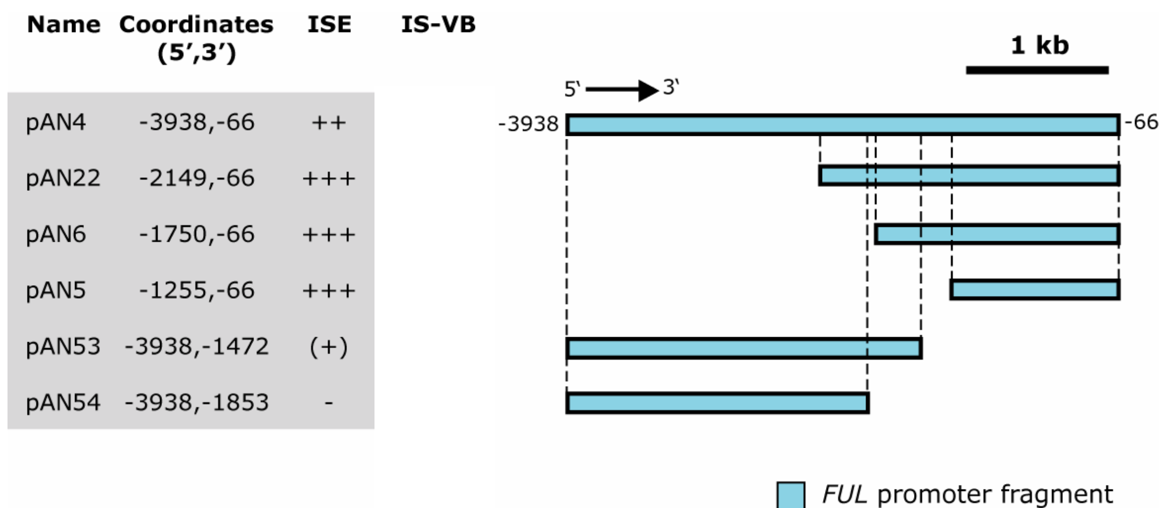


Figure 11. 3' region of *FUL* promoter required for inflorescence stem activity.
(A-F) Whole-mount staining of T2 inflorescences. **(A)** GUS activity in the inflorescence stem and sepal vasculature in pAN39 is similar to pAN4 (as shown in figure 6A).
(B, C, F) Strong GUS activity, similar to pAN4, is also present in the inflorescence stems of the pAN22, pAN6, and pAN5 lines; all of them contain a 5' promoter deletion.
(D) The 3' deleted promoter in pAN53 drives faint GUS activity in the inflorescence stem. **(E)** GUS activity is absent in the inflorescence stem of pAN54 transformants (which contains a larger 3' deletion; see Figure 12), although there is still expression in the vascular tissue of the sepals.

Sepals (se), inflorescence stem (is). Scale bar in (A) is 1mm and applies to (B-F).



Name	Coordinates (5',3')	ISE	IS-VB
pAN4	-3938,-66	++	
pAN22	-2149,-66	+++	
pAN6	-1750,-66	+++	
pAN5	-1255,-66	+++	
pAN53	-3938,-1472	(+)	
pAN54	-3938,-1853	-	

Figure 12. 3' promoter region positively regulates *FUL* expression in the inflorescence stem.
On table to the left are the names of the constructs and the genomic coordinates relative to the *FUL* gene start codon. The column on the far right of the table represent expression levels in the inflorescence stem (ISE) based on whole-mount observations [+++, strong; ++, moderate; +, weak; (+), trace; -, absent]. On the right, a diagram shows the *FUL* promoter fragments for each construct relative to the pAN4 wild-type promoter. The smallest promoter region determined sufficient for *FUL* inflorescence stem expression is located between -1255 and -66 bp upstream the *FUL* start codon.

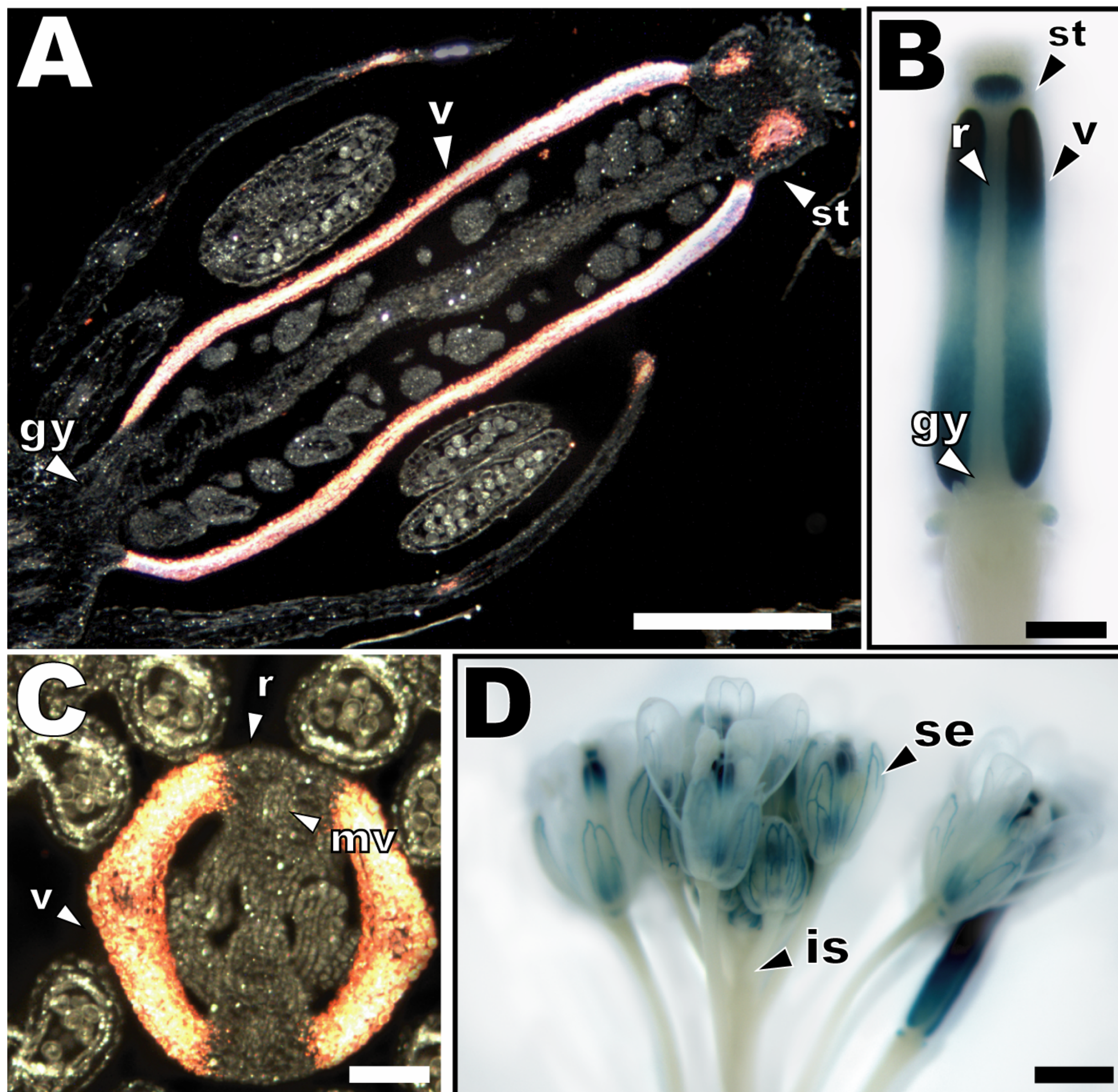


Figure 13. Expression pattern of GUS reporter controlled by reverse-oriented FUL promoter, pANR4.
(A) Longitudinal section of a stage 12 gynoecium from a pANR4 transformant. **(B)** A stage 12 gynoecium from a pANR4 transformant. GUS expression in the valves is similar to pAN4; GUS activity also occurs in the nectaries and vascular tissues of the style.
(C) Lateral section of a stage 11 gynoecium from a pANR4 transformant shows valve-specific GUS activity. **(D)** GUS activity of a pANR4 inflorescence is lowered in the inflorescence stem compared to pAN4. GUS activity also occurs in the vascular tissue of the sepals, like pAN4.

Gynophore (gy), valve (v), style (st), replum (r), medial vascular bundle (mv), sepal (se), inflorescence stem (is). Scale bars in (A) and (B) are 250 µm. Scale bar in (C) is 50 µm. Scale bar in (D) is 1 mm.

APPENDIX 2: TABLES AND TABLE LEGENDS

Table 1. List of oligonucleotides used to create GUS reporter constructs.
The oligonucleotides (oligos) listed were used to generate the FUL reporter constructs. oAN29 and oAN30 were used for site mutagenesis of the 5' CarG box binding domain in the *FUL* promoter. Highlighted are the *BamHI* restriction sites used for cloning.

Oligo Name	Oligo Sequence (5' to 3')
oAN29	CTTTTTGTTCATGTGGTGGAATTTTCCTATATACTATATAGTCTATAG
oAN30	CTATAGACTATATAGTATATAGGAAAATTCCACCACATGAACAAAAAG
oAN39	GGATCCCTTCAGTTAGAAATATCTATTCGATAC
oAN40	GGATCCGTCAAGTTTCGATTCCTCCAAAATTAA
oAN41	GGATCCGTATATTTATTTGAAATAAAGAAAAAT
oAN42	GGATCCGGTCACACACAGAAAAAATAGACAA
oAN44	GGATCCGTCGACTCTCTCTCTTCAAAATC
oAN57	GGATCCCCCGGGCTGCAGGAATT
oAN61	GGATCCGTAGGATTAACACATCACACTAAGC
oAN62	GGATCCCGACGTAAATTTGTTTATGTTGCTC
oAN63	GGATCCAAACGTATTTAATTTCTCTGAATCG
oAN64	GGATCCCGAAGCTTGTGTTGGAAATTTGGG

Table 2. List of *FRUITFULL* GUS reporter transgenic constructs.
For each construct, the *FUL* promoter fragment was made by PCR and cloned into a precursor plasmid (pCR2.1 TOPO vector, Invitrogen). After sequencing, the promoter fragment was excised with BamHI and cloned in front of the GUS reporter gene in the pDW294 vector (Final Plasmid).

Final Plasmid (+PDW294)	Precursor Plasmid (+TOPO)	5' oligo	3' oligo
pAN36	pAN30	oAN39	oAN44
pAN37	pAN31	oAN40	oAN44
pAN38	pAN32	oAN41	oAN44
pAN39	pAN33	oAN42	oAN44
pAN53	pAN48	oAN57	oAN61
pAN54	pAN49	oAN57	oAN62
pAN58	pAN55	oAN57	oAN64
pAN59	pAN56	oAN57	oAN63

Table 3. Unique putatitive motifs found within 682 bp regulatory region necessary for valve-specific *FUL* transcription.
The PLACE program at http://www.dna.affrc.go.jp/PLACE/ was used. In this table are the unique signal motifs in the 682 bp promoter region (region between positions -2952 and -2271) critical for valve-specific expression (refer to Figure 9). The blue-shaded motifs are also found in the pAN59 promoter fragment, which only drives some aspects of the valve-specific *FUL*-like transcription. C = coordinates; Or = orientation [+, forward; -, reverse; +/-, palindromic]; T= number of times a particular motif appears in the wild-type *FUL* promoter.

PLACE Name	Motif Sequence	C	Or	T	Notes
-300CORE	TG(A/C/T)AAA(A/G)(G/T)	-2481	-	1	Binding of DOF proteins; associated with seeds
2SSEEDPROTBANAPA	CAAACAC	-2456	-	2	Associated with seeds
AGMOTIFNTMYB2	AGATCCAA	-2686	+	1	Binding of GATA-type zinc finger proteins; associated with defense
BP5OSWX	CAACGTG	-2752	+	1	Binding of MYC/bHLH proteins
CATATGGMSAUR	CATATG	-2893	+/-	1	Involved in auxin response
CARGATCONSENSUS	$CC(A/T)_6GG$	-2512	+/-	2	CArG-box; binding of MADS-box proteins
LTRECOREATCOR15	CCGAC	-2467	-	3	Binding of CBF/DREB1; associated with stress/ABA
MYBPZM	CC(A/T)ACC	-2331	+	2	Binding of MYB proteins
MYCATRD22	CACATG	-2524	-	2	Binding of MYC/bHLH proteins; associated with stress/ABA
P1BS	GNATATNC	-2505	+/-	1	Associated with stress/starvation
TATCCAOSAMY	TATCCA	-2586	+	1	Binding of MYB proteins; associated with sugar starvation response

Table 4. Select putative motifs found within the pAN5 *FUL* promoter fragment. The PLACE program at http://www.dna.affrc.go.jp/PLACE/ was used. In this table are the promising signal motifs in the 1190 bp promoter region (region between positions -1255 and -66) that is sufficient for *FUL*-like transcription in the inflorescence stem (refer to Figure 12). The blue-shaded motifs are in the forward-orientation and exclusive to the promoter region. C = coordinates; Or = orientation [+, forward; -, reverse; +/-, palindromic]; T= number of times a particular motif appears in the wild-type *FUL* promoter.

PLACE Name	Motif Sequence	C	Or	T	Notes
ANAERO2CONSENSUS	AGCAGC	-1019	+	1	Associated with anaerobic genes
ARFAT	TGTCTC	-89 -641	- +	3	Auxin response element
ASF1MOTIFCAMV	TGACG	-193 -225	- -	3	Binding of bZIP proteins; auxin and stress related
AUXRETGA1GMGH3	TGACGTAA	-193	-	1	Auxin response element
E2FCONSENSUS	$(A/T)TT(C/G)_2C(C/G)_2$	-1182	+	2	E2F target motif
GLUTECOREOS	CTTTCGTGTAC	-792	+	1	Associated with seeds
GT1MOTIFPSRBCS	(G/T)(A/T)GTG(A/G)(A/T)AA(A/T)(A/G)(A/T)	-251	-	1	Associated with shoots and leaves
HEXMOTIFTAH3H4	ACGTCA	-193 -225	+ +	2	Binding of bZIP proteins; associated with meristem
L1BOXATPDF1	TAAATG(C/T)A	-661	-	1	Binding of homeodomain leucine zipper proteins; associated with shoot apical meristem
PYRIMIDINEBOXHVEPB	TTTTTTCC	-225 -910	- -	2	Associated with GA response
RYREPEATBNNAPA	CATGCA	-635	+	1	Associated with seeds
SEBFCONSSTPR10A	(C/T)TGTC(A/T)C	-641	+	1	Associated with silencing
SP8BFIBSP8BIB	TACTATT	-836	+	2	Associated with roots
SREATMSD	TTATCC	-891	-	2	Associated with axillary bud growth
TGACGTVMAMY	TGACGT	-193 -225	- -	2	Associated with seeds and cotyledons
UP2ATMSD	AAACCCTA	-576	+	1	Associated with axillary bud growth
WBBOXPCWRKY1	TTTGAC(C/T)	-805 -1177	- -	2	Binding of WRKY proteins

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